Proteolytic regulation of TRP channels: Implications for pain and neurogenic inflammation

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Summary

1. The protease-activated receptor 2 (PAR_2) is a G protein-coupled receptor (GPCR) expressed by primary afferent neurons. PAR_2 can be activated by inflammatory proteases to induce diverse pathophysiological processes such as neurogenic inflammation and pain. Like other GPCRs, PAR_2 augments these cellular events by initiating signalling pathways to stimulate ion channels, lipid-metabolising enzymes and many other proteins.

2. PAR_2 is differentially regulated by multiple proteases in the body, leading to the potential for protease-driven biased signalling. Whole animal imaging of active proteases using activity-based probes can be used in conjunction with other animal and cellular models to investigate when, where, and how proteases stimulate distinct PAR_2 receptor signalling events to induce specific physiological outcomes.

3. Members of the Transient Receptor Potential (TRP) cation channel family are recognized downstream targets of PAR_2 signalling. The sensitization of TRPV1, TRPA1 and TRPV4 by PAR_2 serves to amplify nociceptive and inflammatory responses to proteases. Our recent findings indicate that PAR_2 can also directly activate TRPV4, which rapidly increases ion influx and signalling events to amplify neurogenic inflammation.

4. Identification of specific PAR_2-stimulated signalling pathways may reveal new therapeutic targets to improve pathophysiological states while leaving physiological PAR_2 and TRPV4 responses intact.

PAR_2 is a key mediator of neurogenic inflammation and pain

The first neurons in the pain pathway are primary spinal afferent neurons, which have cell bodies in dorsal root ganglia (DRG) or trigeminal ganglia (TG). The peripheral nerve terminals of DRG neurons are located within cutaneous and visceral tissue and project centrally to the dorsal horn of the spinal cord; TG neurons innervate the eyes, face and cranium and project to central nerves of the brainstem. Upon activation, these sensory nerves excite central neurons to cause transmission of pain.[1] To allow neurons to detect and respond to a diverse range of stimuli, the neuronal projections express a variety of signalling proteins including ion channels, GPCRs and receptor tyrosine kinases. These channels and receptors are a major target for treatment of chronic pain and inflammatory diseases.

It is well established that proteolytic enzymes can regulate pain and neurogenic inflammation by cleaving protease-activated receptors (PARs), a family of 4 GPCRs (PAR_{1-4}). This stimulation of PARs triggers nociception through an array of cellular events within nerve terminals and surrounding inflammatory tissue, and can modulate the activity of ion channels, such as the thermosensitive transient receptor potential (TRP) superfamily. The broad implications of proteases in disease have previously been reviewed.[2] Here we will focus on the regulation of a neuronally expressed member of the PAR family, PAR_2, and discuss how different proteases can contribute to pro-inflammatory and nociceptive events through PAR_2-dependent stimulation of specific TRP ion channels.

The protease-activated GPCR family

Tissues respond to injury and inflammation through the release and activation of proteases from the circulation, immune cells and epithelial cells. Activated proteases regulate nociception, inflammation, haemostasis and healing by cleaving PARs on multiple cell types.[2] Proteases cleave within the extracellular N-terminal domain of PARs to expose a new N-terminus that activates the receptor as a tethered ligand. Synthetic peptides that mimic this tethered ligand domain, known as an activating peptide (PAR-AP), can directly activate the receptor without the requirement for proteolysis.[3] The complexity of this highly regulated system lies in the ability of PARs to respond to proteolytic attack and subsequently transduce diverse intracellular signalling events. All four PARs are expressed in neuronal and non-neuronal tissues, and each PAR is preferentially cleaved by different subsets of proteases to initiate particular cellular pathways. Hence, the tissue-specific physiological outcome is dependent upon the activated proteases and the cleaved receptors. Thrombin, trypsin and cathepsin G can cleave PAR_1, PAR_3 and PAR_4, which mediate essential events such as inflammation and coagulation. Trypsin, mast cell tryptase, cathepsin-S and kallikreins cleave PAR_2, a major contributor to inflammation and nociception.[4-6] PAR_2 is widely expressed, with multiple functions including control of smooth muscle contractility, immunity and skin barrier formation. From a therapeutic standpoint it is important to understand the effects and role of PAR_2 activation on peripheral and central neurons, where it contributes to neuropeptide release, nerve excitation and inflammatory pain. New concepts are emerging relating to how proteases and PAR_2 impart such signalling specificity.

The identity, origin and role of proteases in inflammatory disease

Understanding the contribution of proteolytic pathways to inflammatory pain requires the identification of contributing proteases, their cellular origin, and their mechanism of action. This is a complex process given the complement of proteases within a host, in addition to exogenous sources such as gastrointestinal bacteria. Proteases have been shown to increase the permeability of cell barriers (*e.g.* of the intestinal mucosa), upregulate immune responses (neutrophil and mast cell recruitment and degranulation) and increase sensory nerve excitation to induce visceral pain and inflammatory disorders.[7-9] While it may be clinically possible to prevent protease attack using protease inhibitors and antagonists of PARs, prevention of all cleavage events across all tissues could be detrimental and does not account for other protease targets contributing to inflammatory pain disorders.

The recent use of protease-specific activity-based probes is a valuable approach for profiling proteases in disease models and therefore identifies harmful proteolytic events.[10-12] Activity-based probes typically comprise a warhead or reactive group that covalently binds and inhibits a protease of interest, a peptide linker and a reporter molecule such as a fluorescent tag or biotin, which is advantageous for non-invasive *in vivo* and *ex vivo* imaging and proteomic identification of active proteases.[12]

Using infrared activity-based probes to specifically target the cysteine cathepsins (Cat) Cat-B, Cat-L and Cat-S, non-invasive imaging reveals the tissue and cellular localization of active cathepsins in disease models. Mice with inflammatory pain induced by colitis have been tested with these probes and revealed active Cat-B, Cat-L and Cat-S in colonic macrophages in spinal neurons and microglia. Cat-S was also secreted and highly abundant in the colonic lumen, which lead to hyperexcitability of DRGs innervating the colon. Importantly, *par*$_2$ and *cat-s* knockout animals demonstrated a causative role for Cat-S in colonic nociceptors *via* zymogen activation or direct cleavage of PAR_2. This provides a functional link between protease specificity, activity, localization and the protease-sensitive PAR_2-dependent contribution to a painful inflammatory disease.[13] Similar studies in mice with inflammatory pancreatitis, revealed the presence of active cathepsins in the pancreas, spinal microglial cells and spinal neurons[14]. Further studies of this nature will provide new functional links between protease release and activity in other inflammatory pain disorders.

TRP channels contribute to the pro-nociceptive actions of PAR2

PAR_2 expressed by primary spinal afferent neurons is a major mediator of neurogenic pain and inflammation due to its ability to cause both local and central effects on the nervous system. A subset of primary afferent (nociceptive) neurons co-expresses PAR_2 and the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP). Cleavage and activation of PAR_2 stimulates neuropeptide release to generate the hallmarks of neurogenic inflammation: SP activates the GPCR neurokinin 1 (NK_1R) on endothelial cells and post-capillary venules, leading to granulocyte infiltration and extravasation of plasma proteins; CGRP binds and activates Calcitonin Receptor-Like Receptor (CLR), to cause vasodilatation of arterioles.[1,15] At the same time, PAR_2 excitation of peripheral nerve terminals can also lead to initiation of action potentials and subsequent release of SP and CGRP from their central projections within the spinal cord. This leads to activation of NK_1R and CLR on spinal neurons and subsequent pain transmission (Figure 1A). Together, these processes are associated with painful inflammation of the intestine, pancreas and joints.[14,15]

Proteases signal to cells *via* the common mechanism of protease attack leading to receptor activation. Stimulation of intracellular signalling events is critical in this process and amplifies the inflammatory pain response. Several lines of evidence indicate that non-selective cation channels such as the thermo-sensitive TRP channels are key targets of these inflammatory signals. Neuronally expressed channels such as TRP Vanilloid 1 and 4 (TRPV1 and TRPV4) and TRP Ankyrin 1 (TRPA1) are co-expressed with PAR_2 on small diameter peptidergic neurons and are downstream targets of PAR_2 and other GPCRs.[16-20] Importantly, these channels can be activated by stimuli generated during injury and inflammation: TRPV1 is activated by protons, noxious temperatures (>43°C) and lipid metabolites;[21-23] TRPV4 is activated by mechanical shear stress, lipid metabolites, osmotic stimuli and warm temperatures >27°C;[24-31] TRPA1 ion flux is increased in response to noxious cold temperatures, reactive oxygen species, and by inflammatory lipids such as 4-hydroxynonenol.[32-34]

For PAR_2, the downstream regulation of TRPs is evident in the pathophysiological outcomes of inflammatory pain models (Table 1). For example, trypsin-stimulated PAR_2 signalling causes thermal and mechanical hyperalgesia in mice, consistent with sensitization of TRPV1 (described below), TRPV4 and TRPA1.[16,17,35] TRPV4 is stimulated by PAR_2 in models of peripheral and visceral pain to induce mechanical hyperalgesia.[20,36] PAR_2-dependent neuropathic pain following chemotherapy (mechanical, heat, or cold hypersensitivity) requires these same thermo-sensitive TRP channels.[37] The magnitude of mechanosensation responses in nociceptive C-fibres pre-treated with the inflammatory mediators prostaglandin E2 and serotonin is also markedly reduced in TRPV4 knockout mice.[38]

GPCR-dependent regulation of TRP channels is now known as a prominent mechanism for the indirect stimulation of TRP channel activity. TRP channels are sensitized and contribute to pro-inflammatory events following the activation of histamine H_2 receptor,[39,40] neurokinin NK_1 and NK_2,[41,42] bradykinin B_2,[43,44] purinergic P2Y1 and P2Y2,[45,46] prostaglandin E_2 and J_2[47-50] and muscarinic receptors.[51]

The importance of the GPCR/TRP axis is also evident in other eukaryotic organisms. TRP channel

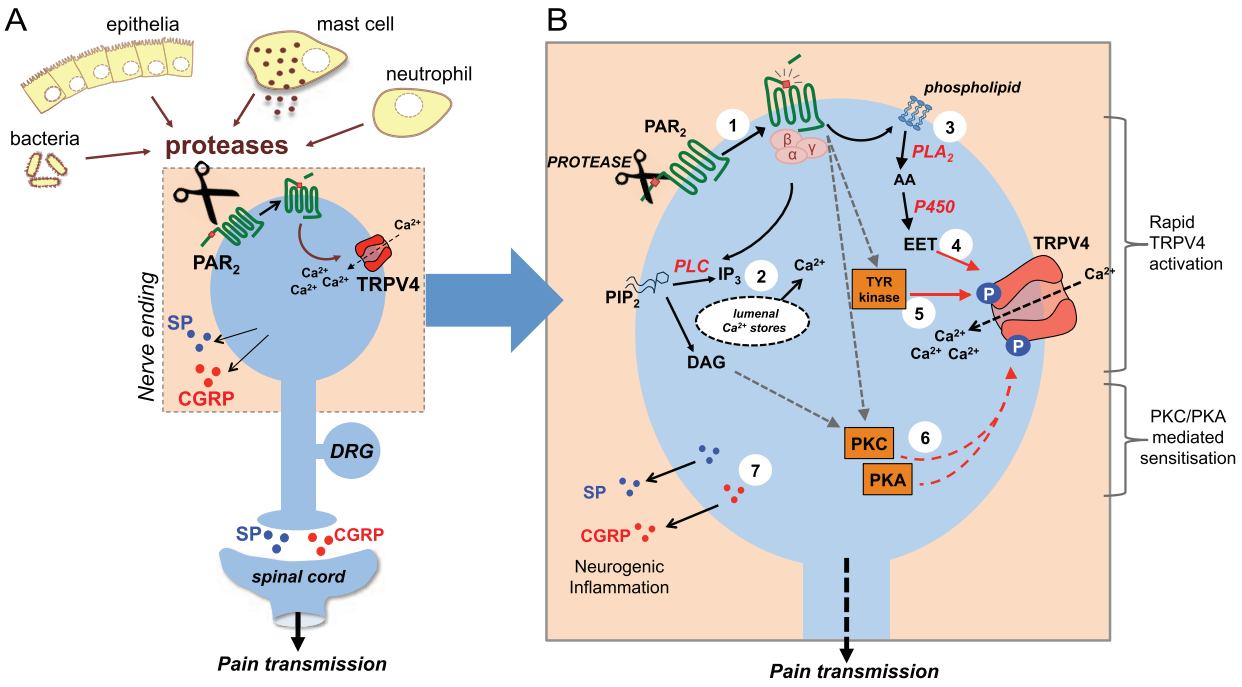

Figure 1. Proposed pathways for PAR_2 and TRPV4 mediated neurogenic inflammation. ***A****. Following inflammation or injury, proteases from multiple cellular sources can cleave PAR_2 on primary afferent nerve terminals (DRG nerve ending). Localized neurogenic inflammation is initiated* via *release of the neuropeptides SP and CGRP. These same peptides contribute to pain transmission* via *stimulation of their respective GPCR on spinal afferents.* ***B****. Following PAR_2 cleavage (step 1), G protein signalling mechanisms lead to the production of second messengers and activation of the TRPV4 ion channel. This includes PLC-dependent PIP_2 hydrolysis into diacylglycerol (DAG) and IP_3 to trigger Ca^{2+} mobilization from intracellular stores (2), PLA_2 mediated breakdown of phospholipids into arachidonic acid (AA; 3) and subsequent metabolism of AA by cytochrome P450 into eicosanoic acid, or EETs (4), which are endogenous activators of TRPV4. A currently unidentified tyrosine kinase can also phosphorylate TRPV4 at residue Tyr110 and contribute to its PAR_2-mediated activation (5). PKC phosphorylation of Ser824 and PKA activity also contribute to TRPV4 sensitization, but it is currently unclear if this contributes to the immediate activation of TRPV4 by PAR_2 in neurons (6). These pathways stimulate neuropeptide release and multiple localized physiological responses (7). Adapted from Poole* et al., *2013.*[19]

activity, for example, has been identified in photoreceptor cells of a *Drosophila melanogaster* mutant, where the *trp* mutant phenotype showed a rapid decay in the photosensitive response to continuous light stimulation. Intense research in the field has since demonstrated that photoreceptor TRP channel gating requires stimulation of the light-sensitive GPCR rhodopsin, activation of membrane-bound phospholipase C (PLC) and lipid signalling pathways. This provided the first known example of a GPCR sensing an environmental stimulus and modulating a TRP channel to facilitate a cellular response.[52] Functional interactions between GPCRs and TRP channels have also been characterized in *C.elegans* and while the mechanisms may differ, common signalling components are evident. This includes PLC-dependent break down of phospholipids into second messengers and upregulation of kinases, which modify channel gating and activity.[53]

Key GPCR pathways for regulation of TRP channels

GPCR signalling requires association with the heterotrimeric α, β and γ G Protein subunits to promote signalling to a range of effectors for the stimulation of diverse cellular events. The C-terminus of PAR_2 can interact with several G protein subtypes ($G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{12/13}$) in addition to important adaptor proteins such as β-arrestins, which show increased affinity for the C-terminus of protease-activated, phosphorylated PAR_2. While arrestins are not the focus of this review, they play key roles in GPCR localization through receptor recognition to facilitate clathrin-mediated internalisation. In the case of PAR_2, receptor internalisation is critical for termination of the protease-activated signals *via* receptor lysosomal targeting for degradation.[59,60] Recent studies have also demonstrated the ability for arrestins to promote additional inflammatory signalling events when receptors are internalized to endosomal compartments.[61] The potential for PAR_2 and other receptors to activate different second messengers depending on their cellular location and

Table 1. TRP channel activity is an important component of PAR_2-mediated hyperalgesia.

TRP channel	Comments	Reference
TRPV1	PAR_2 reduces temperature threshold for TRPV1 activation in HEK, DRG and mice	54
	PAR_2 stimulates the protein kinases PKA and PKC, sensitizing TRPV1 to cause thermal hyperalgesia	17
	PAR_2 activation of PKC sensitizes TRPV1 for Substance P and CGRP release	55
	PAR_2-TRPV1 pathway is associated with thermal hyperalgesia in chronic pancreatitis	56
TRPV4	PAR_2 activation of PLC, PKA, PKC and protein kinase D (PKD) sensitizes TRPV4 to induce neuropeptide release and hyperalgesia	20
	TRPV4 is required for PAR_2-induced mechanical hyperalgesia and excitation of colonic afferents	36,57
	PAR_2 stimulation of PLA_2, P450 and a tyrosine kinase induces sustained TRPV4 activity in DRG neurons and causes neurogenic hyperalgesia in mice	19
TRPA1	TRPA1 potentiates effects of PAR_2 activation in DRG neurons via PLC-mediated PIP_2 hydrolysis	16
	PAR_2-dependent colitis-induced mechanical hyperalgesia requires TRPA1 expression in sensory neurons innervating the colon	58
TRPV1, TRPV4 and TRPA1	Paclitaxel induced PAR_2 signalling sensitizes all three channels to induce neuropathic pain	37

presence of localized proteases is discussed below in "Biased signalling of PAR_2".

GPCR-stimulated $G\alpha_{q/11}$ signalling is an important contributor to rapid changes in intracellular Ca^{2+} and is a well-characterized and prominent pathway for TRP-related activity. A major component of this pathway requires stimulation of PLC for the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). Metabolism of PIP_2 directly relieves tonic inhibition of TRP channels, while DAG stimulates protein kinase C family members to phosphorylate and stimulate TRPV1 and TRPV4.[62-64] Signalling through GTP-bound $G\alpha_s$ subunits stimulates adenylate cyclase activity to increase cAMP levels and PKA activity, also leading to sensitization of TRPV1, TRPV4, and TRPA1.[17,65] These events occur in parallel to a plethora of other signalling processes: Stimulation and nuclear translocation of MAP kinases ERK1/2, activation of tyrosine kinases, transcriptional and Ca^{2+}-dependent signalling events, upregulation of cytokines, and stimulation of metabolic enzymes such as phospholipase A_2 (PLA_2).[66-68] PARs work together with receptor tyrosine kinases (RTKs) to activate PLA_2, and this is an important regulatory process for metabolism of phospholipids into arachidonic acid (AA) leading to increased activity of lipoxygenases, cyclooxygenases and cytochrome P450. Each of these enzymes is responsible for generation of nociceptive lipid agonists of TRP channels, including the TRPV4 agonists epoxyeicosatrienoic acids (5′,6′-EET, 8′,9′-EET), TRPV1 activator leukotrienes B4, and TRPA1-stimulating prostaglandin metabolites.[28,50,69] These signalling pathways represent divergence between GPCRs (>1000 family members) and TRP channels (28 mammalian family members), and exemplify how many physiological outcomes may be achieved through a small number of TRP ion channels.

Together, GPCR signalling in an inflammatory cellular environment provides all of the necessary cues (*e.g.* temperature) and diverse G-protein signalling pathways to stimulate TRP channel activity. Yet, despite the number of signalling events within the PAR_2 signalling network, evidence suggests that there is a high degree of tissue-dependent selectivity for specific ion channels and this is not well understood.

Functional coupling between PAR_2 and TRPV4

Upon investigating a role for TRPV4 in PAR_2-stimulated neurogenic inflammatory pain, we have recently demonstrated that in addition to TRPV4 sensitization, these signalling events also lead to direct functional coupling in both HEK293 cells and nociceptive neurons. While this does not ascribe a physical interaction between PAR_2 and TRPV4, the signalling events involved lead to specific and rapid TRPV4 Ca^{2+} flux, that was not observed for other neuronally-expressed TRP channels.[19] The TRPV4 non-selective cation channel is expressed in afferent nerves and contributes to thermo-, osmo- and mechano-sensation, which is essential for a diverse range of cellular processes including pain and inflammation, vasodilation, ciliary beating frequency, wound healing and muscle contraction (*e.g.* bladder voiding).[70-74] In nerves (*e.g.* innervating the skin and intestine) chronic stimulation of TRPV4 by PAR_2 can lead to mechanical hyperalgesia, however, the molecular events that cause this are not well understood and remain under investigation.

Heterologous expression of TRPV4 in HEK293 cells demonstrates a clear and selective coupling between PAR_2 stimulation and TRPV4-dependent Ca^{2+} flux. Treatment with signalling inhibitors and expression of TRPV4

phosphorylation site mutants has uncovered key players in this direct coupling (Figure 1). Antagonism of PLA_2, P450 and Src family kinases reduced or prevented coupling between PAR_2 and TRPV4. Investigation of phosphotyrosine mutants of TRPV4 (Y110F and Y805F) suggested a specific role for the putative N-terminal phosphorylation site Tyr110 in this process. Further studies will reveal the precise kinase required and how this phosphosite leads to increased channel activity.

Historically, TRPV1 has been the most therapeutically targeted of the vanilloid TRPV family due to its role in threshold pain sensation, such as noxious heat. Consistent with this, co-administration of the TRPV1 agonist capsaicin and PAR_2-AP causes thermal hyperalgesia in mice, which is lost in TRPV1 knockout animals. Sensitization of capsaicin responses with prior PAR_2-AP stimulation is also evident in cultured DRG neurons. PKC (epsilon isoform), PKD and PKA have been identified as significant signalling components of PAR_2. All of these kinases mediate sensitization of TRPV1 channel activity in sensory neurons and in cell lines.[17,65,76] Similarly, protein kinase inhibition in neurons and cell lines can also reduce sensitization of TRPV4, and was, therefore, considered a likely target for rapid PAR_2-dependent modulation of TRPV4 channels.[20] The specific inhibition of PKC in our studies, however, showed no effect on immediate PAR_2-TRPV4 coupling. The PKC phosphorylated residue Ser824 is a key TRPV4 regulatory site that is a requirement for receptor-mediated TRPV4 activation in endothelial cells[24] and contributes to TRP channel sensitization and pain. Despite TRPV studies showing rapid PAR_2-AP induced cell surface translocation of PKC, these data support a long-term role for PKC in neuronal TRPV4 activity that cannot be observed immediately following stimulation of PAR_2 (Figure 1B).[62] PKA was not tested in this study of direct PAR_2-TRP4 functional coupling, although previous studies demonstrate that direct activation of PKA and PKC can lead to TRPV4-dependent mechanical hyperalgesia in mice.

The biased signalling of PAR_2

Biased agonism is an emerging theme in GPCR biology that describes the potential for various ligands to influence GPCR signalling pathways, leading to distinct cellular outcomes.[77] Recently this has been described for PAR_2, not *via* ligand-dependent binding

per se, but following proteolytic cleavage by different proteases. Inflammation leads to activation and release of multiple proteases including neutrophil elastase and proteinase 3, mast cell cathepsin G and tryptase, and it is therefore an important concept for understanding how competing proteolytic attack can influence PAR_2 mediated signalling events in an inflammatory state.

Trypsins, mast cell tryptase and kallikreins cleave PAR_2 at the Arg-Ser site to expose the canonical tethered ligand (SLIGRL sequence for rat PAR_2), leading to $G\alpha_q$ mediated Ca^{2+} mobilization, β-arrestin mediated MAPK signalling, receptor endocytosis and targeting to lysosomes for degradation. Proteinase 3, cathepsin G and elastase cleave the receptor at distinct sites downstream to the tryptic cleavage site and this was initially considered a disarming mechanism for removal of the tethered ligand and prevention of trypsin mediated signalling.[78] While this is true for proteinase 3 and cathepsin G, elastase cleavage initiates distinct signalling events though $G\alpha_{12/13}$ mediated MAPK pathways.[79] This may lead to specific physiological events in inflamed tissue, which may also be temporally controlled by Proteinase 3 or cathepsin G disarming events. Interestingly, activity-based probes have indicated a role for Cat-S in PAR_2 mediated colitis-induced inflammation and may provide an additional, alternative biased protease for PAR_2 signalling. The potential to influence PAR_2 signalling through biased proteases is not completely understood and remains an exciting yet complex area of investigation in protease biology.

Conclusion

PAR_2 is a key contributor to neurogenic inflammation and pain through the upregulation of multiple signalling pathways and, with the identification of biased proteases, the true complexity of these pathways is only now beginning to be understood. The contribution of TRP channels to these pathways is significant. TRP channel knockout mice and inhibitor studies all support a role for non-selective cation channel gating in many physiological processes, including mechanical or thermal hyperalgesia. A major challenge remains in the identification of therapeutic targets that maintain the basal activity of TRP channels and prevent chronic sensitization or stimulation by PAR_2 pro-inflammatory signalling events.

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