Module 11

Cell Stress, Inflammatory Responses and Cell Death

Synopsis

Cells have intrinsic signalling mechanisms that are capable of sensing various deleterious conditions, both normal and pathological, and respond by mounting a variety of stress responses. Examples of normal signals are the cytokines that induce inflammatory responses in cells. Pathological signals include UV and X-ray irradiation, hydrogen peroxide (H_2O_2), abrupt anoxia and physicochemical injury through heat or noxious chemicals. A process of wound healing can function to repair the damage caused by such injuries. In many cases, especially if the stress signal is not too severe, the cell can survive and can even become tolerant to further insults. If cells are growing, such sub-lethal insults can either cause the cell to stop growing temporarily to allow adequate time to repair the damage, or the process of cell proliferation can be stopped more permanently and the cell will enter a state of senescence. Another example of an evolutionarily conserved survival mechanism is autophagy, which enables cells to cope with periods of starvation. If such stresses become too severe, however, the cell dies, either through a process of necrosis, which is rapid and catastrophic, or through a slower and more controlled process that is carried out by a highly regulated process of programmed cell death known as apoptosis.

Although the morphological characteristics of necrosis and apoptosis are clearly distinct, they do share some similarities in that they are induced by similar stimuli and often employ the same signalling mechanism. Necrosis occurs when the cell is overwhelmed by the insult and rapidly disintegrates. The cell volume expands rapidly, the mitochondria become swollen, and the plasma membrane suddenly ruptures, causing release of the cellular contents into the intercellular spaces where they can elicit an inflammatory response. By contrast, apoptosis is a much more orderly affair in that proteases and nucleases within the confines of an intact plasma membrane disassemble the cell that gradually shrinks in size and is then engulfed by neighbouring cells, thus avoiding any inflammatory reactions.

Inflammatory responses

The innate immune system is our first line of defence against invading pathogens. Not only does it initiate a vigorous and rapid inflammatory response to attack foreign pathogens, but it also plays a key role in activating the more slowly developing adaptive immune response. The latter results in the activation of specific B and T cells to extend the host defence system further. The initial line of defence during the innate response is carried out by a complex series of cellular interactions, which is known collectively as the inflammatory response. Such responses are particularly evident during wound healing. The major cellular players are the blood platelets, macrophages, mast cells, neutrophils and endothelial cells (Module 11: Figure inflammation). In the following description of the inflammatory response, emphasis will be placed on the cell signalling pathways that are used to control the participation of these different cell types, as summarized in Module 11: Figure inflammation:

1. Tissue damage. Many inflammatory responses begin with tissue damage, which can activate the complement system to release complement factors that recruit inflammatory cells such as the neutrophils.
2. Endothelial cell damage. A specific form of tissue damage occurs when the endothelial cells are disrupted. The cells are induced to release inflammatory mediators such as thrombin and bradykinin, which are responsible for the redness, pain and swelling as the local blood vessels become dilated and permeable to fluid and blood proteins. Some of these proteins are the complement factors and IgG antibodies that coat the pathogens, marking them out for phagocytosis. Endothelial cells also release sphingosine 1-phosphate (S1P) that can also have effects on vascular permeability.

Please cite as Berridge, M.J. (2014) Cell Signalling Biology; doi:10.1042/csb0001011

3. Blood platelet aggregation and clot formation. The thrombin produced by endothelial cell damage has a major role to play in blood platelet aggregation and formation of the haemostatic plug (Module 11: Figure thrombus formation). With regard to the latter, it is responsible for converting fibrinogen into fibrin, and it also initiates the cascade of reactions that results in cross-linking the fibrin monomers to form a fibrous meshwork that stems the flow of blood. Thrombin contributes to the activation of the Ca^{2+} signal that controls many of the processes of platelet aggregation (Module 11: Figure platelet activation).
4. Endothelial permeability. Endothelial cells control the flow of substances and cells from the plasma into the interstitial space. Under normal circumstances, this flow is fairly restricted. However, during inflammation, a number of mediators, such as thrombin, bradykinin and histamine, can greatly increase this permeability by contracting the cells to open up the paracellular pathway (Module 7: Figure regulation of paracellular permeability).
5. Cell proliferation. During wound healing, there is a considerable amount of cell proliferation to provide new cells for tissue remodelling. Some of this proliferation is driven by the release of platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β) (Module 11: Figure inflammation). Cell proliferation is increased in fibroblasts and other mesenchymal cells. There also is an increase in endothelial cell proliferation as part of the process of angiogenesis to repair the damaged blood vessels. The release of vascular endothelial growth factor (VEGF) plays a critical role in triggering this increase in endothelial cell proliferation (Module 9: Figure VEGF-induced proliferation). Both the platelets and the endothelial cells release sphingosine 1-phosphate, which is one of the lipid messengers formed by the sphingomyelin signalling pathway (Module 2: Figure sphingomyelin signalling).
6. Activation of macrophages. Macrophages persist for months or even years, positioned in a variety of organs where they function as permanent 'sentinels' ready to initiate an inflammatory response through two main mechanisms. Firstly, they can respond to signals coming from the pathogens by releasing a number of inflammatory mediators such as the chemokines. There also is the possibility that macrophages may have receptors capable of detecting uric acid generated from the metabolism of nucleic acids in dying cells. Secondly, they remove pathogens by engulfing them through a process of phagocytosis.

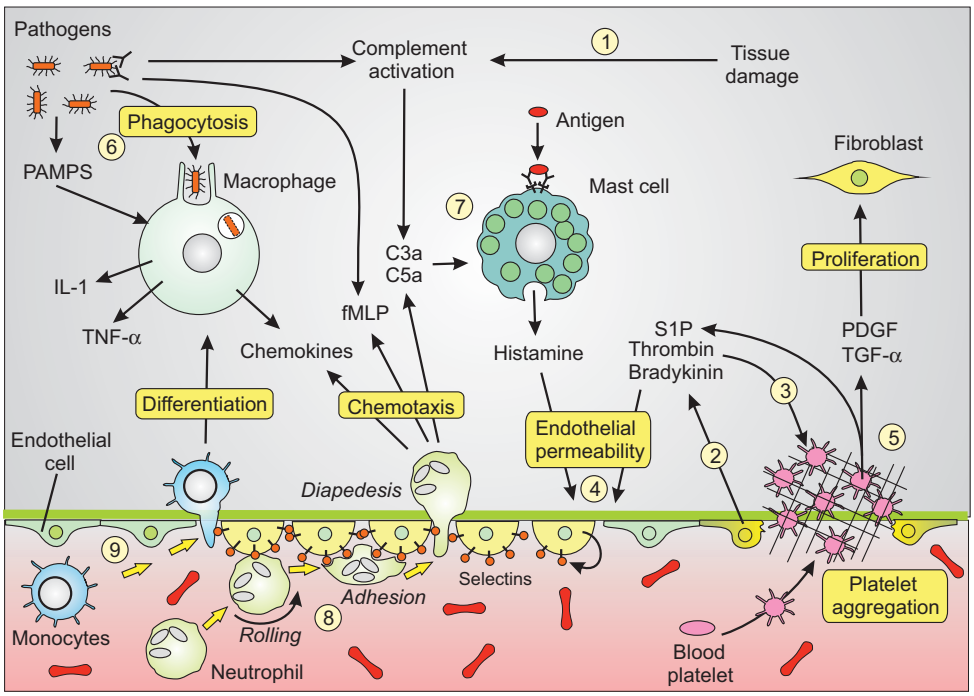
Pathogens initiate macrophage activation by shedding pathogen-associated molecular patterns (PAMPs) (Module 11: Figure formation and action of PAMPs). These PAMPs act through a number of different Toll-like receptors (TCRs) to stimulate the nuclear factor κB (NF-κB) signalling pathway (Module 2: Figure Toll receptor signalling). In the case of macrophages, the PAMPs help to regulate the transcriptional activation of a number of components that contribute to the inflammatory response, such as tumour necrosis factor α (TNFα), interleukin-1 (IL-1) and IL-6 (Module 11: Figure macrophage signalling). PAMPs have a similar action in mast cells (Module 11: Figure mast cell signalling). Pathogens that become coated with antibodies (IgG and IgM) activate the complement system to release complement factors, such as C3a and C5a, which function as chemoattractants for inflammatory cells such as the neutrophils. In addition, the coated pathogens are marked out for phagocytosis by the macrophages (Module 4: Figure phagosome maturation).

7. Activation of mast cells. Resident mast cells play an important role in the initiation of the inflammatory response (Module 11: Figure inflammation). Antigens cross-link the IgE bound to the FcεRIs to trigger many of the mast cell signalling mechanisms that release histamine and other inflammatory mediators (Module 11: Figure mast cell signalling).
8. Neutrophil recruitment and activation. Neutrophils have a relatively short half-life; they circulate in the blood for a few hours before migrating into the surrounding connective tissues, particularly at sites of inflammation, where they function for a few days only. There appears to be two main pathways used by neutrophils to cross the endothelial layer of cells. The conventional view is that they squeeze through the cell junctions. An alternative mechanism is for them to migrate through the neutrophil using a podosome to force a way through the cell. A process of neutrophil chemotaxis then draws these cells to sites of inflammation during which they follow gradients of chemokines, complement factors (C3a and C5a), fMet-Leu-Phe (fMLP) and hydrogen peroxide (H_2O_2) (Module 11: Figure inflammation). fMLP, which is a bacterial breakdown product, is a classical chemoattractant.
9. Monocyte differentiation. Monocytes follow a pathway similar to that which occurs for the neutrophils as they migrate through the endothelial layer to enter the interstitial space, where they differentiate into macrophages.

The inflammatory response is highly regulated and depends upon pro-inflammatory mechanisms that begin early (as described above), but are gradually counteracted by various anti-inflammatory pathways mediated by factors such as cytokines [interleukin-10 (IL-10)], hormones and neurotransmitters [acetylcholine, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP)].

Although the development of an inflammatory response is beneficial, there are instances where the response gets out of hand and begins to be deleterious, owing to an excessive production of inflammatory cytokines such as TNFα, IL-1β and IL-6 that result in oedema and tissue injury. Indeed, acute and chronic inflammation has been associated with a number of disease states, such as sepsis, rheumatoid arthritis, inflammatory bowel disease (which includes Crohn's disease and ulcerative colitis), respiratory distress syndrome, peritonitis and carditis. In the case of the brain, many neurodegenerative diseases may result

Module 11: | Figure inflammation



Summary of the inflammatory response to tissue damage and pathogens.

The innate immune system is triggered by signals derived from tissue damage and invading pathogens to activate cells such as the macrophages, neutrophils, mast cells, blood platelets and endothelial cells that contribute to a co-ordinated series of responses to both remove the pathogens and to repair damaged tissues. The endothelial cells are shown in three states: normal flattened shape (blue), contracted to increase endothelial permeability (light yellow) and damaged cells (bright yellow), where platelet aggregation occurs during formation of the haemostatic plug. Details of these responses are described in the text.

from activation of Toll-like receptor 4 (TLR4) on microglia cells to induce an inflammatory response (Module 7: Figure microglia interactions).

Inflammatory cytokines

There are a number of cytokines and related agents, such as the damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) that act to promote inflammation (Module 1: Figure cytokines). Two of the major cytokines are tumour necrosis factor-α (TNFα) and interleukin-1 (IL-1).

Tumour necrosis factor (TNF)

Tumour necrosis factor (TNF) comes in two forms: TNFα (also known as cachectin because it mediates fever and cachexia) and TNFβ (also known as lymphotoxin). For most purposes, these are considered together as TNF, which is a potent pro-inflammatory cytokine that is responsible for many detrimental effects such as bacterial sepsis, rheumatoid arthritis and Crohn's disease. The pro-form of TNFα is a transmembrane protein that is active in a juxtacrine mode, but also undergoes ectodomain shedding when it is cleaved by TNFα-cleaving enzyme (TACE) to produce soluble TNFα, which can function in both an autocrine and paracrine manner. TNF acts on the TNF receptor (TNF-R) to recruit different signalling pathways (Module 1: Figure cytokines):

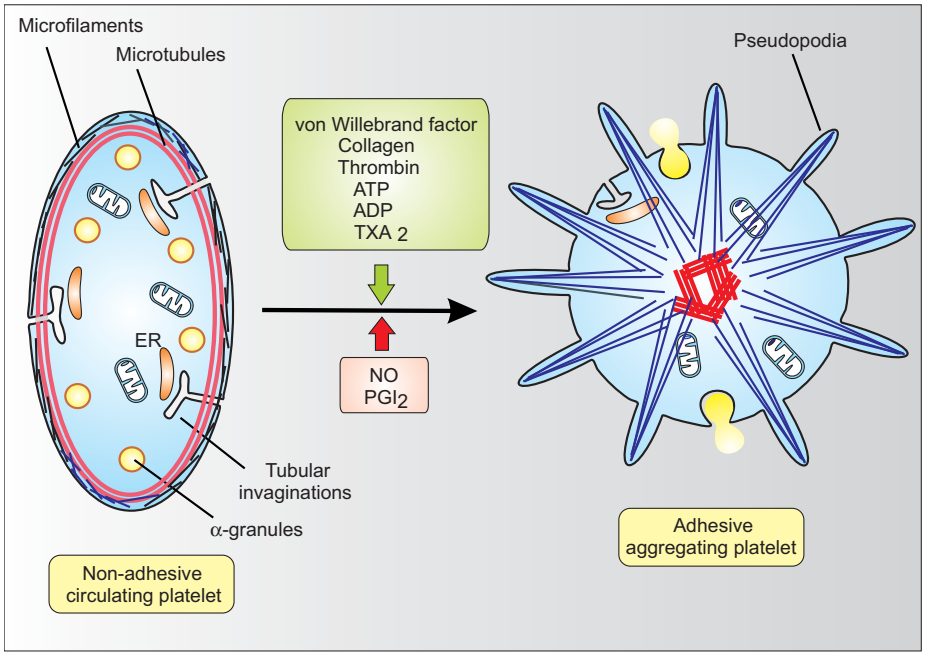
- TNFα activates the nuclear factor κB (NF-κB) signalling pathway (Module 2: Figure NF-κB activation).
- The TNF-R can activate caspase 8 to initiate the extrinsic pathway of apoptosis (Module 11: Figure apoptosis).
- TNFα activates the sphingomyelin signalling pathway (Module 2: sphingomyelin signalling).
- TNFα is released from folliculostellate (FS) cells in response to lipopolysaccharide (LPS)

The TNFα receptor is inactivated through a process of ectoderm shedding. Mutations in the cleavage site of the TNF receptor, which prevents its down-regulation by the ADAM enzyme responsible for its shedding, are the cause of TNF-receptor-associated periodic febrile syndrome (TRAPS).

Damage-associated molecular patterns (DAMPs)

The endogenous damage-associated molecular patterns (DAMPs) are produced by damaged cells and are often referred to as danger signals. Examples of such DAMPs are oxidized low-density lipoprotein (LDL), high-mobility group box 1 (HMGB1) and various adenine nucleotides. The HMGB1, which is also known as amphoterin, is located in the nucleus where it binds to DNA to remodel chromatin structure making it more amenable to transcription. The DAMPs generate inflammatory responses by acting through toll-like receptors (TLRs) that recruit various downstream signalling pathways, such as the

Module 11: | Figure platelet structure



Structure of non-adhesive and adhesive blood platelets.
The anucleate circulating platelet is a non-adhesive biconvex disc whose shape is maintained by a ring of microtubules. Aggregating agents induce a dramatic structural change as the disc transform into an adhesive sphere that aggregate with each other to form a thrombus. The microtubule ring breaks down and the peripheral actin microfilaments are remodelled to form long filaments located within the numerous pseudopodia. The α-granules fuse with the plasma membrane to release their contents as part of the activation process that forms a thrombus (Module 11: Figure thrombus formation)

Toll receptor signalling pathway (Module 2: Figure Toll receptor signalling) or the mitogen-activated protein kinase (MAPK) signalling pathway.

Wound healing

Detection of wounds and the subsequent healing process depends on an orchestrated sequence of cellular events that can be divided into four main phases:

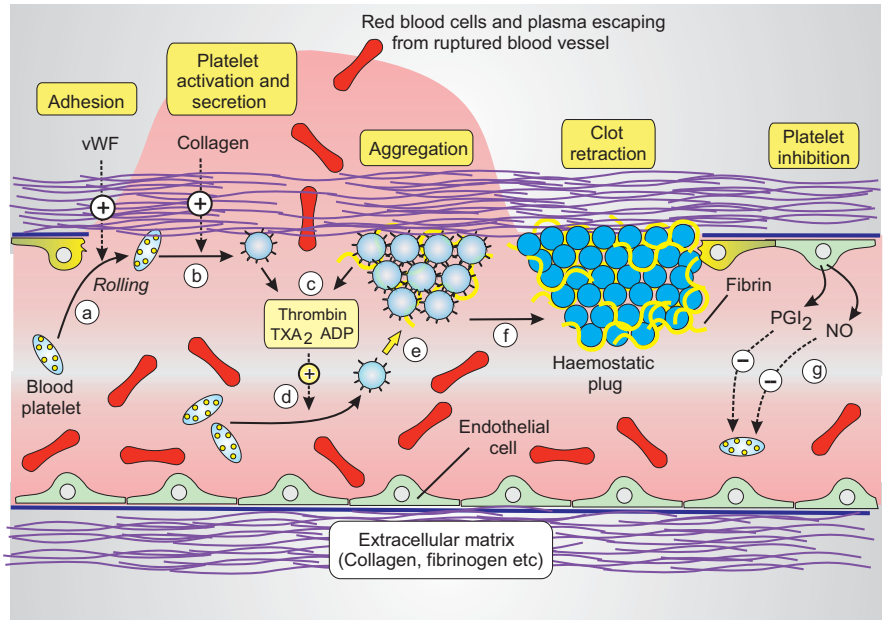
1. Haemostasis. One of the first processes is to stem the flow of blood that is carried out by the blood platelets that rapidly aggregate to form a clot (Module 11: Figure thrombus formation)
2. Inflammation. The presence of cell debris and bacteria in the vicinity of the wound triggers an inflammatory response (Module 11: Figure inflammation). An important feature of this response is the arrival of inflammatory cells, such as the neutrophils, that are drawn in by various attractants such as chemokines, complement factors (C3a and C5a), fMet-Leu-Phe (fMLP) and hydrogen peroxide (H_2O_2).
3. Proliferation. The repair and reconstruction of wound depends on the proliferation of various cell types. The fibroblasts begin to proliferate and secrete collagen. New blood vessels are formed by the process of angiogenesis (Module 9: Figure angiogenesis).
4. Remodelling. During the final remodelling phase, the wound gradually adapts to its more permanent function. Those repair cells that are no longer required are removed and the formation of collagen is refined so that the fibres are realigned along lines where tension forces are maximal.

Blood platelets

Blood platelets that circulate freely in the plasma play a primary role in the repair of damaged blood vessels during wound healing by aggregating to form a clot (thrombus) to prevent haemorrhage (Step 3 in Module 11: Figure inflammation). During thrombus formation, the platelets not only provide the building blocks to construct the haemostatic plug, but also contribute to the plasma coagulation sequence that leads to the formation of the fibrin that stabilizes this plug to stem the flow of blood.

Blood platelets circulate in the blood as small non-adhesive anucleate biconvex discs about 2.5 μm in length (Module 11: Figure platelet structure). The plasma membrane has numerous tubular invaginations, which often come to lie close to segments of endoplasmic reticulum (ER). A ring of microtubules (shown in red) lie in the equatorial plane, and it has been suggested that they function to maintain the disc shape of the circulating platelet. Immediately below the plasma membrane there is a layer of actin microfilaments (shown in black), which make up about 15% of total platelet protein. The cytoplasm has α-granules that store a number of aggregating agents. In response to a range of stimuli, the disc-shaped circulating platelet is transformed into a sphere that has

Module 11: | Figure thrombus formation



Platelet activation and thrombus formation.
When the endothelial cells are damaged, blood leaks out of blood vessel and the platelets are activated by components of the extracellular matrix such as von Willebrand factor (vWF) and collagen. The process of platelet activation follows a sequence of events (a-g) as described in the text.

numerous pseudopodia containing long actin filaments. The α-granules release their contents and this secretion is an important part of the platelet activation processes (Module 11: Figure thrombus formation).

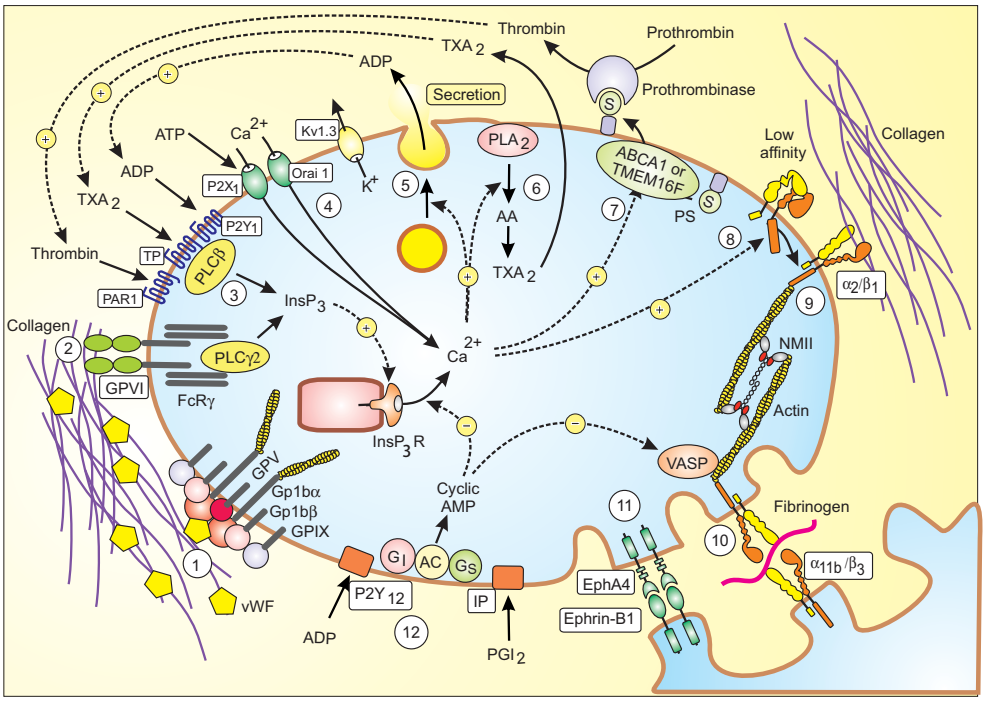
When a blood vessel is damaged, the endothelial cells that line the vessel wall no longer function as a barrier and blood begins to ooze through the extracellular matrix (ECM), and it is this contact between the blood and the ECM that triggers the platelet activation sequence (Steps a–g in Module 11: Figure thrombus formation):

a. One of the first interactions to occur is the adhesion of platelets to ECM components such as von Willebrand factor (vWF) that sits on the surface of the collagen fibres. Since the interaction between WF and the glycoprotein (GP) 1b–GPIX–GPV receptor complex on the surface of the platelet is not particularly strong and is easily made and broken, the platelet 'rolls' over the surface of the ECM.
b. As a result of this rolling over the ECM surface, the glycoprotein receptor (GPIV) on the platelet surface makes more meaningful contacts with glycogen to initiate the process of platelet activation and secretion. During this activation process, the platelets undergo a dramatic change in shape. The non-adhesive disc is transformed into a spherical shape with numerous pseudopodia and becomes highly adhesive, enabling the activated cells to aggregate.
c. In addition to the change in shape, the activated platelets begin to secrete the contents of their α-granules to release molecules such as ATP and ADP that play such an important role in the subsequent aggregation response. Presumably, the available collagen surfaces are rapidly encrusted with activated platelets, and additional aggregating stimuli are necessary to activate incoming platelets to build up the clot.
d. The release of ADP and the formation of other activators such as the eicosanoid thromboxane A_2 (TXA_2) and thrombin create a local environment that provides a positive-feedback loop whereby the initial platelets activated on the collagen surface are able to activate platelets as they flow into the region of the developing clot.
e. The incoming platelets activated by the soluble mediator (ADP, thrombin and TXA_2) are highly adhesive and rapidly stick to the developing clot. The development of the platelet aggregate is facilitated by both integrin signalling and by the ephrin (Eph) receptor signalling system. Fibrin fibres begin to appear around the aggregated platelets
f. Once the clot reaches a certain size, a clot retraction process occurs whereby a concerted contraction of the platelets somehow pulls the aggregated cells and fibrin closer together to form a water-tight seal (the haemostatic plug).
g. To ensure that the positive-feedback processes responsible for rapid platelet activation and clot formation do not get out of hand, the surrounding endothelial cells orchestrate a negative-feedback process by releasing prostaglandin I_2 (PGI_2) and nitric oxide (NO) to switch off further platelet activation.

This platelet activation sequence is controlled by a number of signalling pathways as illustrated in Module 11: Figure platelet activation:

1. When platelets come into contact with the extracellular matrix (ECM) at the sites of vascular injury, one of the first interactions to occur is for the GP1b–GPIX–GPV complex on the platelet surface to interact with vWF that sits on the surface of collagen. The GP1b–GPIX–GPV complex consists of four different GPs. There are two GPIbα subunits, two GPIbβ subunits, two GPIX subunits and a single GPV subunit. A disulphide bond links together the GPIbα and GPIbβ subunits. The GPIbα subunit seems to be particularly important because it has the binding sites for vWF and its cytoplasmic tail is connected to the cytoskeleton. The interaction between vWF and GP1b–GPIX–GPV is relatively weak and this enables the platelet to roll over the surface of collagen (see Step a in Module 11: Figure thrombus formation) and this presumably sets the stage for the next interaction. Bernard-Soulier syndrome has been linked to mutations in some of the components of the GP1b–GPIX–GPV complex.
2. One of the key steps in platelet activation sequence is the interaction between collagen and the collagen receptor GPVI (Module 11: Figure platelet activation). GPVI has two immunoglobulin-like domains, a transmembrane helix and a short cytoplasmic domain. The receptor function of GPVI depends upon a close association with the Fc receptor γ (FcRγ) chains, which have the immunoreceptor tyrosine-based activation motifs (ITAMs) that provide the docking sites to assemble the transducing mechanism. Signal transduction begins when the non-receptor protein tyrosine kinases Fyn and Lyn, which are bound to GPVI through their Src homology 3 (SH3) domains, phosphorylate the ITAMs on the FcRγ chains to provide binding sites for phospholipase Cγ2 (PLCγ2). The resulting activation of the inositol 1,4,5-trisphosphate ($InsP_3$)/Ca^{2+} signalling cassette (Module 2: Figure $InsP_3$ and DAG formation) appears to be a major control mechanism for platelet activation (see below).
3. Activation of $InsP_3$ and the release of Ca^{2+} are also used by a number of the other aggregating agents such as ADP, thrombin and TXA_2 which all act through G protein-coupled receptors (GPCRs) that activate phospholipase Cβ (PLCβ).
4. Platelets express the P2X1 isoform of the ionotropic P2X receptors (Module 3: Figure P2X receptor structure), that provide an influx of external Ca^{2+} that contributes to platelet activation (Module 11: Figure platelet activation). In addition, they express the Orai 1 channel that responds to store depletion by an increase in Ca^{2+} entry (Module 3: Figure SOC signalling components). The membrane depolarization induced by these Ca^{2+} channels is counteracted by the presence of Kv1.3 channels that hyperpolarize the membrane to maintain the driving force for Ca^{2+} entry.
5. One of the signalling functions of Ca^{2+} is to stimulate the release of the α-granules that release aggregating agents such as ADP, which thus provide positive-feedback processes by stimulating further Ca^{2+} release through Step 4 as described above.
6. Another important positive amplification step is the formation of TXA_2, which is produced by the Ca^{2+}-dependent activation of phospholipase A_2 (PLA_2). The resulting arachidonic acid (AA) is converted into TXA_2 (Module 1: Figure eicosanoids), which feeds back to activate the TP receptor. The AA is also converted into PGI_2, which builds upper towards then end of the aggregation process to activate an inhibitory pathway (Step 12 in Module 11: Figure platelet activation).
7. An increase in Ca^{2+} may contribute to phospholipid scrambling that result in phosphatidylserine (PS) transfer from the inner to outer plasma membrane leaflet, which then contributes to formation of the tenase and prothrombinase complexes responsible for generating thrombin. Scott syndrome may result from mutations in ABCA1, which is one of the ATP-binding cassette (ABC) transporters responsible for the transfer of PS. Another protein that has been implicated in phospholipid scrambling is the Ca^{2+}-sensitive transmembrane protein 16F (TMEM16F). A mutation of TMEM16F has been linked to Scott syndrome.
8. Platelets express two integrin receptor types, α_2/β_1 and α_{11b}/β_3, which can link to collagen and fibrinogen respectively (Module 1: Figure integrin heterodimeric complexes). The former interaction plays an important role in the initial attachment to collagen, whereas the α_{11b}/β_3 interaction with fibrinogen functions later to bind the aggregating platelets together (Module 11: Figure platelet activation). The collagen receptor GPVI plays an important role in activating the integrin receptors early in the activation process through the *inside-out* mechanism whereby intracellular signals coming from other receptors induce a conformational change in the integrin receptors that greatly enhance their affinity for external ligands (Module 1: Figure integrin receptor). In the case of platelets, the α_2/β_1 receptor binds collagen whereas the α_{11b}/β_3 receptor binds fibrinogen.
9. The high-affinity α_2/β_1 receptor is activated by collagen (Module 11: Figure platelet activation), which not only increase the platelet adhesiveness to the ECM, but also activates integrin signalling mechanisms that remodel the actin cytoskeleton that contributes to formation of the pseudopodia of the activated platelet (Module 11: Figure platelet structure
10. The activated α_{11b}/β_3 integrin binds to fibrinogen to help establish cell–cell interactions between the aggregating platelets (Module 11: Figure platelet activation). In addition, the activated integrin receptors can assemble a large signalling complex capable of both remodelling the actin cytoskeleton and inducing a number of signalling pathways (Module 6: Figure integrin signalling). The development of actin fibres stabilized by non-muscle myosin II (NMII) extend across the cell and function in clot retraction (Step f in Module 11: Figure thrombus formation). Glanzmann's thrombasthenia is caused by mutations in the β_3 integrin subunit.

Module 11: | Figure platelet activation



Platelet activation signalling pathways.

Platelets are sensitive to a large number of stimuli that act through a wide range of receptors. The numbers refer to the receptors and signalling pathways responsible for controlling the processes of thrombus formation, which is summarized in Module 11: Figure thrombus formation.

11. The aggregating platelets communicate with each other through the bidirectional ephrin (Eph) receptor signalling system (Module 1: Eph receptor signalling). This ephrin signalling system can generate a number of signals that appear to contribute to platelet activation. One of its actions may be to phosphorylate the β_3 integrin subunit to enhance the activity of the α_{11b}/β_3 integrin receptor (Module 11: Figure platelet activation).
12. The cyclic AMP signalling pathway has an important function in platelet aggregation by activating a negative-feedback process to ensure that the positive-feedback processes responsible for rapid platelet activation and clot formation do not get out of hand (Step g in Module 11: Figure thrombus formation). The inhibitor action of cyclic AMP is not clear, but it may act by inhibiting the Ca^{2+} signal (Module 11: Figure platelet activation). Another function of cyclic AMP is to activate the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), which is a member of the Ena/vasodilator-stimulated phosphoprotein (VASP) family (Module 4: Figure Ena/VASP family) resulting in a decrease in the actin-dependent processes associated with clotting. One of the potent activators of cyclic AMP formation is PGI_2 acting on its IP receptor (Module 1: Figure eicosanoids). ADP acting on the $P2Y_{12}$ receptor is coupled to the G protein $G\alpha i_2$, which acts to inhibit adenylyl cyclase (Module 2: Table heterotrimeric G proteins). ADP thus has two effects: it stimulates Ca^{2+} signalling through the $P2Y_1$ receptor (Step 3), while simultaneously lowering the level of the inhibitory cyclic AMP signal by acting on the $P2Y_{12}$ receptor.

Mast cells

Mast cells are widely distributed throughout the body, where they are positioned close to blood vessels and epithelial cell layers. They are particularly evident in locations that are exposed to the outside world, such as the lung, gastrointestinal tract and skin. Since they take up permanent residence in these locations, they are always on hand to respond to infections that most commonly occur during wounding. Basophils have a similar function to mast cells, except that they circulate in the blood and are recruited to sites of infection. Once they arrive and settle down, basophils begin to function much like mast cells.

Mast cells play a central role in the innate immune response and are particularly important in helping to recruit other cells, such as the basophils, neutrophils and lymphocytes (Module 11: Figure inflammation). In the case of skin infections, mast cells contribute to the mediators of the inflammatory soup that act on sensory neurons to cause pain (Module 10: Figure inflammatory soup). This role in inflammatory responses depends on the capability of the mast cells to release an enormous range of mediators (Module 11: Figure mast cell signalling). There are three main mast cell release mechanisms.

Mast cells have been implicated in a number of allergic diseases, such as asthma, and they also contribute to

chronic inflammatory conditions such as atherosclerosis, vasculitis and rheumatoid arthritis.

Mast cell release mechanisms

Mast cells release mediators through three main mechanisms (Module 11: Figure mast cell signalling):

- Mast cell granule release
- Mast cell release of lipid-derived mediators
- Mast cell synthesis and release of inflammatory cytokines and immunoregulators

Mast cell granule release

A characteristic feature of mast cells is the large number of secretory granules that fill the cytoplasm. These large vesicles contain many different mediators, including biogenic amines (histamine and 5-hydroxytryptamine), growth factors and enzymes. The earliest response to stimulation of mast cells is the fusion of these vesicles with the plasma membrane. Since the granules are so large, not all the granules have access to the membrane, so they can fuse with each other to produce profiles that resemble a bunch of grapes.

Mast cell release of lipid-derived mediators

Mast cells have the capacity to rapidly synthesize prostaglandins and leukotrienes from the arachidonic acid (AA) released following activation of phospholipase A_2 (PLA_2). The pathways for forming these eicosanoids are shown in Module 1: Figure eicosanoids.

Mast cell synthesis and release of inflammatory cytokines and immunoregulators

In addition to the rapid release of granules and formation of lipid-derived mediators, mast cells also have a slower, but more prolonged, release of a bewildering array of cytokines and immunoregulators, as shown in Module 11: Figure mast cell signalling. This slower release mechanism depends upon activating the genes responsible for coding all of these mediators.

These multiple secretory processes are controlled by a number of different mast cell signalling mechanisms.

Mast cell signalling mechanisms

Mast cells have a large number of signalling mechanisms to control the different mast cell release mechanisms (Module 11: Figure mast cell signalling). A number of different stimuli engage signalling pathways that can either activate or inhibit secretion.

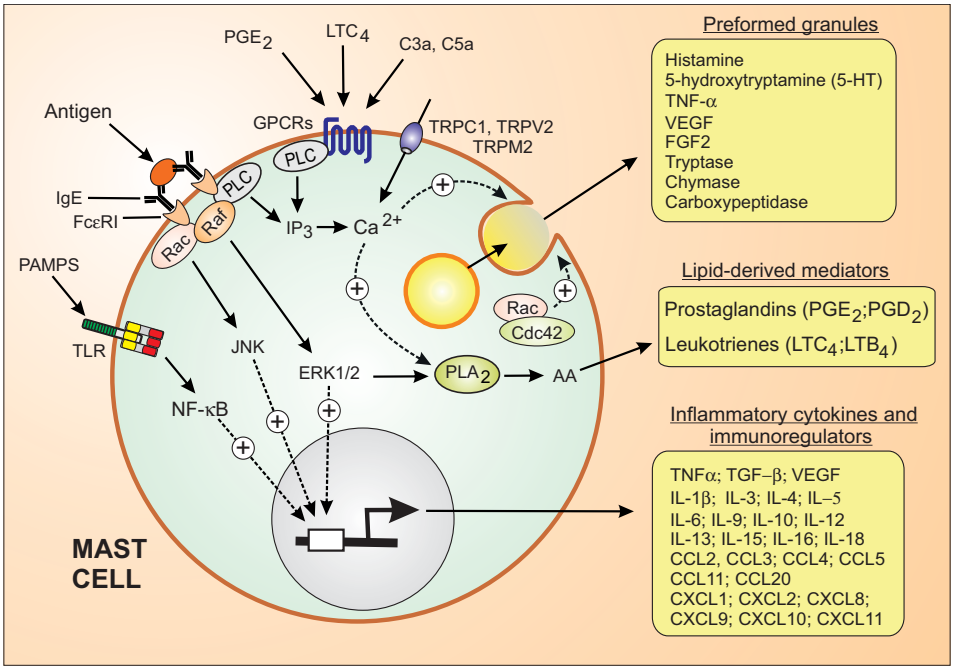
Mast cell FcεRI signalling pathway

The FcεRI is responsible for activating signalling pathways that control all three secretory processes (Module 11: Figure mast cell signalling). It acts through the phosphoinositide signalling pathway to produce inositol 1,4,5-trisphosphate ($InsP_3$), which mobilizes the Ca^{2+} that contributes to the release of secretory granules, which is very dependent on the monomeric G proteins Rac and Cdc42 (see below). This release of internal Ca^{2+} is augmented by various Ca^{2+} entry channels that belong to the transient receptor potential (TRP) ion channel family, such as canonical TRP 1 (TRPC1), vanilloid TRP 2 (TRPV2) and melastatin-related TRP 2 (TRPM2). The increase in Ca^{2+} also contributes to the activation of phospholipase A_2 (PLA_2), which release the precursor arachidonic acid that is converted into the prostaglandins and leukotrienes (Module 1: Figure eicosanoids). The FcεRI also relays information out through different mitogen-activated protein kinase (MAPK) signalling pathways to regulate all three secretory processes. It uses the extracellular-signal-regulated kinase (ERK) pathway to contribute to the activation of both PLA_2 and the transcriptional events responsible for the synthesis of inflammatory cytokines and immunoregulators. It also activates transcription through the c-Jun N-terminal kinase (JNK) pathway. The link between the receptor and JNK seems to depend on the monomeric G proteins Cdc42 and Rac. The latter also play a critical role in controlling granule release, which is unusual because it is very dependent on GTP. Rac and Cdc42 seem to function by preparing the granules for release, whereas Ca^{2+} functions more as a modulator of exocytosis.

The way in which the FcεRI is coupled to these different signalling pathways is shown in Steps 1–7 in Module 11: Figure FcεRI mast cell signalling:

1. The FcεRI consists of four subunits: an α subunit, a β subunit and two γ subunits that are connected together through a disulphide bridge. The α subunit has a very high affinity for IgE, which is permanently bound to the FcεRI complex. Signalling begins when a bivalent antigen binds to two IgE molecules to bring together two receptor complexes that then interact to initiate the process of signal transduction.
2. These FcεRI subunits lack enzyme activity, and thus have to recruit various transducing elements, such as the non-receptor tyrosine kinases Fyn, Lyn and Syk. Lyn plays an early role by phosphorylating immunoreceptor tyrosine-based activation motifs (ITAMs) (the red bars on the β and γ subunits). These ITAMs are the same as those used during signal transduction by the T cell receptor (TCR) (Module 9: Figure TCR signalling). Another similarity to T cell signalling is that the FcεRI also makes use of the scaffolding proteins LAT (linker of activated T cells) and Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76). The phosphorylated ITAMs on the FcεRI then recruit Syk, which is also activated by Lyn phosphorylation (Module 11: Figure FcεRI mast cell signalling).
3. Activated Syk has a number of actions. It phosphorylates the scaffolding proteins LAT and SLP-76 to provide binding sites for a number of signalling components. It also phosphorylates phospholipase Cγ (PLCγ1) and Bruton's tyrosine kinase (Btk), which interact with each other during the activation of PLCγ1.
4. The activated PLCγ1 hydrolyses $PtdIns4,5P_2$ to form both $InsP_3$ and diacylglycerol (DAG).
5. Phosphorylation of LAT recruits growth factor receptor-bound protein 2 (Grb2), which sets up a nucleation

Module 11: | Figure mast cell signalling



The function and mechanisms of mast cell signalling.

Mast cells release an enormous number of components that fall into three separate groups that are controlled by different signalling mechanisms. The preformed granules are released quickly through a typical Ca^{2+}-dependent exocytotic mechanism. The lipid-dependent mediators that are produced from the arachidonic acid (AA) following stimulation of phospholipase A_2 (PLA_2). Finally, a whole range of inflammatory cytokines and immunoregulators are produced and released as a result of increases in gene transcription. The signalling mechanism that controls these release processes is described in the text. This figure represents only those signalling pathways that activate release processes. The signalling pathways that inhibit release are described in Module 11: Figure mast cell inhibitory signalling.

centre to assemble the signalling components that result in the formation of phosphorylated ERK1/2, which activates both PLA_2 and gene transcription.

6. Phosphorylated LAT recruits SLP-76 and Grb2-related adaptor protein (GADS) to set up a signalling complex that activates monomeric G proteins such as Vav and Rac that stimulate JNK, and they also function to stimulate granule release.
7. The FcεRI can also stimulate the PtdIns 3-kinase (PI 3-K) signalling pathway. The Fyn that is recruited to the activated receptor phosphorylates Gab2 and Btk, which contribute to the activation of PI 3-K, which then phosphorylates PtdIns4,5P_2 to form the lipid messenger PtdIns3,4,5P_3.

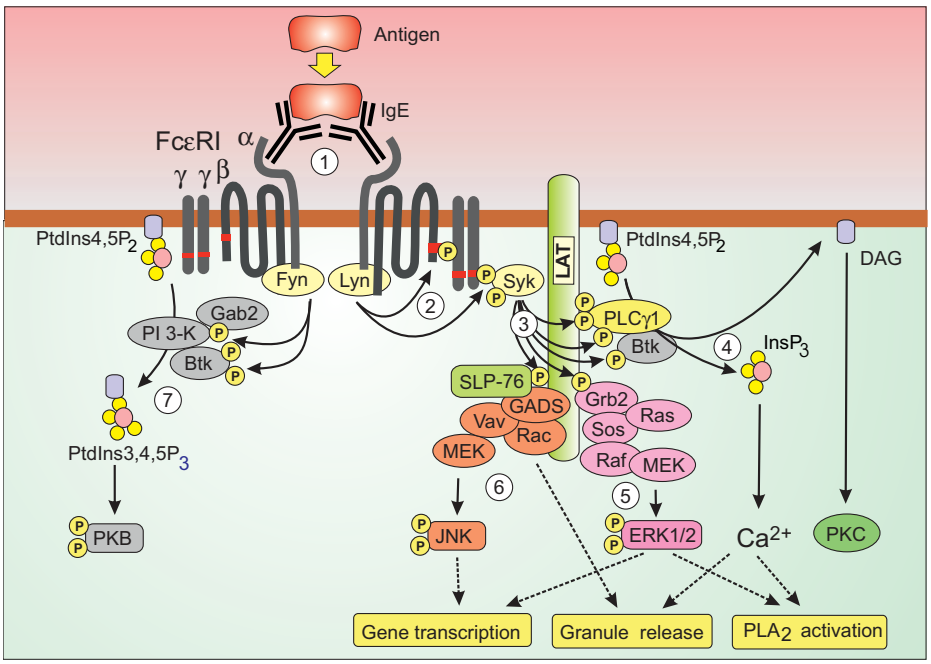
Mast cell GPCR signalling pathways

Mast cells express a number of G protein-coupled receptors (GPCRs) that function to either stimulate or inhibit mast cell secretory functions. The stimulatory actions of the GPCRs are shown in Module 11: Figure mast cell signalling. For example, stimuli such as PGE_2, leukotriene C_4 (LTC_4) and the complement factors C3a and C5a act through these GPCRs, which are coupled to the G_q phospholipase Cβ (PLCβ) signalling pathway that generates inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). The $InsP_3$ mobilizes Ca^{2+}, which contributes to granule release and also functions to stimulate phospholipase A_2 (PLA_2). On the other hand, noradrenaline (norepinephrine) stimulates β_2-adrenoceptors that act through G_s and adenylyl cyclase (AC) to generate cyclic AMP, which inhibits some of these secretory pathways (Steps 5 and 6 in Module 11: Figure mast cell inhibitory signalling). The mode of action of cyclic AMP is not clear, but there are indications that it may act to inhibit certain aspects of the Ca^{2+} signalling system.

Mast cell Toll receptor signalling pathway

Mast cells are sensitive to many of the fragments coming from various pathogens that are known as pathogen-associated molecular patterns (PAMPs) (Module 11: Figure formation and action of PAMPs). These PAMPs act through the Toll receptor signalling pathway that engages the nuclear factor κB (NF-κB) signalling pathway to stimulate gene transcription (Module 2: Figure Toll receptor signalling). Mast cells are somewhat unusual in that they do not express the CD14 co-receptor, but they appear to use a soluble CD14 present in plasma to facilitate the transfer of PAMPs on to the Toll-like receptors (TLRs). The PAMPs such as lipopolysaccharide (LPS) can activate transcription independently of the other cellular processes, such as the release of granules or the lipid-derived messengers.

Module 11: | Figure FcεRI mast cell signalling



The mast cell FcεRI complex relays information to a number of signalling pathways.
The FcεRI complex is composed of α, β and γ subunits that lack enzymatic activity. Signal transduction is carried out by non-receptor tyrosine kinases (Fyn, Lyn and Syk) that phosphorylate different elements that then recruit the signalling components of a number of pathways to control phospholipase A_2 (PLA_2) activation, granule release and gene transcription, as described in the text.

Mast cell FcγRIII signalling pathway

The FcγRIII receptor on mast cells exerts an inhibitory action on FcεRI through Steps 1–4 in Module 11: Figure mast cell inhibitory signalling:

1. FcγRIII can bind IgE and is thus drawn into the receptor complex containing FcεRI, where it begins to exert its inhibitory action.
2. The Lyn attached to FcεRI phosphorylates immunoreceptor tyrosine-based inhibitory motifs (ITIMs; purple region on FcγRIII). These phosphorylated residues then provide binding sites for various negative regulators.
3. The Src homology 2 (SH2) domain-containing inositol 5-phosphatase (SHIP), which is one of the inositol polyphosphate 5-phosphatases, dephosphorylates the lipid second messenger $PtdIns3,4,5P_3$.
4. The Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1) acts by reversing the phosphorylations responsible for activating Syk, which relays information to a number of signalling pathways (Module 11: Figure FcεRI mast cell signalling).

Macrophages

There are two distinct macrophage types: M1 macrophages sometimes known as 'killers' and the M2 'healer' macrophages.

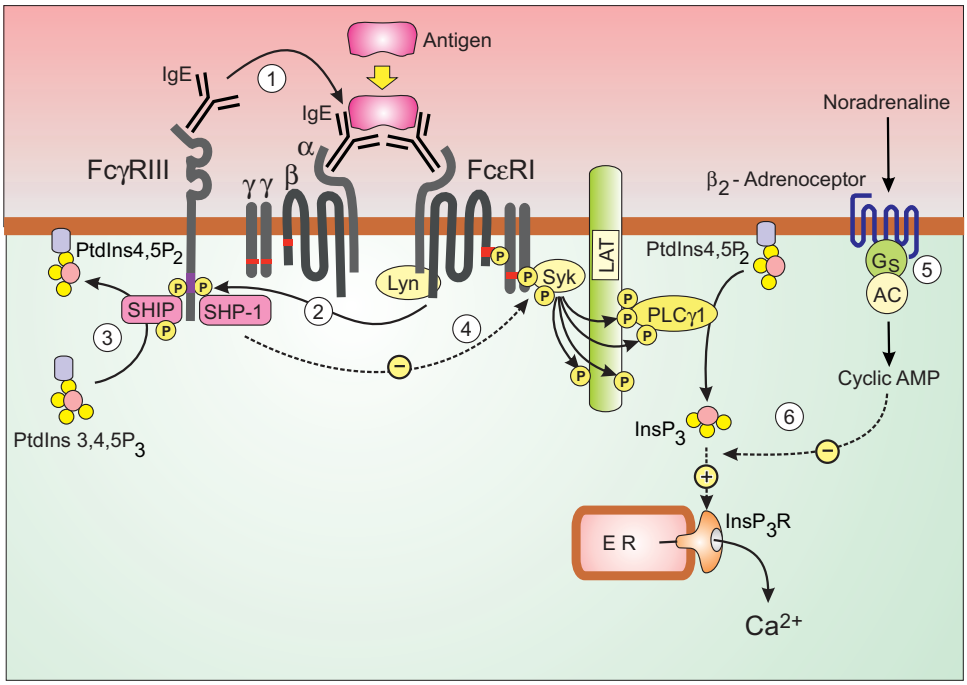
The M1 macrophages function to increase inflammatory responses and can kill pathogens. They respond to interferon-γ (IFNγ) and to pathogen-associated molecular patterns (PAMPs) such as LPS to initiate inflammatory responses by acting through the Toll receptor signalling pathway (Module 2: Figure Toll receptor signalling) to release inflammatory cytokines, chemokines and immunoregulators (Module 11: Figure inflammation). The M1 macrophages have high levels of iNOS to generate nitric oxide (NO) to combat bacterial and viral infections and to destroy tumour cells.

M2 macrophages ('healers'), ingest dead and damaged host cells and micro-organisms such as bacteria and protozoa through a process of phagocytosis, are activated by interleukin-4 (IL-4) and interleukin-13 (IL-13). They also play a role in enhancing collagen synthesis by releasing transforming growth factor β (TGF-β).

Pathogen-associated molecular patterns (PAMPs)

The pathogen-associated molecular patterns (PAMPs) are specific components of invading pathogens that are used to stimulate resident macrophages to initiate an inflammatory response (Module 11: Figure formation and action of PAMPs). Many of the PAMPs are derived from the surface coat of the pathogens, whereas others are unique nucleic acid sequences. For example, viruses do not contain many PAMPs because both the viral coat protein and lipids are derived from the host. However, they do provide double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) fragments that can be detected by the virus recognition and antiviral response system (Module 2: Figure virus recognition).

Module 11: | Figure mast cell inhibitory signalling



Inhibition of mast cell release by FcγRIII and β_2-adrenoceptors.
Release of inflammatory mediators by mast cells is inhibited by FcγRIII and β_2-adrenoceptors. The former bind IgE and are drawn into the FcεRI complex, where they are phosphorylated by Lyn and this enables them to bind the phosphatases Src homology 2 (SH2) domain-containing inositol 5-phosphatase (SHIP) and SH2 domain-containing protein tyrosine phosphatase-1 (SHP-1) that can dephosphorylate the lipid messenger PtdIns3,4,5P_3 and phosphorylated proteins respectively. The β_2-adrenoceptors produce cyclic AMP that appears to inhibit the Ca^{2+} signalling pathway.

One of the most active ingredients of the PAMPs is lipopolysaccharide (LPS), which is composed of lipid A derived from the external membrane of Gram-negative bacteria, and lipoteichoic acid from Gram-positive bacteria. At the sight of infection, there will therefore be a complex pattern of pathogen-derived molecules, which is then interpreted by a corresponding pattern of TLRs (Module 11: Figure formation and action of PAMPs). These TLRs then recruit various downstream signalling pathways, such as the Toll receptor signalling pathway (Module 2: Figure Toll receptor signalling) or the mitogen-activated protein kinase (MAPK) signalling pathway, to generate an inflammatory response to match the kind of pathogens that are invading the organism. Modulation of inflammatory responses is carried out by a number of inflammatory regulators that exert both pro- and anti-inflammatory responses.

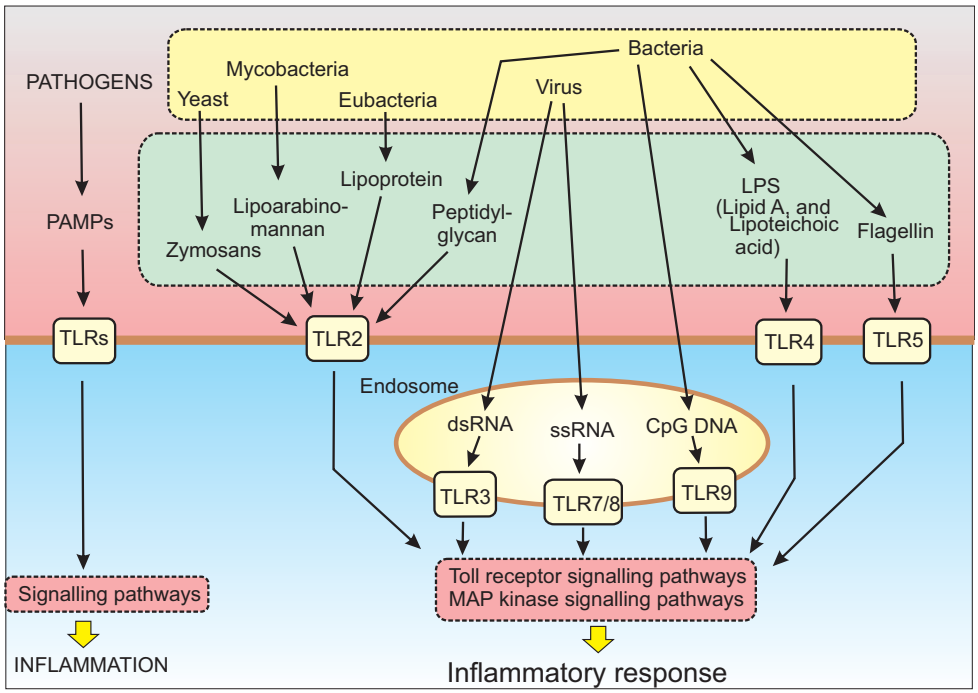
Modulation of inflammatory responses

The Toll receptor signalling pathway (Module 2: Figure Toll receptor signalling) responds to PAMPs by activating both nuclear factor κB (NF-κB) signalling and the p38 pathway to induce the transcription of a large number of inflammatory mediators (Module 11: Figure macrophage signalling). Some of these gene products, such as cyclooxygenase 2 (COX-2), act to increase the formation of eicosanoids (Module 1: Figure eicosanoids). The COX-2 increases the conversion of arachidonic acid (AA) into prostaglandin E_2 (PGE_2), which sets up a negative-feedback loop because PGE_2 acts through cyclic AMP to inhibit transcription (see below).

The ability of the Toll receptor signalling pathway to elicit an inflammatory response can be modulated by a number of regulatory mechanisms, which are either pro- or anti-inflammatory (Module 11: Figure macrophage signalling). Regulation of the macrophage response is highly dynamic in that there are processes that act to speed up the onset of the response, which are then counteracted by an anti-inflammatory response that comes into play to ensure that the response does not get out of hand. Some of these positive- and negative-feedback responses are endogenous (i.e. they occur within the macrophage), whereas others are imposed from the outside. Understanding this dynamic balance is of considerable interest with regard to pathology in that they reveal novel strategies for the discovery of anti-inflammatory drugs.

The cyclic AMP signalling pathway is particularly important in carrying out some of the anti-inflammatory responses. The macrophage responds to a number of agonists, such as noradrenaline, vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating polypeptide (PACAP) and PGE_2, which are coupled to the formation of cyclic AMP. The latter acts through protein kinase A (PKA) to not only inhibit mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) kinase kinase 1 (MEKK1), thereby reducing the

Module 11: | Figure formation and action of PAMPs



The role of pathogen-associated molecular patterns (PAMPs) in triggering the inflammatory response.
Unique components derived from different pathogens make up the pathogen-associated molecular patterns (PAMPs) that activate the Toll-like receptors (TLRs) located on macrophages and some other cell types. These TLRs are located either on the plasma membrane or on the endosomal membranes. The TLRs, such as TLR4, act through the Toll receptor signalling pathway (Module 2: Figure Toll receptor signalling). The TLRs on endosomal membranes function in virus recognition (Module 2: Figure virus recognition). CpG DNA, cytidine-phosphate-guanosine DNA; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; MAP kinase, mitogen-activated protein kinase.

phosphorylation of the basal transcription factor TATA-box-binding protein (TBP), but it also phosphorylates and activates cyclic AMP response element-binding protein (CREB), which enters the nucleus to compete with NF-κB for a binding site on CREB-binding protein (CBP). In this way, cyclic AMP inhibits the formation of inflammatory mediators.

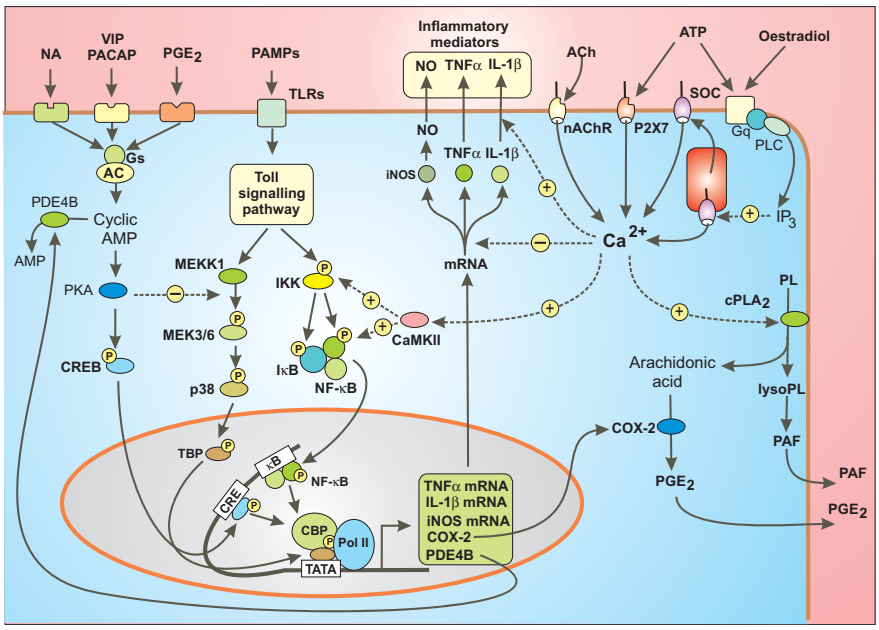
The inhibitory action of cyclic AMP is terminated by phosphodiesterase PDE4B, which is one of the genes activated by the TLRs, which thus sets up an internal positive-feedback loop. By increasing the expression level of PDE4B, the macrophage will reduce the inhibitory effect of cyclic AMP, thereby enhancing the inflammatory response (Module 11: Figure macrophage signalling).

An increase in the level of Ca^{2+} brought about by a number of receptor mechanisms has both pro- and anti-inflammatory effects. These different actions of Ca^{2+} may depend on the way the Ca^{2+} signal is presented both in time and space. These Ca^{2+} signals are generated either by activation of receptor-operated channels (ROCs) such as the nicotinic acetylcholine receptors (nAChRs) or the purinergic P2X7 receptor. In addition, Ca^{2+} is released from internal stores by inositol 1,4,5-trisphosphate ($InsP_3$) generated by G protein-coupled receptors (GPCRs) activated by ATP or oestradiol. One of its anti-inflammatory responses depends upon inhibition of the synthesis of inflammatory mediators. On the other hand, Ca^{2+} can also be pro-inflammatory by promoting gene transcription by stimulating the phosphorylation of inhibitor of NF-κB (IκB) kinase (IKK) and NF-κB, which enhances the release of inflammatory mediators such as interleukin 1β (IL-1β).

Neutrophils

Neutrophils are one of the major cell types contributing to innate immunity, which is the first line of defence against invading pathogens. During an inflammatory response, the neutrophils move out of the blood and migrate towards the site of infection (see Step 8 in Module 11: Figure inflammation). Neutrophils are guided towards these inflammatory sites by responding to gradients of chemokines, complement factors (C3a and C5a) or fMet-Leu-Phe (fMLP). They sense these gradients while still in the blood vessel, and start their journey through a typical response that begins with them attaching to the surface of the activated endothelial cells. The neutrophils then begin to roll along the surface by interacting with molecules of P-selectin, which is packaged within Weibel–Palade bodies and released on to the endothelial cell surface following activation by histamine (i.e. during Step 4). As the attachment to the selectins strengthens, the increase in adhesion causes the neutrophils to flatten out in preparation for the processes of diapedesis, during which they squeeze their way through the gaps that have appeared between the endothelial cells. When the neutrophils have passed through

Module 11: | Figure macrophage signalling



Modulation of inflammatory responses.
Pathogen-associated molecular patterns (PAMPs) acting through Toll-like receptors (TLRs) use the Toll receptor signalling pathway to activate the transcriptional processes that result in the release of inflammatory mediators such as nitric oxide (NO), tumour necrosis factor α (TNFα) and interleukin 1β (IL-1β). These inflammatory signalling pathways can be modulated by both the cyclic AMP and Ca^{2+} signalling pathways, as described in the text. PAF, platelet-activating factor; (lyso)-PL, (lyso)-phospholipid.

the endothelium, a process of neutrophil chemotaxis draws them towards the sites of inflammation.

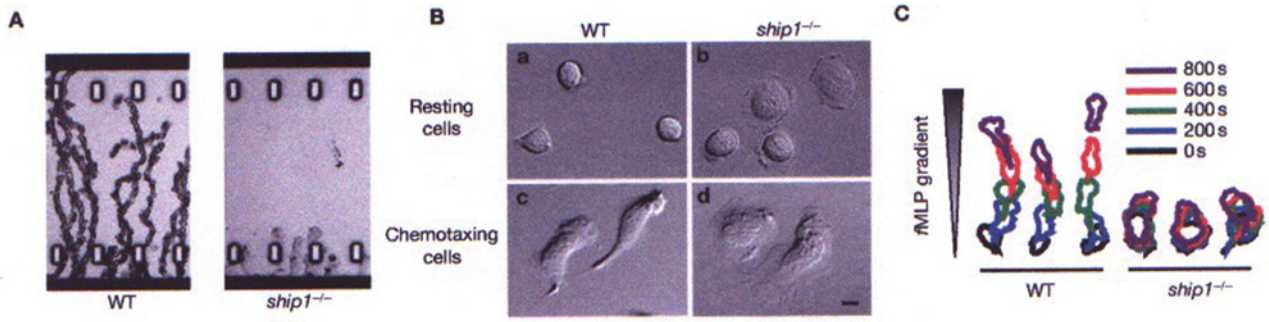
Neutrophil chemotaxis

Resting neutrophils are spherical, but once they detect a chemoattractant, they rapidly develop a polarity characterized by an elongated shape with a pseudopod at the front and a bulbous uropod at the rear (Module 11: Figure neutrophil chemotaxis). These two regions have a characteristic organization of actin filaments. At the front, there is an actin network that helps to push out the pseudopod, whereas the uropod has an actin/myosin II network that functions to contract and pull up the rear end as the cell moves forward. There also is a microtubule organizing centre (MTOC) that aligns the microtubules in the direction of movement and helps to stabilize cell polarity. The microtubules may also be important to directing the flow of vesicles that enter the cell by endocytosis at the rear of the cell and are released at the front end. Another important aspect of motility, which concerns adhesion to the matrix, is apparent when cells are viewed from the side. In order for cells to move over a matrix, they have to strike a balance between motility and adhesion. They require a certain level of adhesiveness to provide the traction to move forward. This delicate adhesion/motility balance seems to depend on the integrins that attach to the surface at the front and detach at the back. The flow of vesicles mentioned earlier may provide a mechanism for moving integrin receptors from the back to the front.

The process of chemotaxis enables neutrophils to seek out the sites of inflammation by moving up a chemoattract gradient. This directed movement is clearly evident when the position of neutrophils are recorded at 30 s intervals as they move up a chemoattractant gradient in an EZ-Taxiscan chamber (see panel A in Module 11: Figure SHIP1 and PIP_3 polarity). A number of external stimuli function as chemoattractants (Module 11: Figure neutrophil chemotaxis). One of the main attractants is the tripeptide fMet-Leu-Phe (fMLP), which can attract neutrophils even when applied in a very shallow gradient such that the front of the cell experiences a concentration that is just 1–2% higher than that experienced at the back end. Since the receptors for fMLP are distributed equally over the surface, the cell has to detect this small difference in concentration to develop a polarity that enables them to move towards the source of the gradient. The chemoattractant gradient will result in more receptors being occupied at the front compared with the back, and this small difference in occupancy is somehow translated into a polarized cell capable of migrating in a directed way towards the source of the gradient. In effect, the difference in receptor occupancy is transduced into an internal 'compass' that is then used to direct the motile machinery to move in one direction. Just how this compass is set up is still somewhat of a mystery.

In addition to following a pre-existing chemoattractant gradient such as fMLP, neutrophils also create a localized gradient of ATP and its breakdown product adenosine that are concentrated at the front of the cell (yellow halo in Module 11: Figure neutrophil chemotaxis). Hemichannels in the region of the pseudopod release ATP, some of which is converted into adenosine. ATP and adenosine then feedback, in an autocrine manner, to activate receptors at the front thereby contributing to chemotaxis by amplifying the early polarity signalling processes.

Module 11: | Figure SHIP1 and PIP_3 polarity



Src homology 2 (SH2) domain-containing inositol 5-phosphatase 1 (SHIP1) controls neutrophil polarity and motility.

A. Wild-type (WT) and Src homology 2 (SH2) domain-containing inositol 5-phosphatase 1 (SHIP1) $^{-/-}$ neutrophils were placed in an EZ-chamber and their positions were monitored at 30 s intervals as they responded to a gradient of fMet-Leu-Phe (fMLP). The WT cells moved in a relatively straight line up the gradient, whereas the SHIP1 $^{-/-}$ cells moved more slowly. B. When viewed at higher magnification, the WT cells were rounded, but assumed an elongated shape with a pseudopod at one end when placed in a fMLP gradient. By contrast, the SHIP1 $^{-/-}$ neutrophils remained rounded with pseudopodia appearing all round the cell. If recorded over a longer period, these cells did develop a partial polarity and there was some motion up the gradient (see panel C). Reproduced by permission from Macmillan Publishers Ltd: Nishio, M., Watanabe, K., Sasaki, J., Taya, C., Takasuga, S., Iizuka, R., Balla, T., Yamazaki, M., Watanabe, H., Itoh, R., Kuroda, S., Horie, Y., Forster, I., Mak, T.W., Yonekawa, H., Penninger, J.M., Kanaho, Y., Suzuki, A. and Sasaki, T. (2007) Control of cell polarity and motility by the PtdIns(3,4,5)P_3 phosphatase SHIP1. Nat. Cell Biol. 9:36-44. Copyright (2007); http://www.nature.com/ncb; see Nishio et al. 2007.

The signalling pathways activated during the onset of chemotaxis have been divided into the following sequence of events (Module 11: Figure neutrophil chemotactic signalling):

- Early gradient-sensing mechanisms (setting the compass)
- Amplification of early polarity signalling
- Actin assembly, pseudopod formation and uropod contraction

Early gradient-sensing mechanisms (setting the compass)

One of the most impressive aspects of chemotaxis is the way the various signalling components are polarized in order to direct different motile processes such as actin assembly at the front and actin/myosin II assembly and contraction at the back of the cell. In Module 11: Figure neutrophil chemotactic signalling, the major signalling components have been positioned as near as possible to their functional locations within the cell. An expression of the polarity of signalling components is the location of the Rho family (Cdc42, Rac and Rho) of monomeric G proteins. Cdc42 and Rac are active at the front, whereas Rho functions at the back. Just how this differential activation of G proteins is set up may hold the key to understanding the gradient sensing mechanism that establishes the compass. One hypothesis considers that a G protein signalling and chemotactic orientation mechanism provides the compass. The proposed polarized function of these G proteins is illustrated within the yellow arrow in Module 11: Figure neutrophil chemotactic signalling). Another suggestion is that a relationship between Ca^{2+} signalling microdomains and chemotactic orientation is the basis for the compass. Migrating neutrophils have unusual Ca^{2+} signals. In addition to a standing gradient of Ca^{2+}, which is high at the rear and low at the front (see the red shading of the cells in Module 11: Figure neutrophil chemotaxis), there also are localized Ca^{2+} flickers that are restricted to the front of the cell. The latter may play a role in cell orientation.

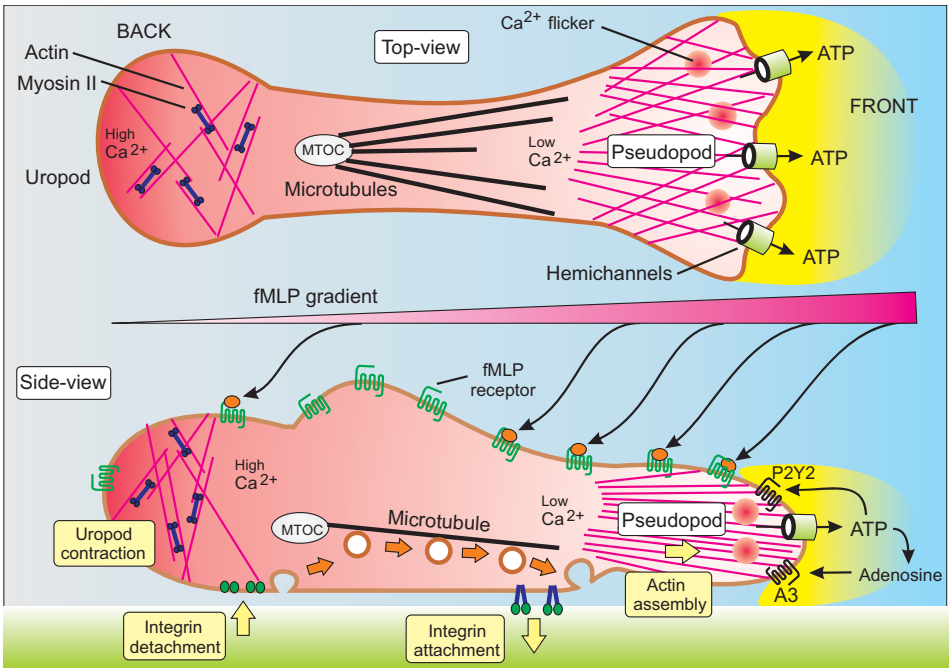
These two mechanisms, G protein or Ca^{2+} signalling, are not mutually exclusive and they may interact with each other to provide a robust orientation system to guide cells as they migrate along a chemotactic gradient.

G protein signalling and chemotactic orientation mechanism

The fMet-Leu-Phe (fMLP) receptor, which sets up the compass, is a typical G protein-coupled receptor (GPCR) that acts through heterotrimeric G proteins (Module 2: Figure heterotrimeric G protein signalling). Another expression of the signalling polarity that develops during chemotaxis is the fact that fMLP receptors at the front are coupled to G_i, whereas those at the back operate through $G_{12/13}$ (Module 11: Figure neutrophil chemotactic signalling). Just why fMLP receptors at the front couple to G_i, whereas those at the back select $G_{12/13}$ is a mystery and is clearly something that must be closely linked to setting up the compass because so many other events follow from the differential activation of the different G proteins at either end of the cell. Signalling events at the front will be considered first before turning to what happens at the back end.

When fMLP binds to its receptor, it dissociates the G protein into its two components $G\alpha_i$ and $G\beta\gamma$. The function of the former is unclear, but the $G_{\beta\gamma}$ subunit seems to play a critical role in chemotaxis where it has two actions: it can activate the guanine nucleotide exchange factors (GEFs) that switch on the monomeric G proteins and it can also activate PtdIns 3-kinase (Module 2: Figure heterotrimeric G protein signalling). The Cdc42 signalling mechanism (Module 2: Figure Cdc42 signalling) and the Rac signalling mechanism (Module 2: Figure Rac signalling) are particularly active at the front of the cell. For example,

Module 11: | Figure neutrophil chemotaxis



Structural organization of neutrophil motility and chemotaxis.
The top view shows a neutrophil moving up a gradient of a chemoattractant such as fMet-Leu-Phe (fMLP). The exploratory pseudopod at the front has an extensive actin meshwork. The actin and myosin II located at the rear enables the uropod to contract to propel the cell forwards. The microtubule-organizing centre (MTOC) aligns the microtubules in the direction of movement and helps to stabilize cell polarity. The side view illustrates how polarity is established by fMLP acting on receptors to promote actin assembly and pseudopod formation at the front and contraction of the uropod at the rear. During movement, integrins attach at the front and detach at the rear. The yellow halo at the front represents a local concentration of ATP released from the cell through hemichannels. The ATP and its hydrolytic product adenosine feedback in an autocrine manner to activate P2Y2 and A_3 purinergic receptors that function to amplify the chemotactic response. Information on the standing Ca^{2+} gradient (see the red shading showing the high level at the back and the low level at the front) and the Ca^{2+} flickers was taken from Wei et al. (2009).

this Gβγ-dependent activation of Cdc42 GEFs such as α-Pix (Module 2: Table monomeric G protein toolkit) convert inactive Cdc42.GDP into active Cdc42.GTP, may be an essential part of the compass. A similar mechanism is responsible for producing active Rac.GTP, which drives actin assembly, as described later.

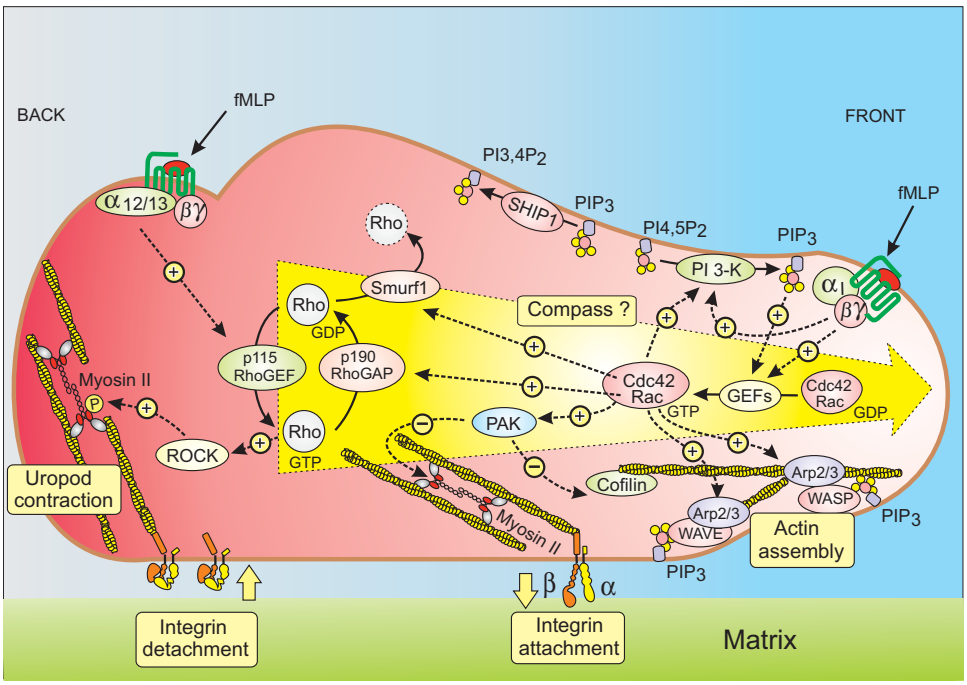
Different signalling mechanisms operate at the back of the cell to activate the monomeric G protein Rho (Module 11: Figure neutrophil chemotactic signalling). The fMLP receptors dissociate the heterotrimeric $G_{12/13}$ into the two components $G\alpha_{12/13}$ and Gβγ. The $G\alpha_{12/13}$ then activates the p115-RhoGEF (Module 2: Table monomeric G protein toolkit) to convert inactive Rho.GDP into active Rho.GTP. The fact that Rho activity is confined to the back may depend not only on its activation through $G\alpha_{12/13}$ at the back, but also on the fact that it is activity seems to be switched off by Cdc42 and Rac at the front. A number of mechanisms have been proposed for this inhibition of Rho. For example, Rac may inhibit Rho by stimulating the p190-RhoGAP that inactivates Rho.GTP by promoting the hydrolysis of GTP to form the inactive Rho.GDP. On the other hand, the degradation of Rho in the front of the cell may be facilitated by Cdc42. The Cdc42 located in the front of the cell interacts with the E3 ubiquitin ligase Smad ubiquitin-regulatory factor 1 (Smurf1), which then promotes ubiquitination and degradation of Rho in the front of the cell thus leaving high levels of Rho at the back. Smurf1 is a component of the Smad signalling toolkit (Module 2: Table Smad signalling toolkit)

Although the polarization of early signalling events leading to Cdc42 and Rac activation at the front and Rho at the back appear to be enough to initiate the gradient sensing mechanism, they are not sufficient to drive the large-scale morphological changes necessary to drive cell motility. The next step appears to be an amplification of early polarity signalling.

Ca^{2+} signalling microdomains and chemotactic orientation

Neutrophils may use Ca^{2+} signalling as an orientation mechanism. There are two components to neutrophil Ca^{2+} signalling. First, migrating neutrophils have a standing gradient of Ca^{2+} with low concentrations at the leading end and higher concentrations at the rear end (Module 11: Figure neutrophil chemotaxis). The latter may play a role in driving the cytoskeletal events that occur at the back to trigger uropod contraction to drive the cell forwards in the direction of the gradient (Module 11: Figure neutrophil chemotactic signalling). Secondly, there are brief localized pulses of Ca^{2+} called flickers that are another example of an elementary Ca^{2+} event. These localized Ca^{2+} flickers depend on Ca^{2+} entering the cell through TRPM7 channels, which are thought to be stretch-activated channels that open when the membrane is deformed as occurs at

Module 11: | Figure neutrophil chemotactic signalling



Neutrophil chemotactic signalling mechanisms.
Chemotaxis of neutrophils in response to a gradient of a chemoattractant such as fMet-Leu-Phe (fMLP) that elicits different signalling mechanism at the front and back. The large yellow arrow encloses those signalling components that may function as the compass responsible for setting up the polarity that drives directed cell movement. At the front, fMLP acts through guanine nucleotide exchange factors (GEFs) to activate both Cdc42 and Rac. The activated Cdc42 and Rac initiates an amplification loop by stimulating phosphoinositide 3-kinase (PI 3-K) that establishes a high level of the lipid second messenger PtdIns3,4,5P_3 (PIP_3) in the front of the cell. The localized PIP_3 together with Cdc42 and Rac, then act together to assemble actin in the pseudopod. At the rear of the cell, the fMLP receptor activates Rho that then stimulates the Rho kinase (ROK) to induce contraction of the uropod.

the leading edge where the membrane is being thrown out to form the pseudopods. The TRPM7 channels gate a small amount of Ca^{2+} that is then amplified by activating the type 2 Ins 1,4,5P_3 receptors (Ins1,4,5P_3R2s) responsible for generating these flickers. When the direction of the chemotactic gradient is altered, the flickers are concentrated on the side of the cell that turns towards the gradient suggesting that these local pulses of Ca^{2+} may play a role in cell orientation.

Neutrophils express P2Y2 receptors, which are activated by ATP that is released from the front of the cell (Module 11: Figure neutrophil chemotaxis), are coupled to $G_{q/11}$ that normally stimulates phospholipase C (PLC) to form inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). This formation of $InsP_3$ may thus be important in maintaining this Ca^{2+}-mobilizing messenger at a level that will keep the Ins1,4,5P_3R2s sufficiently sensitive to respond to the small pulses of trigger Ca^{2+} introduced by the TRPM7 channels. This possibility is consistent with the observation that the deletion of P2Y2 receptors causes a marked loss in gradient sensing (see the right-hand panel in Module 11: Figure purinergic chemotaxis).

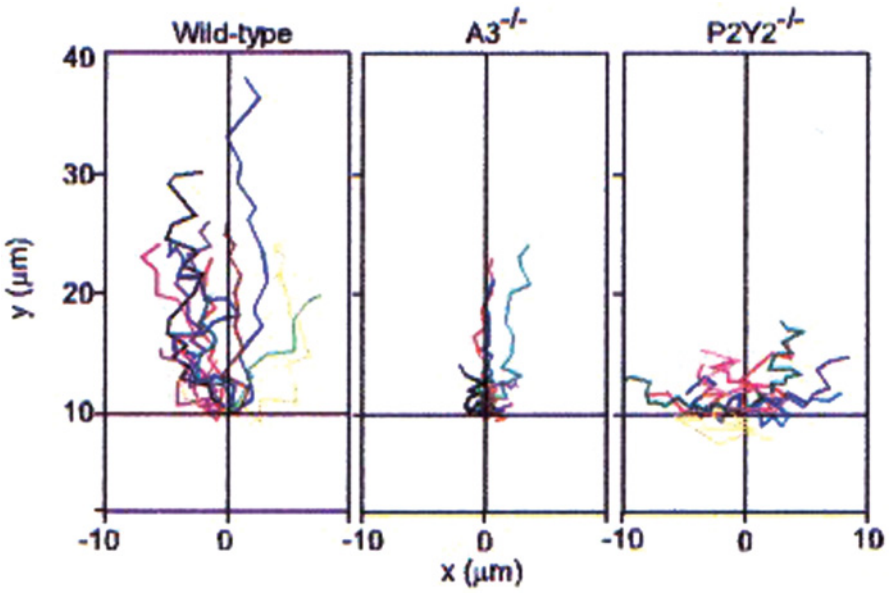
Amplification of early polarity signalling

Amplification of the early gradient-sensing signals seems to be an important feature of neutrophil chemotaxis. At least two amplification steps have been identified. One of these steps depends on the rapid and polarized formation of the lipid second messenger PtdIns3,4,5P_3 at the leading edge. The other is the release of ATP, which is hydrolysed to adenosine, and these two purinergic stimuli feedback in an autocrine manner to enhance the early chemotactic signals (Module 11: Figure neutrophil chemotaxis).

One of the earliest indicators of polarity in neutrophils, which are responding to a chemoattractant gradient, is the rapid formation of the lipid second messenger PtdIns3,4,5P_3 (PIP_3 in Module 11: Figure neutrophil chemotactic signalling) at the front of the cell. While Gβγ is able to induce some activation of the PtdIns 3-kinase, the real surge in the formation of PIP_3 seems to depend upon various positive-feedback loops. For example, there are indications that Rac.GTP can activate PtdIns 3-kinase and this will lead to further formation of PIP_3, which in turn will lead to more Rac.GTP formation. Also there are indications that once actin starts to be formed at the leading edge, it can recruit PtdIns 3-kinase to induce more PIP_3 formation, resulting in a large accumulation of this signalling lipid in the membrane at the front of the cell. Although these various feedback mechanisms need to be defined more precisely, there are indications that the early appearance of PIP_3 localized to the leading edge is a critical feature of chemotaxis.

This localized build up of PIP_3 at the front of the cell is very dependent on the activity of one of the type II inositol polyphosphate 5-phosphatases called Src homology 2 (SH2) domain-containing inositol phosphatase 1

Module 11: | Figure purinergic chemotaxis



Disruption of chemotaxis by deletion of P2Y2 and A_3 purinergic receptors.
The first panel shows how wild-type (WT) mouse neutrophils migrate in a directed way towards a source of fMet-Leu-Phe (fMLP). The middle panel shows that neutrophils lacking the adenosine A_3 receptor ($A3^{-/-}$) retain a sense of direction, but their motility is severely impaired. On the other hand, deletion of the P2Y2 receptor ($P2Y2^{-/-}$) does not affect motility, but there is a loss of gradient sensing. Reproduced from Chen, Y., Corriden, R., Inoue, Y., Yip, L., Hashiguchi, N., Zinkernagel, A., Nizet, V. Insel, P.A., and Junger, W.G. (2006) ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. Science 314:1792-1795; with permission from AAAS; http://www.sciencemag.org; see Chen et al. 2006.

(SHIP1), which hydrolyses PIP_3. As the PIP_3 formed at the front diffuses within the plane of the membrane towards the rear of the cell, it is hydrolysed, and this helps to maintain the sharp anterior/posterior gradient of this signalling lipid. If SHIP1 is knocked out in mice, PIP_3 increases throughout the membrane, and there is less evidence of cellular polarity (Module 11: Figure SHIP1 and PIP_3 polarity). The $SHIP1^{-/-}$ neutrophils were not able to polarize correctly; they retained their spherical shape and appeared to put out pseudopodia in all directions. When their position and shapes were recorded over a period of 800 s, it is clear that the wild-type (WT) cells have an elongated shape and moved in a direct line, whereas the $SHIP^{-/-}$ cells are much more rounded and move very little (see panel C in Module 11: Figure SHIP1 and PIP_3 polarity). Since the $SHIP^{-/-}$ cells can still display some polarity, it seems that the polarized distribution of PIP_3 may not be so important in setting up the compass, but it is critical for the mechanism that translates this polarity signal into directional movement through actin assembly, pseudopod formation and uropod contraction.

Another major amplification step depends upon the release of ATP from hemichannels that are activated by the membrane deformation of the pseudopods at the front of the cell (Module 11: Figure neutrophil chemotaxis). Some of the ATP is hydrolysed by ectoenzymes such as CD39 and CD73 to form adenosine. In this way, the migrating cell sets up its own chemotactic gradient that contributes to both the gradient sensing and motility components of chemotaxis. Both ATP and adenosine feed back in an autocrine manner to stimulate G protein-coupled receptors (GPCRs). ATP and adenosine appear to have slightly different effects on the chemotactic response. ATP, which acts through P2Y2 receptors, contributes to gradient sensing whereas adenosine that act through A_3 receptors enhance cell motility (Module 1: Table G protein-coupled receptors).

The P2Y2 receptors, which are activated by ATP, are coupled to $G_{q/11}$ that normally stimulates phospholipase C (PLC) to form inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) (Module 2: Figure $InsP_3$ and DAG formation). It is not clear yet whether these two second messengers function in neutrophil chemotaxis. Certainly, the formation of $InsP_3$ would contribute to the Ca^{2+} signalling microdomains and chemotactic orientation mechanism by enhancing the likelihood of generating the Ca^{2+} flickers (Module 11: Figure neutrophil chemotaxis). The dissociation of $G_{q/11}$ into $G\alpha_{q/11}$ and $G\beta\gamma$ could also be significant because the $G\beta\gamma$ subunit could feed into the putative compass to augment the $\beta\gamma$ subunits introduced by the fMet-Leu-Phe (fMLP) receptor (Module 11: Figure neutrophil chemotactic signalling). Each of these possibilities would be in line with the observation that the deletion of P2Y2 receptors causes a marked loss in gradient sensing (see right hand panel in Module 11: Figure purinergic chemotaxis).

Adenosine has a different action to ATP in that it seems to contribute to cell motility. Deletion of the adenosine A_3 receptors does not effect gradient sensing, but it does markedly reduce motility (see the middle panel in Module 11: Figure purinergic chemotaxis). The A_3 receptors are not uniformly distributed over the surface, as are the P2Y2

receptors, but are concentrated at the anterior end. Just how this receptor polarity is established is unclear. There also is little information on the mode of action of these A_3 receptors, which are known to be coupled to G_i, i.e. the same G protein used by fMLP. It would be interesting to learn more about the action of these P2Y2 and A_3 receptors, as this could provide more insights into the signalling mechanisms that control chemotaxis.

Actin assembly, pseudopod formation and uropod contraction

The polarization of signalling elements into the front and back of the cell, as described above, provides the localized instructions to protrude a pseudopod at the front and to contract the uropod at the rear (Module 11: Figure neutrophil chemotactic signalling). The activation of Cdc42 and Rac at the front of the cell orchestrate the assembly of an actin network that contributes to protruding the pseudopod. The ways in which the Cdc42 signalling mechanisms function to assemble actin are described in Module 2: Figure Cdc42 signalling. Similarly, the ways in which the Rac signalling mechanisms function to assemble actin are described in Module 2: Figure Rac signalling. These two G proteins have subtly different actions on the process of actin remodelling (Module 4: Figure actin remodelling). Cdc42.GTP stimulates Wiskott–Aldrich syndrome protein (WASP) and actin-related protein 2/3 complex (Arp2/3 complex) to form long actin filaments, whereas Rac.GTP favours the formation of branches (Module 11: Figure neutrophil chemotactic signalling). Both Cdc42 and Rac activate p21-activated protein kinase (PAK), which is highly concentrated in the front of the cell where it has a number of functions. It acts through LIM kinase 1 (LIM-K1) to phosphorylate cofilin, thus preventing it from cutting actin so that assembly can occur. PAK also phosphorylates myosin light chain kinase (MLCK), resulting in a decrease in the activity of the myosin II motor at the front of the cell. During movement, integrins attach at the front and detach at the rear. Actin filaments together with myosin form the cables that attach to the integrin receptors and it is important that this contractile system remains quiescent at the front of the cell. Activation of Cdc42 and Rac thus ensures that actin assembly is rapid at the front of the cell to facilitate the protrusion of the pseudopod and to provide stable attachments.

Different cytoskeletal events occur at the back to trigger contraction of the uropod to drive the cell forwards in the direction of the gradient. This local contraction is controlled by Rho, which is activated at the back during the operation of the early gradient-sensing mechanisms (setting the compass). There are a number of Rho signalling mechanisms (Module 2: Figure Rho signalling). In the case of neutrophil chemotaxis, the main function of Rho.GTP is to activate contraction by stimulating Rho kinase (ROK) (Module 11: Figure neutrophil chemotactic signalling). ROK induces a signalling cascade that results in the activation of non-muscle myosin IIA (NMIIA) to trigger contraction of the uropod.

In summary, the localized action of Cdc42 and Rac at the front assembles the actin network that forms the pseudopod. While at the back of the cell, Rho acts to stimulate the myosin II–actin network to contract the uropod to propel the cell forward. A challenge for the future is to determine the exact nature of the signalling mechanisms that constitutes the compass that orchestrates these directed motile mechanisms that are responsible for neutrophil chemotaxis.

Senescence

Senescence is a process whereby cells lose the ability to proliferate. They can survive for a prolonged period in a state of suspended animation with regard to cell division. There are two main states of senescence that are arrived at by separate mechanisms (Module 11: Figure senescence). There is replicative senescence, which depends upon the loss of telomeres, and stress-induced senescence, which is produced by various cell stresses. One of these stresses is caused by the activation of oncogenes that are potent activators of stress-induced senescence. Senescence-associated β-galactosidase (SA-β-gal) is a biochemical marker used to detect senescence.

Replicative senescence

Normal mammalian cells have a finite capacity to divide, as was first recognized when cells were placed in culture. For example, when human cells from a young individual are placed in culture, they will divide about 50 times before stopping. Cells from older individuals will divide less. The number of divisions appears to be determined by the telomeres located at the ends of the chromosomes. These telomeres, which are essential for DNA synthesis, are made up of approximately 20 repetitive TTAGGG tracts followed by a single strand overhang on the 3′ G-rich strand. Since these telomeres shorten during each round of cell division, the number of repeats decline to a point where DNA synthesis is no longer possible and the cell enters a state of replicative senescence (Module 11: Figure senescence).

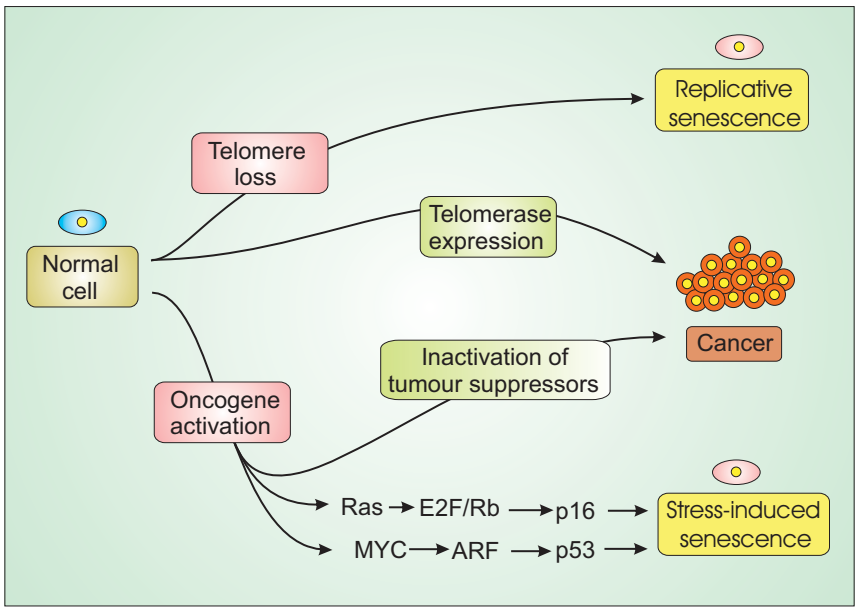
Such replicative senescence is avoided by stem cells or by cancer cells by the expression of the enzyme telomerase, which is able to maintain the telomeres by replicating the telomere tracts. One of the prerequisites for a cancer to develop is therefore the expression of telomerase, thus enabling the cancer cell to divide repeatedly without running the risk of replicative senescence.

Stress-induced senescence

As the name implies, stress-induced senescence is activated by various cell stresses such as DNA damage or through the appearance of oncogenes that begin to activate proliferation. The latter is particularly relevant to the onset of cancer, because such stress-induced senescence is an important mechanism to suppress the early stages of cancer. For example, this form of senescence has now been described in a number of precancerous tissues such as nevi (skin moles) and early-stage prostate abnormalities.

Stress-induced senescence is activated by components of the proliferative pathways that normally act to stimulate proliferation. This apparent paradox of the same

Module 11: | Figure senescence



Mechanisms of cell senescence.
Normal cells can transform into a non-proliferating senescent state through two main mechanisms. Loss of telomeres results in replicative senescence. A variety of cellular stresses, including the activation of oncogenes, results in stress-induced senescence. The latter is induced by two main pathways. Oncogenes such as Ras and Myc can activate tumour suppressors, such as p16 and alternative reading frame (ARF), that act through E2F/retinoblastoma protein (Rb) and p53 respectively to divert cells into stress-induced senescence. These senescence pathways are avoided or switched off during the development of cancer cells. The expression of telomerase avoids the replicative senescence pathway, whereas the inactivation of the tumour suppressors such as Rb, p16 or p53 prevents the emerging cell from being diverted into stress-induced senescence.

signalling mechanisms being able to activate both senescence and proliferation can probably be resolved by the fact that these two outcomes may have different thresholds. Normal levels of signalling can activate the proliferative pathways that lead to cyclin D activation (Module 9: Figure proliferation signalling network), whereas abnormally high signals are required to activate the pathways that deflect the cell towards stress-induced senescence (Module 11: Figure senescence).

There appear to be two main signalling pathways that are activated by excessive proliferative signalling to switch on stress-induced senescence. One pathway depends upon the tumour suppressor $p16^{INK4a}$ that is activated by the E2F/retinoblastoma protein (Rb) complex. The onset of senescence may be facilitated by Suv39h1, which binds to Rb and methylates histones to enhance formation of heterochromatin that will silence DNA. The other pathway depends upon the tumour promoter alternative reading frame (ARF) that activates p53. The latter plays a key role in preventing cancer, since it can drive cells towards either senescence or apoptosis (Module 9: Figure proliferative signalling network).

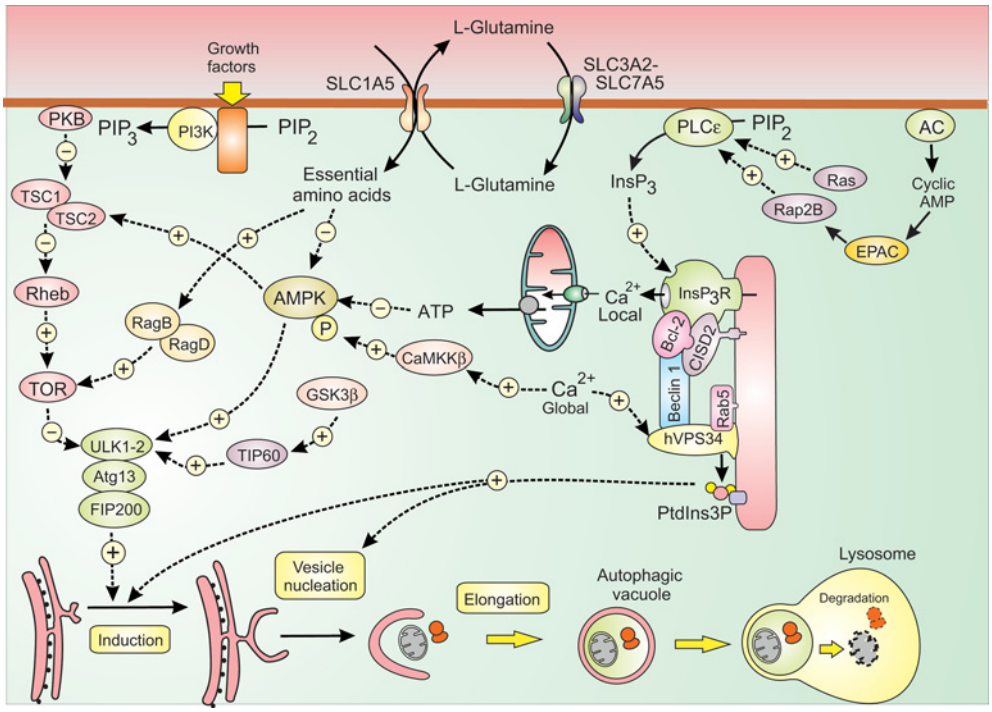
Immortalization is the term given to a process whereby cultured cells escape senescence and acquire the ability to grow in culture indefinitely. The frequency of spontaneous immortalization is species-specific, being very efficient in rodent cells, but occurs rarely in human and avian cells. There appear to be a limited number of genes that change during immortalization. An alteration in p53 is often associated with immortalization.

Autophagy

Autophagy, which means self-eating, has a number of functions in both normal cellular physiology and in various pathological processes, especially those related to nutrient starvation and hypoxia. Its normal day-to-day role is to remove both damaged organelles and proteins. Autophagy is also activated by various stressful stimuli and most attention will focus on how this process is activated by a decline in energy metabolism due to a loss of nutrients or hypoxia.

Cell survival depends critically on a continuous supply of energy. There is a metabolic energy network that manages the energy status of the cell (Module 7: Figure metabolic network). During periods of starvation, this energy network can be maintained for a considerable period by using energy stored in reserves such as fat and glycogen. However, when these easily accessible reserves are depleted, survival can be prolonged by a process of autophagy whereby the cell begins to catabolize its cytoplasmic components (Module 11: Figure autophagy). The autophagic process depends on an orderly sequence of events that begins with the process of induction whereby a small region of the endoplasmic reticulum (ER) and perhaps also the mitochondria begin to form a cup-like bud, which is defined by a local accumulation of the phospholipid PtdIns3P. After this cup detaches itself from the ER/mitochondria, a process of vesicle nucleation enlarges this cup to form a membrane envelope that engulfs large volumes of the cytoplasm containing organelles such as ribosomes and mitochondria to form an autophagic

Module 11: | Figure autophagy



Control of autophagy.

Autophagy is the large-scale degradation of cytoplasmic components. During an induction process, a membrane protrusion buds off from the ER and begins to enlarge (vesicle nucleation) to form a cup-shaped vesicle that then elongates and breaks away from the ER to form an isolation membrane that engulfs mitochondria and ribosomes to form an autophagic vacuole, which then fuses with a lysosome where degradation occurs. These membrane events are controlled by a variety of signalling pathways many of which are channelled through the target of rapamycin (TOR) to activate the induction processes. Another pathway depends on various signalling processes that are directed towards the class III PI 3-kinase hVPS34 to generate PtdIns3P that acts to control the process of vesicle nucleation. For further details of the way these signalling pathways control autophagy see Module 11: Figure autophagy signalling mechanisms.

vacuole. This autophagic vacuole then fuses with lysosomes to form an autophagosome, where all the macromolecular components are broken down into metabolites that can be fed to the mitochondria to provide ATP for survival. Much of the control of autophagy is carried out by a large family of proteins coded for by autophagy-related genes (Atg). With regard to cell signalling, therefore, the problem is to understand how the signalling pathways that have been implicated in the control of autophagy have an impact on the function of this ensemble of Atg proteins.

Autophagy is controlled by a number of signalling pathways (Module 11: Figure autophagy). The major control is carried out by the target of rapamycin (TOR), which is regulated both by growth factors and by the energy state of the cell (Module 9: Figure target of rapamycin signalling). Growth factors acting through class I PtdIns 3-kinase (PI 3-K) stimulate the PtdIns 3-kinase signalling pathway (Module 2: Figure PtdIns 3-kinase signalling) to maintain the ability of TOR to inhibit autophagy. As part of this mechanism, the PtdIns3,4,5P_3-dependent activation of PKB inhibits the TSC1/TSC2 complex that serves to remove the inhibition of Rheb that is responsible for the activation of TOR (Module 11: Figure autophagy).

Under conditions where energy becomes limiting, the inhibitory effect of TOR declines and autophagy begins. Many of the factors that induce autophagy act by reducing TOR activity through various mechanisms. An important signal for autophagy is a decrease in the cell's energy state and this is transmitted through the AMP signalling pathway (Module 2: Figure AMPK control of metabolism). One reflection of energy state is the level of essential amino acids that enter the cell through specific carriers. Glutamine enters through a high-affinity transporter SLC1A5 and its build up within the cell is then used by the heterodimeric bidirectional transporter SLC7A5/SLC3A2 to exchange this glutamine for essential amino acids (Module 11: Figure autophagy). The presence of essential amino acids acts through the GTPase Rag family, which are a members of the Ras family of small monomeric G proteins (Module 2: Table monomeric G protein toolkit). A RagB/RagD heterodimer interacts with TOR and directs it to the surface of the late endosomal/lysosomal compartment where TOR activity is maintained to inhibit autophagy. The amino acids also act to inhibit the activity of AMPK, but when essential amino acids are limiting this inhibition is reversed and AMPK can activate TCS2 to inhibit Rheb and TOR to induce autophagy. A similar AMPK-dependent mechanism occurs when the level of ATP falls since the resulting increase in AMP stimulates AMPK resulting in the inhibition of TOR through the same sequence of events just described for the decline in essential amino acids.

There is an AMPK-independent autophagy signalling pathway that occurs when cells are deprived of growth

factors, which depends on glycogen synthase kinase-3β (GSK3β) phosphorylating Tat-interactive protein 60 (TIP60), which acetylates ULK1 on lysine residues 162 and 606 to initiate autophagy (Module 11: Figure autophagy).

The next question to consider is how TOR acts to regulate the induction of autophagy by acting on members of the Atg family. In the case of the early induction step, the action of TOR is to control three components of this Atg family: ULK1, Atg13 and FAK family interacting protein of 200 kDa (FIP200) (Module 11: Figure autophagy signalling mechanisms). Under nutrient-rich conditions, active TOR phosphorylates and inactivates ULK1 and Atg13 to inhibit autophagy. When nutrients decline and TOR is inactive, these inhibitory phosphates are removed and the ULK1/Atg13/FIP200 complex is able to initiate autophagy by activating the initial induction step when membrane is removed from the ER to initiate the formation of the autophagic vacuole. This early membrane trafficking step is also facilitated by Atg14L that acts together with the integral membrane protein p150 to stimulate the class III PI 3-Kinase hVPS34 to produce the PtdIns3P that defines the ER membrane that is being budded off to form the autophagic vacuole (Module 2: Figure localized inositol lipid signalling). This hVPS34/p150 complex is then carried over to function in the next phase of vesicle nucleation and elongation.

Vesicle nucleation is driven by a large macromolecular complex that depends on the formation of PtdIns3P by hVPS34. This lipid is a messenger that operates in the PtdIns3P signalling cassette that functions in the control of a number of vesicle trafficking processes. During this vesicle nucleation process, hVPS34 can be activated by two mechanisms. First, Rab5 interacts with and stimulates hVPS34. Secondly, another member of the Atg family called Beclin-1 is an important component of a large regulatory complex that also controls the activity of hVPS34 (Module 11: Figure autophagy). A number of proteins bind to Beclin-1 to regulate its activity both positively and negatively. The positive regulators include activating molecule in Beclin-1 regulator 1 (ambra-1), Rubicon and UV radiation resistance-associated gene protein (UVRAG) (Module 11: Figure autophagy signalling mechanisms). Beclin-1 inhibition depends on its interaction with the $InsP_3$ receptor ($InsP_3R$) through a mechanism that is modulated by a number of other proteins such as Bcl-2 and by CDGSH iron sulfur domain-containing protein 2 (CISD2). The ability of Beclin-1 to inhibit hVPS34 depends on its interaction with the nexus formed by Bcl-2, CISD2and the $InsP_3R$. Phosphorylation of Bcl-2 by JNK1 causes Beclin-1 to dissociate from Bcl-2 resulting in the activation of hVPS34 and the formation of the PtdIns3P necessary to activate vesicle nucleation. One of the functions of PtdIns3P is to recruit the early autophagic protein WPP-domain interacting protein 1 (WIP-1), which is a serine/threonine protein phosphatase that is also known as protein phosphatase 1D magnesium-dependent (PPM1D). The function of PtdIns3P in vesicle nucleation in autophagy is very reminiscent of endosome vesicle fusion to early endosomes that occurs during endocytosis (Module 4: Figure endosome vesicle fusion).

The myotubularin MTMR14, which is also known as Jumpy, inactivates the messenger function of PtdIns3P by hydrolysing it back to PtdIns (Module 11: Figure autophagy signalling mechanisms). Inactivation of MTMR14 enhances the onset of either basal or starvation-induced autophagy and thus highlights the significance of PtdIns3P in vesicle nucleation. A missense mutation in MTMR14 has been identified in centronuclear myopathy (CNM).

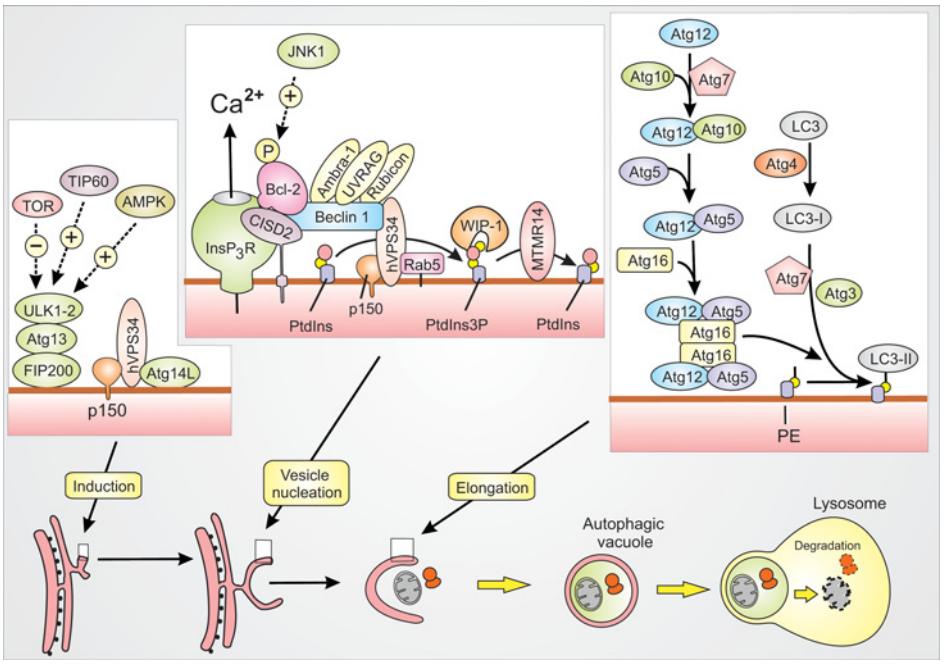
In addition to playing a role in modulating the activity of hVPS34, the $InsP_3R$ also functions to release internal Ca^{2+} that may also play a role in controlling autophagy (Module 11: Figure autophagy). There are a number of mechanisms for activating the phospholipase C isoforms to elevate the level of $InsP_3$ (Module 2: Figure PLC structure and function). For example, formation of $InsP_3$ by phospholipase Cε (PLCε) has been implicated in the control of autophagy. This PLCε can be activated through Ras (Module 2: Figure Ras signalling) or through the EPAC/Rab2b components of the cyclic AMP signalling pathway (Module 2: Figure cyclic AMP signalling). The $InsP_3$-induced release of Ca^{2+} has been implicated in a number of autophagy control mechanisms. However, the relationship between Ca^{2+} signalling and autophagy is somewhat contradictory in that Ca^{2+} has been reported to be both stimulatory and inhibitory.

Ca^{2+} signalling and autophagy

Such positive and negative actions of Ca^{2+} are not uncommon and often reflect a dual role depending on the location and concentration of Ca^{2+}. Such a dual mechanism might explain the contradictory actions of Ca^{2+} in autophagy with local low levels of Ca^{2+} having an inhibitory effect, whereas higher global levels, often associated with stress, may stimulate autophagy. For example, there are reports that a constitutive release of Ca^{2+} from the ER may operate to inhibit autophagy. This constitutive Ca^{2+} release by the $InsP_3R$, which is controlled by the Bcl-2/CISD2 complex, may create a local elevation of Ca^{2+} that operates within the confines of the ER-mitochondrial shuttle (Module 5: Figure ER/mitochondrial shuttle) to maintain mitochondrial energy metabolism and the increase in ATP formation will reduce AMPK activation to prevent autophagy (Module 11: Figure autophagy). The intimate communication between the ER and mitochondria means that this Ca^{2+} signalling is highly localized and may not be detected by some of the other cellular Ca^{2+}-sensitive processes. The continuous leakage of Ca^{2+} also ensures that the concentration of Ca^{2+} in the lumen of the ER is kept relatively low.

The other reports of Ca^{2+} being able to activate autophagy may be explained when conditions favour more global elevations of Ca^{2+} that act on a different set of targets. One of these targets is CaMKKβ that acts to stimulate AMPK (Module 2: Figure AMPK control of metabolism), which will result in inhibition of TOR and the induction of autophagy. Another action of this global Ca^{2+} is to stimulate hVPS34 to increase the formation of the PtdIns3P

Module 11: | Figure autophagy signalling mechanisms



Autophagy signalling mechanisms.
A family of autophagy-related gene (Atg) products carry out the signalling mechanisms that activate autophagy. When the target of rapamycin (TOR) is switched off, the ULK1/Atg13/FIP200 complex is activated to stimulate the induction of autophagy. Vesicle nucleation is activated by a macromolecular complex that controls the hVPS34 responsible for forming PtdIns3P. Elongation depends on two ubiquitin-like pathways carried out by a series of Atg proteins that result in the conjugation of the Atg protein LC3-I to phosphatidylethanolamine (PE) to form LC3II. See the text for further details.

that plays a role in driving the early membrane trafficking events. Such a scenario is supported by experiments on mice where CISD2 has been knocked out and the constitutive local release of Ca^{2+} seems to be reduced and this results in higher luminal Ca^{2+} levels and larger global Ca^{2+} signals that trigger autophagy.

Following vesicle nucleation, the developing membrane cup begins to elongate and this process seems to be regulated by two ubiquitin-like pathways carried out by a series of Atg proteins (Module 11: Figure autophagy signalling mechanisms). The conjugation of Atg5 to Atg12 is carried out by Atg7 (E1-like enzyme) and Atg10 (E2-like enzyme). The Atg5 conjugate then interacts with Atg16 to form a large complex containing Atg5/Atg12/Atg16 tetramers. The other pathway begins with microtubule-associated protein 1A/1B-light chain 3 (LC3) that is hydrolyzed at its C-terminus by the protease Atg4B to form LC3-I. The latter then undergoes a series of conjugation reactions carried out by Atg7 and Atg3 resulting in the LC3-I being attached to phosphatidylethanolamine (PE) to form LC3-II. The Atg5/Atg12/Atg16 complex can also participate in the conversion of LC3-II into LC3-III, which plays a role in the elongation step.

There is a significant relationship between autophagy and Alzheimer's disease.

Apoptosis

Apoptosis is particularly evident during development, when unwanted cells are pruned during organogenesis. For example, fingers and toes begin to form when cells are selectively removed between developing digits. The developing nervous system also produces a large excess of both neurons and oligodendrites, many of which are destroyed as the neural circuitry is formed. Massive apoptosis occurs in the developing immune system when autoreactive T- and B cells are destroyed. The completion of development does not mark the end of apoptosis, because it continues to play a vital role in the adult organism during the normal turnover of cells. For example, the human body spawns several million new cells every second. If cell numbers are to remain constant, several million cells must die every second. This massive turnover is necessary to replace aged and damaged cells. For example, the cells of the hair bulb die by apoptosis during the catagen phase of each hair follicle cycle (Module 8: Figure hair follicle cycle).

Any distortion of this precise homoeostasis between cell birth and cell death can have very severe consequences. Autoimmune diseases and cancer are associated with a decrease in apoptosis. Conversely, an increase in apoptosis occurs during neurodegenerative and neuromuscular diseases, ischaemia/reperfusion damage during a stroke or heart failure, and with various infectious diseases such as AIDS and those caused by toxin-producing microorganisms.

The orderly programme of cell death is controlled by an apoptotic signalling network. There are two major pathways for initiating apoptosis: an extrinsic pathway that responds to external signals such as the Fas ligand (FasL) and tumour necrosis factor α (TNFα), and an

intrinsic pathway where a process of Ca^{2+}-induced apoptosis is induced following an alteration in the normal operation of the endoplasmic reticulum (ER)/mitochondrial Ca^{2+} shuttle. These two pathways activate the caspase cascade responsible for carrying out the orderly cell death programme. The Bcl-2 superfamily, which has both pro- and anti-apoptotic members, plays a major role in regulating apoptosis, with much of its activity focused on the intrinsic pathway where Ca^{2+}-induced apoptosis is an important element. The Bcl-2 superfamily control of Ca^{2+} signalling plays an important role in regulating apoptosis, and features significantly in the relationship between apoptosis and cancer.

Control of apoptosis is regulated by both hormonal and transcriptional processes. The hormonal modulation of apoptosis is carried out by a number of the cell signalling pathways activated by growth factors and hormones that act either directly through the covalent modification of pre-existing components of the apoptotic system (e.g. the Bcl-2 superfamily) or by activating gene transcription to alter the balance between the pro- and anti-apoptotic regulatory components. This balance is also regulated by transcriptional processes activated during p53-induced apoptosis, which is one of the primary mechanisms used by this tumour suppressor to destroy developing cancer cells.

Apoptotic signalling network

A complex apoptotic signalling network controls the fateful decision of whether a cell survives or dies. The signalling system is made up of a number of discrete processes that are linked together into an integrated network (Module 11: Figure apoptosis):

1. Cytokines such as the Fas ligand (FasL) and tumour necrosis factor α (TNFα) act on death receptors that engage the extrinsic pathway, which has two major outputs (Module 11: Figure TNFα apoptotic signalling).
2. It activates caspase 8, which is one of the initiator caspases of the caspase cascade.
3. It can also recruit the intrinsic pathway by activating Bid, which is one of the pro-apoptotic members of the Bcl-2 superfamily. This conversion of Bid into tBid can also be carried out by cathepsin D that is activated by ceramide during sphingomelin signalling (Module 2: Figure sphingomyelin signalling).
4. The initiator caspases (e.g. caspases 8, 9 and 10) activate the executioner caspases (e.g. caspases 3, 6 and 7) that are responsible for driving the processes of apoptosis.
5. The intrinsic pathway depends upon an interaction between the endoplasmic reticulum (ER) and the mitochondria. These two organelles are tied together through an endoplasmic reticulum (ER)/mitochondrial Ca^{2+} shuttle, which seems to play a major part in determining how Ca^{2+} functions together with the Bcl-2 superfamily to regulate the initiation of apoptosis. Both organelles can provide output signals that can activate apoptosis. A process of endoplasmic reticulum (ER) stress signalling generates a number of output signals, one of which is the activation of caspase 12 (Module 2: Figure ER stress signalling), which feeds into the caspase cascade. However, most of the output signals from the intrinsic pathway come from the mitochondria, which release a variety of apoptotic factors, such as cytochrome *c* and a second mitochondrial derived activator of caspase (SMAC) that feed into the caspase cascade, and apoptosis-inducing factor (AIF) and endonuclease G (EndoG), which feed into the caspase-independent pathway.
6. The Bcl-2 superfamily contains both pro- and anti-apoptotic factors that play a major role in modulating the intrinsic pathway.
7. A number of cell signalling pathways can modulate the apoptotic signalling network. This hormonal modulation of apoptosis is carried out by a variety of classical cell signalling pathways or through various stress stimuli that act by either modulating the performance of pre-existing Bcl-2 family members or altering the expression of pro- and anti-apoptotic factors.
8. Some of the signalling pathways modulate apoptosis by adjusting the activity of the Bcl-2 superfamily [e.g. protein kinase B (PKB) phosphorylation of Bad; see Module 11: Figure Bcl-2 family functions].
9. Some of the pathways can regulate the transcription of components of the apoptotic signalsome. A particularly important aspect of this remodelling process is that it can alter the balance between the pro- and anti-apoptotic factors, thereby altering the sensitivity of cells to apoptosis.
10. Genotoxic stress resulting in the activation of the transcription factor p53, which functions as a tumour suppressor by increasing the expression of a large number of apoptotic factors (Module 4: Figure p53 function).

Extrinsic pathway

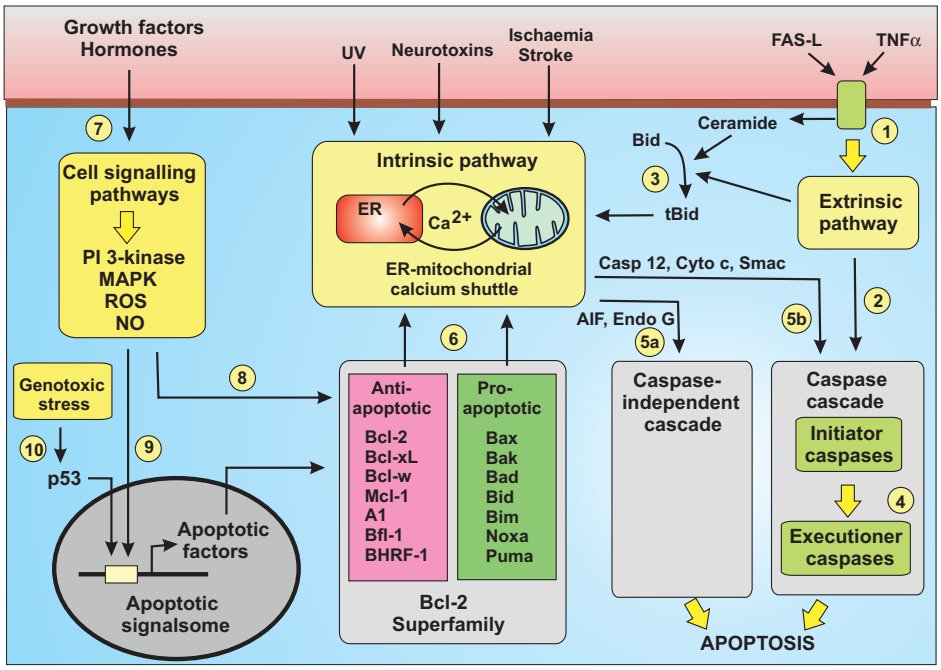
The extrinsic pathway is activated by external signals such as the Fas ligand (FasL) and tumour necrosis factor α (TNFα), which are typical cytokines (Module 1: Figure cytokines). The FasL and TNFα act through death receptors.

The DR6 receptor, which is another member of the superfamily of tumour necrosis factor receptors (TNF-Rs), functions to control apoptosis in neurons (see step 11 in Module 12: Figure amyloid cascade hypothesis).

Death receptors

The best-characterized death receptors belong to the tumour necrosis factor α (TNFα) receptor family that also mediate inflammatory responses (Module 1: Figure cytokines). Members of this receptor family, such as the TNF receptor (TNF-R) and Fas, have cysteine-rich extracellular domains, while their cytosolic regions contain a death domain (DD). When these death receptors engage their ligands, they form trimers and are then able to activate various signalling pathways. One of these is the nuclear factor κB (NF-κB) signalling pathway (Module 2: Figure NF-κB activation). A separate pathway is used to activate

Module 11: | Figure apoptosis



The apoptotic signalling network.
The onset of apoptosis is controlled by a number of interconnecting processes. Particular attention is focused on how these different processes interact with each other as outlined in the text.

the extrinsic pathway, as outlined in Steps 1–6 in Module 11: Figure TNFα apoptotic signalling:

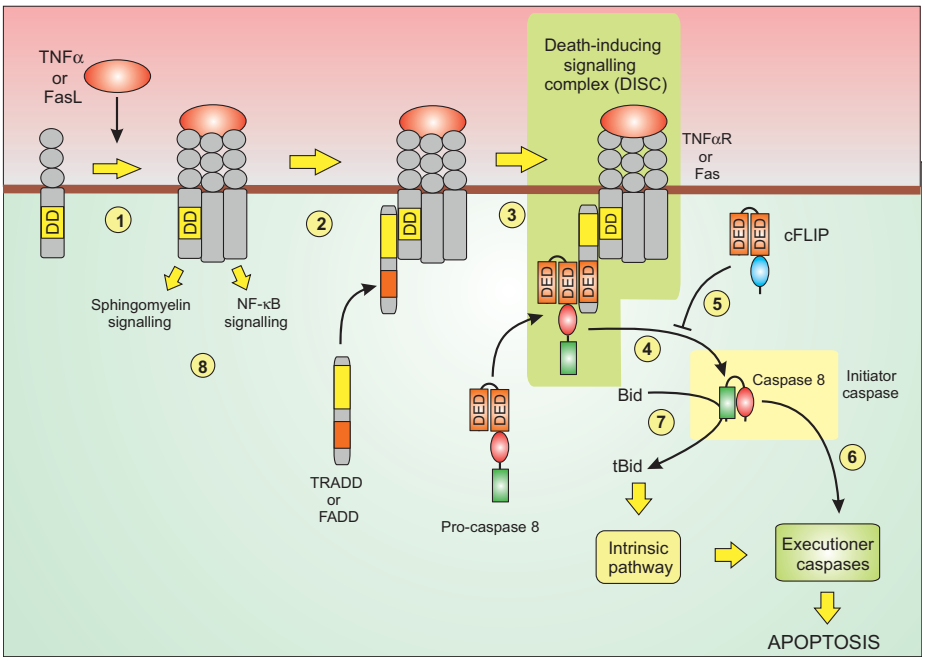
1. Apoptotic signals such as TNFα and FasL bind to the trimeric TNFα receptor family (TNFα or Fas) to form a platform that then binds a variety of adaptor proteins capable of relaying information to different signalling pathways.
2. Activation of death receptors results in the recruitment of adaptor proteins such as tumour-necrosis-factor-receptor-associated death domain (TRADD) or Fas-associated death domain (FADD) that bind to the death domain (DD) on the trimeric TNFα receptor.
3. The next event is the recruitment of pro-caspase 8 to form the death-inducing signalling complex (DISC).
4. The pro-caspase 8 is cleaved to release caspase 8, which can have two functions depending on the cell type. In some cells, such as lymphocytes, there is a direct activation of the caspases downstream of caspase 8 (Step 5). In other cells, such as hepatocytes and pancreatic β-cells, there is an additional caspase cascade amplification pathway that depends on the formation of tBid (Step 6). The X-chromosome-linked inhibitor of apoptosis protein (XIAP) seems to play a prominent role in determining which of these pathways is activated.
5. Apoptosis can be inhibited by cellular FLICE-inhibitory protein (cFLIP), which acts by binding to the death-inducing signalling complex (DISC).
6. Caspase 8 then functions as an initiator caspase to feed in to the executioner caspases. Through these rapid protein–protein interactions, the death receptors can activate the caspase cascade within seconds, causing the cell to die within hours.
7. Caspase 8 can also convert Bid into tBid, which feeds into the intrinsic pathway. In addition, the ceramide formed during sphingomyelin signalling can activate cathepsin D that carries out a similar conversion of Bid into tBid.
8. In addition to this direct fast-track signalling pathway to the caspase cascade, these death receptors can also activate other signalling systems, such as the NF-κB signalling pathway (Module 2: Figure NF-κB activation) and the sphingomyelin signalling pathway (Module 2: Figure sphingomyelin signalling).

Cellular FLICE-like inhibitory protein (cFLIP)

Cellular FLICE-inhibitory protein (cFLIP), which is also known as the Casp8 and FADD-like apoptosis regulator and is encoded by the *CFLAR* gene, inhibits apoptosis by binding to the death-inducing signalling complex (DISC) that consists of a member of the TNFα receptor family (TNFαR or Fas), an adaptor such as FADD or TRADD and pro-caspase 8 (Module 11: Figure TNFα apoptotic signalling). Such a mechanism may act to prevent the apoptosis of B-cells in the germinal centre during the process of B-cell differentiation in the lymph node (Module 8: Figure B cell maturation signalling). Resistance of breast cancer tumour cells to the TNF-related apoptosis-inducing ligand (TRAIL) may depend on the expression of cFLIP.

The expression of cFLIP seems to be regulated by the NF-κB signalling pathway.

Module 11: | Figure TNFα apoptotic signalling



Tumour necrosis factor α (TNFα) activation of the extrinsic apoptotic pathway.
Apoptotic ligands such as tumour necrosis factor α (TNFα) or Fas ligand (FasL) bind to members of the trimeric TNFα receptor family (TNFαR or Fas) to initiate the extrinsic signalling cascade, as described in the text.

Intrinsic pathway

The mitochondria, which are an essential component of the intrinsic apoptotic pathway, harbour an array of apoptotic factors. Release of these stored apoptotic factors from the mitochondria are triggered by inputs from either signalling pathways (e.g. ceramide from the sphingomyelin signalling pathway) or from various stresses (UV, DNA damage, chemotherapeutic agents and neurotoxins) (Module 11: Figure apoptosis). This intrinsic pathway is also responsible for inducing apoptosis following cardiac ischemia or stroke when cells are overwhelmed by Ca^{2+}. Indeed, Ca^{2+}-induced apoptosis is a significant component of the intrinsic pathway. The latter is also regulated by the Bcl-2 superfamily of apoptotic regulators

In response to these various stimuli, apoptotic factors are released into the cytoplasm, where they feed into the caspase cascade to induce apoptosis (Step 5 in Module 11: Figure apoptosis). Two of the major factors released from the mitochondria are cytochrome *c* and apoptosis-inducing factor (AIF). The cytochrome *c* combines with apoptosis-activating factor 1 (Apaf-1) in the cytoplasm to form a large scaffolding complex called the apoptosome, which draws in and activates pro-caspase 9, which then activates pro-caspase 3 to form caspase 3 (Module 11: Figure Bcl-2 family functions).

As cells undergo apoptosis they often release nucleotides such as ATP and UTP that function as 'find-me' signals to attract phagocytes that come in to clear up the debris. Caspase 3 may play a role in generating this find-me signal by cleaving the cytoplasmic region of pannexin 1 to induce the release of ATP. Studies on neutrophil chemotaxis have revealed that ATP can function as a chemotactic signal (Module 11: Figure neutrophil chemotaxis).

Ca^{2+}-induced apoptosis

Intracellular Ca^{2+} is an important regulator of both cell proliferation and apoptosis. This example of Ca^{2+} having opposite effects in cells can be explained by the way that this signal is presented in both time and space. During normal signalling, Ca^{2+} is usually presented as brief transients, where information is encoded in the temporal properties of the transients (Module 6: Figure encoding oscillatory information). Following each transient, there is a corresponding flux through the mitochondria through the operation of the endoplasmic reticulum (ER)/mitochondrial Ca^{2+} shuttle (Module 5: Figure ER/mitochondrial shuttle). Normally, most of the Ca^{2+} stored in the cell resides in the ER, except during signalling, when some of the Ca^{2+} released from the ER takes up temporary residence in the mitochondria. However, if this equilibrium position is changed such that there is a large transfer of Ca^{2+} from the ER to the mitochondria, a process of Ca^{2+}-induced apoptosis is triggered through activation of the intrinsic pathway. A number of stress signals can repartition this stored Ca^{2+} such that it moves from the ER lumen into the mitochondrion. For example, inhibition of the sarco/endo-plasmic reticulum Ca^{2+}-ATPase (SERCA) pump by thapsigargin leads to a decrease in luminal Ca^{2+} concentration and activation of endoplasmic reticulum (ER) stress signalling. In addition, the shift of Ca^{2+} from the ER into the mitochondrion leads to an elevation of Ca^{2+} in the mitochondrial matrix, which

results in activation of the mitochondrial permeability transition pore (mPTP). Another consequence of mitochondrial Ca^{2+} loading is to stimulate the generation of reactive oxygen species (ROS), which have also been linked to formation of the mPTP (Module 5: Figure mitochondrial Ca^{2+} signalling).

The Bcl-2 superfamily control of Ca^{2+} signalling may depend upon their ability to regulate the leak of Ca^{2+} from the ER, which will thus reduce the probability of mPTP opening because less Ca^{2+} will be transferred to the mitochondria.

Bcl-2 superfamily

The Bcl-2 superfamily of apoptotic regulatory factors is a primary regulator of the intrinsic pathway. This highly dynamic internal regulatory system has both pro- and anti-apoptotic family members (Module 11: Figure apoptosis). Some of this dynamism depends upon the Bcl-2 superfamily domain structure and function, which is dominated by the Bcl-2 homology (BH) domains that enable family members to interact with each other (Module 11: Figure Bcl-2 superfamily domain structure). Such Bcl-2 superfamily interactions enable the anti-apoptotic factors to curb the activity of the pro-apoptotic factors, and the balance between these two opposing factors is critical in determining whether a cell lives or dies. This critical balance can be altered through the hormonal modulation of apoptosis (Step 7 in Module 11: Figure apoptosis), which operates either in the short term through post-translational modifications such as phosphorylation/dephosphorylation reactions, or in the longer term by the transcriptional control of the Bcl-2 superfamily. The Bcl-2 superfamily control of Ca^{2+} signalling is another way in which these apoptotic regulators may control apoptosis.

In addition to these regulatory functions, the Bcl-2 superfamily may also have a structural role in that they may contribute to the formation of channels in the outer mitochondrial membrane (OMM) responsible for the release of the apoptogenic factors.

Bcl-2 superfamily domain structure and function

The Bcl-2 superfamily has a domain structure that is dominated by the Bcl-2 homology (BH) domain (Module 11: Figure Bcl-2 superfamily domain structure). Many of the family members also have a hydrophobic C-terminal region resembling a transmembrane (TM) domain, which may assist in the attachment of these factors to the membranes of the mitochondria and endoplasmic reticulum where they exert their actions.

The superfamily is divided into the anti-apoptotic and pro-apoptotic families. The anti-apoptotic factors, such as Bcl-2 and Bcl-X_L, have four BH domains and most have a TM domain except for A1. The pro-apoptotic factors are divided into the multidomain (BH) and BH3-only families. Examples of the latter are Bad, Bid, Bim, Noxa and Puma, which act as sensors to pick up information from various inputs that are then relayed to the multidomain (BH) factors such as Bak and Bax, which function as effectors responsible for the permeabilization of the outer mitochondrial membrane (Module 11: Figure Bcl-2 family functions). The importance of Bak and Bax, which have been referred to as a 'gateway to the intrinsic pathway', is evident from the fact that their removal renders cells resistant to a variety of death signals, such as a Ca^{2+} overload, ceramide and oxidative stress. It is not therefore surprising to find that many of the other Bcl-2 family members act in one way or another to modulate the activity of these two pro-apoptotic factors. An important feature of the modulatory function within the Bcl-2 superfamily is the way they interact with each other through direct protein–protein interactions. The α-helical BH3 domain of one protein inserts into a specific binding site formed by the BH1, BH2 and BH3 domains of another Bcl-2 family member.

Bcl-2

B cell leukaemia-2 (Bcl-2) is a prominent anti-apoptotic factor. Two mechanisms have been proposed to explain this inhibitory action of Bcl-2. One mechanism depends upon the ability of Bcl-2 to bind to pro-apoptotic factors such as Bax, thereby preventing them from forming channels in the outer mitochondrial membrane (Module 11: Figure Bcl-2 family functions). The second mechanism, which is somewhat more controversial, depends upon growing evidence of a Bcl-2 superfamily control of Ca^{2+} signalling.

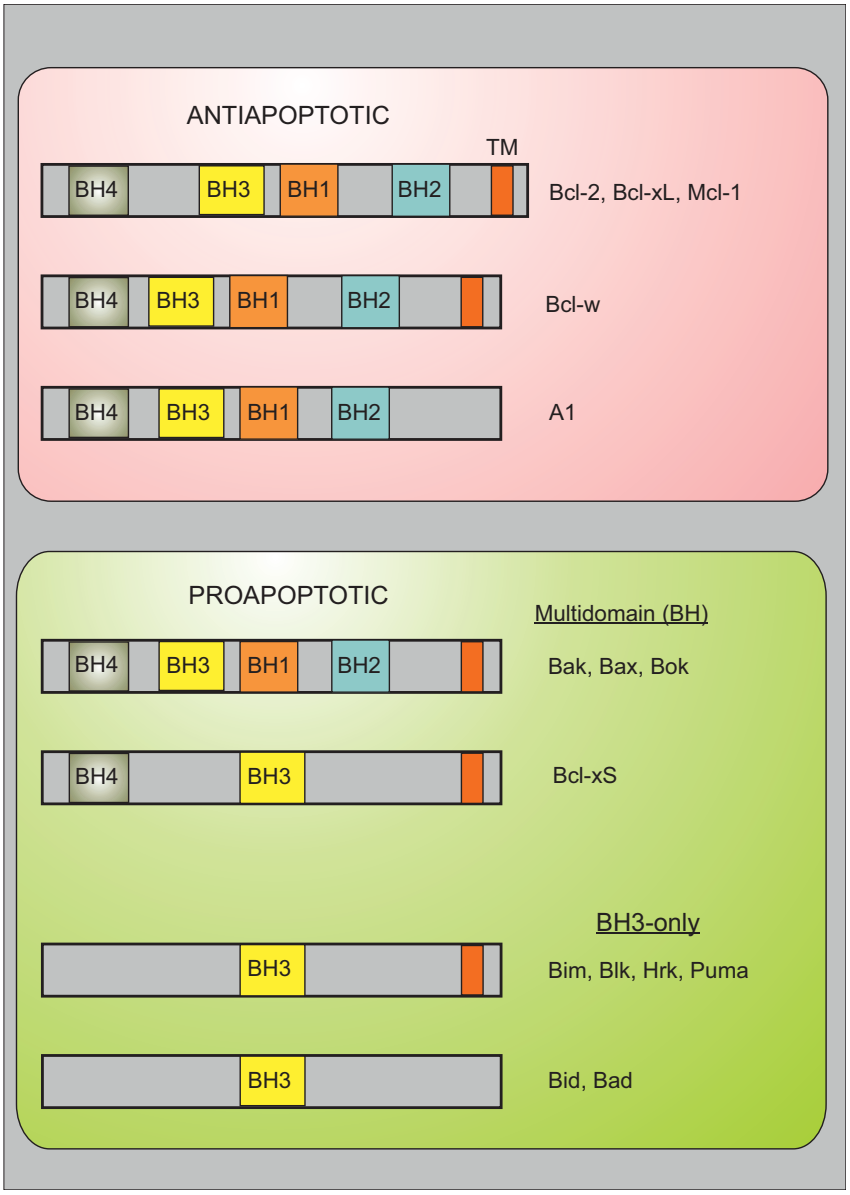
The transcription factor microphthalamia-associated transcription factor (MITF) regulates the expression of Bcl-2 and this contributes to the survival of melanocytes during melanogenesis (Step 5 in Module 7: Figure melanogenesis).

One of the functions of Bcl-2 is to contribute to inositol 1,4,5-trisphosphate receptor ($InsP_3R$) modulation (Module 3: Figure $InsP_3R$ regulation) and this may play a role in a number of cellular processes and some neural diseases:

- Bcl-2 interacts with Beclin-1 to regulate the process of autophagy (Module 11: Figure autophagy).
- Bcl-2 gene single nucleotide polymorphisms (SNPs) have been associated with a risk of developing manic depressive illness.
- There may be a protective role for Bcl-2 in Alzheimer's disease (AD). The calcium hypothesis of Alzheimer's disease (AD) suggests that memory loss may be caused by an elevation of the resting level of Ca^{2+} and some of this may result from an increase in $InsP_3$-dependent Ca^{2+} release (Module 12: Figure amyloids and Ca^{2+} signalling). The ability of Bcl-2 to reduce the symptoms of Alzheimer's disease (AD) may be explained by its ability to reduce the release of Ca^{2+} by inhibiting $InsP_3$ receptors.

The expression of Bcl-2 is repressed by miR-15 and miR-16. These two miRs are often deleted or down-regulated in many cases of chronic lymphocytic leukaemia (CLL).

Module 11: | Figure Bcl-2 superfamily domain structure



Domain structure of the Bcl-2 superfamily.
The function of the different domains is described in the text. Many members of the superfamily have a transmembrane (TM) domain that enables them to insert into membranes.

Bcl-X_L

The Bcl-X_L anti-apoptotic factor functions much like Bcl-2 to inhibit apoptosis by curbing the activity of Bak (Module 11: Figure Bcl-2 family functions).

BH3-only family

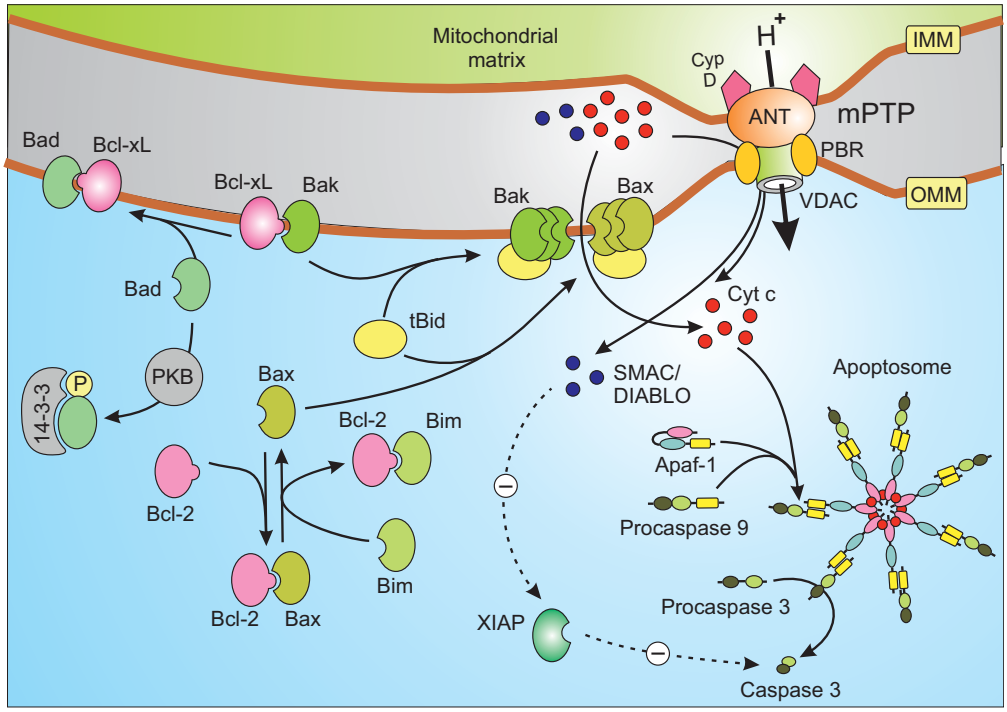
Bad

Bad promotes apoptosis by binding to Bcl-X_L, which is a potent inhibitor of cell death. Following phosphorylation by protein kinase B (PKB) or protein kinase A (PKA), Bad binds instead to 14-3-3 protein, thereby releasing the Bcl-X_L that then inhibits apoptosis by binding to the pro-apoptotic factor Bak (Module 11: Figure Bcl-2 family functions).

Bid

BH3-interacting domain death agonist (Bid) is constitutively expressed in the cytoplasm, where it has a specific role in linking together the extrinsic and intrinsic pathways (Step 3 in Module 11: Figure apoptosis). Caspase 8, which is activated by the extrinsic pathway (Module 11: Figure TNFα apoptotic signalling), cleaves Bid to a C-terminally truncated fragment (tBid), which translocates to the mitochondrion where it triggers the activation of Bak and Bax by inducing the oligomerization and permeabilization of the mitochondrial membrane (Module 11: Figure Bcl-2 family functions). This conversion of Bid into tBid can also be carried out by cathepsin D that is activated by ceramide during sphingomyelin signalling (Module 2: Figure sphingomyelin signalling).

Module 11: | Figure Bcl-2 family functions



Intrinsic apoptotic events at the mitochondrion responsible for the release of cytochrome *c* and the formation of the apoptosome.
A critical event of the intrinsic pathway is the release of cytochrome *c* (Cyt c) through mechanisms that are still being investigated. One hypothesis considers that formation of the mitochondrial permeability transition pore (mPTP) provides an avenue for cytochrome *c* to pass across the outer mitochondrial membrane (OMM). Another hypothesis is that Cyt c passes across channels formed by the polymerization of pro-apoptotic factors such as Bak and Bax, which are members of the Bcl-2 superfamily. Whether or not Bax and Bak can polymerize depends on a complex web of interactions with other members of the Bcl-2 superfamily. Bax is kept quiescent by being bound to the anti-apoptotic factor Bcl-2, but the inhibitory action can be negated by the pro-apoptotic factor Bim. Likewise, Bak is inhibited by Bcl-X_L, but this inhibition can be reversed by Bad. The ability of Bad to remove Bcl-X_L is regulated by the PtdIns 3-kinase signalling pathway, which acts through protein kinase B (PKB) that phosphorylates Bad, which is then taken out of action by being bound to 14-3-3 protein.

The tBid can also induce apoptosis by facilitating the release of cytochrome *c* by disrupting the optic atrophy 1 (OPA1) barrier that closes off the opening of the mitochondrial cristae (Module 5: Figure OPA1 and mitochondrial cristae remodelling).

Bim

The Bcl-2 interacting mediator of cell death (Bim) acts as a pro-apoptotic factor by binding to and neutralizing anti-apoptotic factors such as Bcl-2 and Bcl-X_L.

Noxa

The expression of Noxa is enhanced by the pro-apoptotic transcription factor p53 (Module 9: Figure proliferation signalling network). Noxa can also be induced by activating transcription factor 4 (ATF4) following endoplasmic reticulum (ER) stress (Module 2: Figure ER stress signalling).

Puma

Like Noxa, the expression of p53 up-regulated modulator of apoptosis (Puma) is increased by the pro-apoptotic transcription factor p53, and thus contributes to p53-induced apoptosis (Module 9: Figure proliferation signalling network). PUMA can also be induced by activating transcription factor 4 (ATF4) following endoplasmic reticulum (ER) stress (Module 2: Figure ER stress signalling).

Multidomain (BH) family

Bak

Bak is normally resident in the outer mitochondrial membrane (OMM), where it exerts its pro-apoptotic function by oligomerizing to form channels through which apoptogenic factors such as cytochrome *c* are released. The activity of Bak depends upon the balance of other pro- and anti-apoptotic factors. Bak activity can be neutralized by binding to Bcl-X_L. If the Bak/Bcl-X_L dimer is disrupted by the removal of Bcl-X_L by Bad, the monomeric Bak can oligomerize through a process that can be facilitated by tBid (Module 11: Figure Bcl-2 family functions).

Bax

(Bax) is a pro-apoptotic factor that is highly homologous with Bak and acts in much the same way in that it oligomerizes to form channels in the mitochondrial membrane. As its name implies, it can form dimers with the anti-apoptotic protein Bcl-2, and this inhibits its activity (Module 11: Figure Bcl-2 family functions).

Bcl-2 superfamily control of Ca^{2+} signalling

It has been known for some time that members of the Bcl-2 superfamily not only bind to the mitochondria, but are also found on the endoplasmic reticulum (ER), where they may act to modulate Ca^{2+} signalling by adjusting the operation of the endoplasmic reticulum (ER)/mitochondrial Ca^{2+} shuttle (Module 5: Figure ER/mitochondrial shuttle). There is general agreement that Bcl-2 seems to act by reducing the amount of Ca^{2+} being transferred from the ER to the mitochondria, but there is some controversy as to the exact mechanism. The opposing views are centred on the question of whether or not Bcl-2 acts to reduce the level of Ca^{2+} in the ER lumen. Some consider that Bcl-2 promotes the passive leak of Ca^{2+} through the inositol 1,4,5-trisphosphate ($InsP_3$) receptor, which then reduces the amount of Ca^{2+} stored in the lumen. Such a mechanism has been implicated in local Ca^{2+} signalling and autophagy (Module 11: Figure autophagy). The alternative view is that Bcl-2 acts by altering the sensitivity of the $InsP_3$ receptor, thereby reducing the amount of Ca^{2+} being released during stimulation. Despite this uncertainty as to the exact mechanism, there does seem to be some agreement that Bcl-2 plays an important role in regulating apoptosis by interacting with the $InsP_3$ receptor to reduce the load of Ca^{2+} that is imposed upon the mitochondrion by the ER during cell signalling. The pro-apoptotic factors Bak and Bax can promote Ca^{2+}-induced apoptosis by binding Bcl-2, thereby removing the ability of this anti-apoptotic factor to reduce Ca^{2+} signalling.

Bcl-2 has been shown to interact with the $InsP_3$ receptor to control the phosphorylation of Ser-1755, which seems to be critical for altering the release of Ca^{2+}. The Bcl-2 may act either by promoting the activity of the protein kinase A (PKA)/A-kinase-anchoring protein (AKAP) complex that carries out the phosphorylation or by removing the calcineurin (CaN) that dephosphorylates Ser-1755.

Bcl-2 gene single nucleotide polymorphisms have been associated with a risk of developing bipolar disorder.

The calcium hypothesis of Alzheimer's disease (AD) suggests that memory loss may be caused by an elevation of the resting level of Ca^{2+} and some of this may result from an increase in $InsP_3$-dependent Ca^{2+} release (Module 12: Figure amyloids and Ca^{2+} signalling). The ability of Bcl-2 to reduce the symptoms of Alzheimer's disease may be explained by its ability to reduce the release of Ca^{2+} by inhibiting $InsP_3$ receptors.

Caspase cascade

Signals coming either from the death receptors or from the mitochondria activate the caspase cascade (Module 11: Figure apoptosis). A family of caspases, which exist in the cell as dormant proenzymes, respond to the various induction signals by initiating a proteolytic cascade that produces active heterodimers responsible for the final degradation phase. The sequence begins with the initiator caspases (e.g. caspases 8, 9 and 10). Caspases 8 and 10 contain a tandem motif of death effector domains (DEDs), whereas caspase 9 has a caspase-recruitment domain (CARD) (Module 11: Figure TNFα apoptotic signalling). These initiator caspases are responsible for activating the executioner caspases (e.g. caspases 3, 6 and 7). The latter then cleave specific polypeptides [e.g. poly(ADP-ribose) polymerase, DNA-dependent protein kinase, lamins and actin]. Caspase 3 cleaves the inhibitor of caspase-activated DNAase (iCAD) to release CAD that begins to hydrolyse DNA. Through this co-ordinated sequence of protein and DNA hydrolytic events, the cell begins its regulated disassembly.

Inhibitor of apoptosis family of proteins (IAPs)

There are five members of the inhibitor of apoptosis (IAP) family of proteins, which function to inhibit specific caspases. A prominent member of the family is X-chromosome-linked inhibitor of apoptosis protein (XIAP), which is a potent inhibitor of caspases 3, 7 and 9, can prevent cell death induced by TNF-α, Fas and UV light. This inhibitory activity of XIAP is, in turn, inhibited by the second mitochondrial-derived activator of caspases (SMAC), which is also known as the direct IAP-binding protein with low pI (DIABLO). SMAC/DIABLO is released from the mitochondria to enhance apoptosis by reducing the activity of XIAP (Module 11: Figure Bcl-2 family functions).

A t(11;18)(q21;q21) chromosomal translocation of the MALT1 gene to the genomic locus of the inhibitor of apoptosis gene cIAP to give an *API2-MLT* fusion gene has been linked to MALT lymphomas.

Another member of the IAP family is the baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5, also known as survivin) that is controlled by the hippo signalling pathway (Module 2: Figure hippo signalling pathway).

Caspase-independent pathway

Some of the factors such as apoptosis-inducing factor (AIF) and endonuclease G (EndoG) released by the mitochondria during the operation of the intrinsic pathway can activate apoptosis independently of caspases (Step 5a in Module 11: Figure apoptosis). These two factors translocate into the nucleus, where they function to degrade DNA.

Hormonal modulation of apoptosis

Apoptosis is tightly regulated by a number of signalling mechanisms that operate to either adjust the activity of pre-existing components or to alter the expression levels of these components (Steps 8 and 9 in Module 11: Figure apoptosis). Both mechanisms can be controlled by the PtdIns 3-kinase signalling pathway, which plays a major role as a negative regulator of apoptosis. One of its functions depends on the phosphorylation of the pro-apoptotic factor Bad on Ser-136, which increases its interaction with 14-3-3 protein and thus prevents Bad from inactivating Bcl-X_L (Module 11: Figure Bcl-2 family functions). PtdIns 3-kinase signalling can also modulate apoptosis by altering the expression of the pro-apoptotic factor Bim (Module 4: Figure FOXO control mechanisms). Survival factors use this signalling pathway to remove Forkhead box O (FOXO) from the nucleus, thereby reducing the expression of Bim. When survival factors are removed and

signalling through the PtdIns 3-kinase pathway is decreased, FOXO returns to the nucleus to begin the transcription of Bim, which will help to bias the repertoire of apoptotic factors towards apoptosis.

The operation of the anti-apoptotic factor Bcl-2 is also influenced by phosphorylation. For example, the phosphorylated form of Bcl-2 is less effective in binding to Bim and Bax. The dephosphorylation of Ser-70 on Bcl-2 by calcineurin (CaN) seems to be necessary for its anti-apoptotic activity. Phosphorylation may also influence the Bcl-2 superfamily control of Ca^{2+} signalling, because Bcl-2 is better able to modulate endoplasmic reticulum (ER) Ca^{2+} signalling when it is dephosphorylated.

Pathogens such as *Shigella flexneri* and *Salmonella enterica* serotype Typhimurium, which cause bacillary dysentery and food poisoning respectively, manipulate this hormonal adaptation of apoptosis to prolong the survival of their host cells.

References

Inflammation

Benoist, C. and Mathis, D. (2002) Mast cells in autoimmune disease. Nature 420:875–878.

Bieber, J. and Chi, D.S. (2001) The human mast cell: functions in physiology and disease. Front. Biosci. 6:D1109–D1127.

Marshall, J.S. (2004) Mast-cell responses to pathogens. Nat. Rev. Immunol. 4:787–799.

Krishnaswamy, G., Kelley, J., Johnson, D., Youngberg, G., Stone, W., Huang, S.-K., Martin, P. and Leibovich, S.J. (2005) Inflammatory cells during wound repair: the good, the bad and the ugly. Trends Cell Biol. 15:599–606.

Nathan, C. (2002) Points of control in inflammation. Nature 420: 846–852.

Tracey, K.J. (2002) The inflammatory reflex. Nature 420:853–859.

Blood platelets

Brass, L.F., Zhu, L. and Stalker, T.J. (2005) Minding the gaps to promote thrombus growth and stability. J. Clin. Invest. 115:3385–3392.

Furie, B. and Furie, B.C. (2005) Thrombus formation *in vivo*. J. Clin. Invest. 115:3355–3362.

Gachet, C. (2006) Regulation of platelet functions by P2 receptors. Annu. Rev. Pharmacol. Toxicol. 46:277–300.

Nieswandt, B. and Watson, S.P. (2003) Platelet–collagen interaction: is GPIV the central receptor? Blood 102:449–461.

Nurden, A.T. and Nurden, P. (2006) Inherited disorders of platelets: an update. Curr. Opin. Hematol. 13:157–162.

Mast cells

Benoist, C. and Mathis, D. (2002) Mast cells in autoimmune disease. Nature 420:875–878.

Galli, S.J., Kalesnikoff, J., Grimbaldeston, M.A., Piliponsky, A.M., Williams, C.M.M. and Tsai, M. (2005) Mast cells as "tunable" effectors and immunoregulatory cells: recent advances. Annu. Rev. Immunol. 23:749–786.

Krishnaswamy, G., Kelley, J., Johnson, D., Youngberg, G., Stone, W., Huang, S.-K., Bieber, J. and Chi, D.S. (2001) The human mast cell: functions in physiology and disease. Front. Biosci. 6: 1109–1127.

Marshall, J.S. (2004) Mast-cell responses to pathogens. Nat. Rev. Immunol. 4:787–799.

Neutrophils

Charest, P.G. and Firtel, R.A. (2007) Big roles for small GTPases in the control of directed cell movement. Biochem. J. 401:377–390.

Chen, Y., Corriden, R., Inoue, Y., Yip, L., Hashiguchi, N., Zinkernagel, A., Nizet, V., Insel, P.A. and Junger, W.G. (2006) ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. Science 314:1792–1795.

Nishio, M., Watanabe, K., Sasaki, J., Taya, C., Takasuga, S., Iizuka, R., Balla, T., Yamazaki, M., Watanabe, H., Itoh, R., Kuroda, S., Horie, Y., Forster, I., Mak, T.W., Yonekawa, H., Penninger, J.M., Kanaho, Y., Suzuki, A. and Sasaki, T. (2007) Control of cell polarity and motility by the PtdIns(3,4,5)P_3 phosphatase SHIP1. Nat. Cell Biol. 9:36–44.

Van Haastert, P.J.M. and Devreotes, P.N. (2004) Chemotaxis: signalling the way forward. Nat. Rev. Mol. Cell. Biol. 5:626–634.

Wei, C., Wang, X., Chen, M., Ouyang, K., Song, L.-S. and Cheng, H. (2009) Calcium flickers steer cell migration. Nature 457:901–905.

Senescence

Campisi, J. (2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbours. Cell 120:513–522.

Schmitt, C.A. (2003) Senescence, apoptosis and therapy: cutting the lifelines of cancer. Nat. Rev. Cancer 3:286–295.

Shay, J.W. and Roninson, I.B. (2004) Hallmarks of senescence in carcinogenesis and cancer therapy. Oncogene 23:2919–2933.

Autophagy

Baehrecke, E.H. (2005) Autophagy: dual roles in life and death? Nat. Rev. Mol. Cell Biol. 6:505–510.

Lum, J.J., DeBerardinis, R.J. and Thompson, C.B. (2005) Autophagy in metazoans: cell survival in the land of plenty. Nat. Rev. Mol. Cell Biol. 6:439–448.

Intrinsic pathway

Henry-Mowatt, J., Dive, C., Martinou, J.-C. and James, D. (2004) Role of mitochondrial membrane permeabilization in apoptosis and cancer. Oncogene 23:2850–2860.

Mayer, B. and Oberbauer, R. (2003) Mitochondrial regulation of apoptosis. News Physiol. Sci. 18:89–94.

Ca^{2+}-induced apoptosis

Kristián, T. and Siesjö, B.K. (1998) Calcium in ischemic cell death. Stroke 29:705–718.

McConkey, D.J. and Orrenius, S. (1997) The role of calcium in the regulation of apoptosis. Biochem. Biophys. Res. Commun. 239:357–366.

Orrenius, S., Zhivotovsky, B. and Nicotera, P. (2003) Regulation of cell death: the calcium–apoptosis link. Nat. Rev. Mol. Cell Biol. 4:552–565.

Simpson, P.B. and Russell, J.T. (1998) Role of mitochondrial Ca^{2+} regulation in neuronal and glial cell signalling. Brain Res. Rev. 26:72–81.

Bcl-2 superfamily

Distelhorst, C.W. and Shore, G.C. (2004) Bcl-2 and calcium: controversy beneath the surface. Oncogene 23:2875–2880.

Oakes, S.A., Scorrano, L., Opferman, J.T., Bassik, M.C., Nishino, M., Pozzan, T. and Korsmeyer, S.J. (2005) Proapoptotic BAX and BAK regulate the type 1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum. Proc. Natl. Acad. Sci. U.S.A. 102:105–110.

Palmer, A.E., Jin, C., Reed, J.C. and Tsien, R.Y. (2004) Bcl-2-mediated alterations in endoplasmic reticulum Ca^{2+} analysed with an improved genetically encoded fluorescent sensor. Proc. Natl. Acad. Sci. U.S.A. 101:17404–17409.

Scorrano, L., Oakes, S.A., Opferman, J.T., Cheng, E.H., Sorcinelli, M.D., Pozzan, T. and Korsmeyer, S.J. (2003) BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science 300:135–139.

