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Evolution of Cell-Autonomous Effector Mechanisms in Macrophages versus Non-Immune Cells

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Summary

Specialized adaptations for killing microbes is synonymous with phagocytic cells including macrophages, monocytes, inflammatory neutrophils and eosinophils. Recent genome sequencing of extant species, however, reveals analogous antimicrobial machineries exist in certain non-immune cells and also within species that ostensibly lack a well-defined immune system. Here we probe the evolutionary record for clues about the ancient and diverse phylogenetic origins of macrophage killing mechanisms and how some of their properties are shared with cells outside the traditional bounds of immunity in higher vertebrates such as mammals.

INTRODUCTION

Microorganisms maintain an evolutionary advantage over their slowly replicating eukaryotic hosts. High mutation rates, rapid doubling times and free genetic exchange between microbial species often place a considerable burden on the infected host to counter virulence escape mechanisms. This selective pressure has driven the acquisition of numerous eukaryotic defense strategies to protect host genome integrity and promote survival at the level of the individual cell (1). These cell-autonomous effector mechanisms, often considered unique to the immune cells of advanced metazoans, have in fact been largely inherited and repurposed from our eukaryotic ancestors (Fig. 1). For example, phagocytosis developed as a trophic mechanism in unicellular amoebae long before its adaptation as a tool for immunity in the specialized ‘immune-like’ cells of early invertebrates (2, 3). Amebocytes, haemocytes and coelomocytes present in lower organisms likewise predate professional phagocytes in animals with their ability to bind, engulf and kill foreign microorganisms (4).

The appearance of multicellular organization ~600 million years ago (Mya), coupled with the evolutionary arms race between host and microbe, saw the eventual emergence of a dedicated immune system (4). With this came a remarkable degree of specialization to

counter the temporal and genetic advantage held by pathogens. In animals, hematopoietic cells developed extensive machinery to detect and respond to microbial and cellular host signatures through unique immunoreceptors. Such receptors endow immune cells with a capacity to survey, sequester, and ultimately destroy microbial pathogens as well as produce paracrine and autocrine signaling molecules that invoke drastic changes in local tissue microenvironments (5).

With the advent of multicellularity, however, came a new challenge: pathogen cell tropism. Here the increased number and diversity of host cell types offered potential refuge for taxonomically distinct microbes that target selected lineages for replication. As a consequence, intrinsic defense mechanisms also emerged in non-immune cells as well (1,6). Many of these restriction factors evolved from the defense arsenal of lower organisms like that seen for the classical immune system, and are thus operative in most nucleated cells. For example, nitric oxide synthases (NOSs) serve an antimicrobial function not just in mammalian macrophages but also in hepatocytes, neurons, fibroblasts and smooth muscle (6, 7). These mammalian NO-mediated killing mechanisms were, in turn, presaged by those found in flies (8), crustaceans (9), and even Gram-positive bacteria (10).

In this chapter, we probe the evolutionary record for clues about the ancient and diverse phylogenetic origins of macrophage killing mechanisms. We demonstrate how some of their properties are shared with cell lineages outside the traditional bounds of immunity in higher vertebrates such as mammals and speculate on their historical legacy for cell-autonomous defense.

PHYLOGENETIC ORIGINS OF MACROPHAGE KILLING MECHANISMS

Amoeboid Defenses

The phylum Amoebozoa emerged soon after the divergence of plants, forming a sister group to animals and fungi that provide a glimpse into the effector mechanisms operating before the divergence of Metazoans (11–13). These unicellular organisms are highly phagocytic cells in perpetual contact with bacteria in the environment. Much of the basic machinery and signal transduction pathways of phagocytosis are evolutionarily conserved between amoeba and vertebrate macrophages, reflecting the ancient origins of this process (14). Model amoeba like *Dictyostelium discoideum* can offer invaluable insights into specific bacterial killing mechanisms that have been retained in the common ancestor of plants and animals. The production of superoxide radicals (O_2^-) by the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) complex that becomes targeted to the phagosome during phagocytosis is one such example (15). In mammalian macrophages, this machinery is a potent antimicrobial pathway and there is some evidence for respiratory burst activity in *Acanthamoebae* (16). *Dictyostelium* strains lacking NADPH oxidases, however, still display normal phagocytosis and bacterial killing profiles (17). Thus *Dictyostelium* reveals the existence of other cell-intrinsic killing mechanisms besides oxidant defense. For these amoebae, the bulk of bacterial restriction appears reliant on vacuole acidification, when acidic vacuoles containing the V-H⁺ ATPase and lysosomal hydrolases fuse with the contractile vacuole to generate the phagolysosome (18–20). Mammalian macrophages also enlist phagolysosomal killing in certain settings as well (6).

A survey of the *Dictyostelium* genome reveals an extensive repertoire of pore-forming peptides that resemble human defensins (21), and the amoebapore used by *Entamoeba histolytica* is a homologue of the NK-lysin produced by cytotoxic T lymphocytes (22). Indeed, the amoebapore shares an unusual pattern of cysteines with other saposin-like proteins found across phylogeny from nematodes to mammals (23). Like mammalian antimicrobial defensins, these peptides use highly charged residues to permeabilize the bacterial membrane and cause bacteriolysis. In Amoebozoa, they are delivered to the contractile vacuole to aid in bacterial killing before degradation by the lysosomal hydrolases. Among these hydrolases is lysozyme which plays a key role in host defense in animals. Several lysozyme-encoding genes are present within the *D. discoideum* genome, belonging to either the bacteriophage T4 or conventional (C-type) families that exhibit preferential activity against Gram-positive bacteria (14).

Whereas amoebae engulf, kill, and digest bacteria primarily as a food source, they can occasionally serve as hosts for parasitic bacteria like *Legionella* and *Mycobacterium* that enter during phagocytosis to survive intracellularly. Accordingly, amoebae have evolved effector mechanisms that attempt to restrict the growth of these facultative bacteria. Natural Resistance Associated Membrane Protein (NRAMP)1, a divalent metal transporter that is widely distributed from bacteria to humans and found on pre-lysosomal vesicles prior to vacuole acidification (24), is deployed against both *Mycobacteria* and *Legionella* spp. to combat infection. Eukaryotic NRAMPs transport Fe^{2+} and Mn^{2+} (25, 26) whereas bacterial homologs solely transport Mn^{2+} (27). In mammals, two NRAMP isoforms exist - NRAMP1 (SLC11A1) and NRAMP2 (SLC11A2) - with the former expressed exclusively within the endocytic pathway of macrophages and the latter exhibiting plasma membrane expression across many tissue types (25).

The *Dictyostelium* genome likewise encodes two *NRAMP* genes with NRAMP1 orthologous to mammalian NRAMP1 as it contains a single N-terminal intron conserved in all eukaryotes (28) and is localized to phagosomal membranes. This transporter is essential for amoeboid defense against *Mycobacteria* and *Legionella* since genetic disruption of NRAMP1 renders *Dictyostelium* more susceptible to these pathogenic bacilli (29, 30). Interestingly, phagocytosis and digestion of non-pathogenic bacteria is unimpaired in the NRAMP1 mutant, suggesting it is positioned solely for defense against virulent organisms (29). *Dictyostelium* NRAMP1 arguably represents the first appearance of a so-called “nutriprive” mechanism during evolution, using the proton gradient in the phagosome to electrogenically pump Fe^{2+} out of the bacteria-containing vacuole (31). This depletion restricts the intracellular growth of *Legionella* and *Mycobacteria* that normally replicate within the phagosome by interfering with recruitment of the V-H^+ ATPase and concomitant vacuole acidification.

In addition to the free-living form that feed on soil bacteria, *Dictyostelium* is a social amoeba, inasmuch that during starvation conditions, solitary amoebae aggregate into large groups to form a multicellular slug. This slug can migrate for several days before forming a fruiting body complete with a cellular stock and environment-resistant spores (32). During the multicellular slug-stage, the selective pressure to avoid parasitic exploitation is increased (33). Remarkably, this pressure appears to have driven a subgroup of these amoebae, termed

sentinel cells (S-cells), to develop immune-like functions complete with Toll/Interleukin-1 receptor domain signaling pathways (34) and the ability to deploy extracellular DNA nets (or traps) that can sequester and kill bacteria (35). Thus, amoebae enlist both offensive and defensive effector mechanisms that are conserved throughout the evolution of mammals, implying that the ability to kill bacteria, either as a food source or for self-defense, emerged well before the appearance of metazoans.

Invertebrate Defenses

The rise of multicellularity during the Paleozoic era (541-252 Mya) brought about both an expansion of amoeboid effector mechanisms and the evolution of a new group of effector molecules that protect larger multicellular animals from infection. Contrary to popular belief, both invertebrates and vertebrates employ specialized cell types that harness the phagocytic and antimicrobial abilities of amoebae to function as specialized “immune-like” progenitors (36). The most primitive groups, the sponges and cnidarians, carry single specialized cell types, amoebocytes or interstitial cells respectively, that perform a phagocytic function in their respective organ (3). The evolutionary importance of these cell types is reflected by their rapid diversification in more advanced invertebrates (echinoderms and urochordates) where both granulocyte- and macrophage-like cells become apparent (37–39). However, even in invertebrate animals, these defense mechanisms are not exclusive to immune-like cells. Antimicrobial effector mechanisms are found in organs that are not traditionally considered to have evolved immune-like functions, such as the epithelia of reproductive, respiratory and digestive tracts in *Drosophila* (40) or the midgut epithelium of the *Anopheles gambiae* mosquito (41).

Together with phagocytosis, antimicrobial peptides (AMPs) represent the most fundamental defense mechanism across all phylogeny and comprise the main effector arm of the invertebrate immune system. In contrast with Amoebozoa where AMP production is constitutive (42), pathogens or their products selectively induce AMPs in complex invertebrates such as *C. elegans* (43) and *D. melanogaster* (44). AMPs can be produced by phagocytic cells, such as in arthropods, annelids, and urochordates to aid bacterial destruction (36, 45), or by somatic cells of the fat body and epithelia of renal tubules and the digestive tract in *Drosophila* (8, 40). Here flies infected with entomopathogenic fungi trigger signaling via the Toll and Imd pathways to elicit AMPs with specific antifungal activities (46). In addition to their role in cell-autonomous defense, AMPs serve as the systemic arm of the invertebrate immune response, some circulating in the hemolymph for several days.

Invertebrate phagocytes readily produce reactive oxygen (ROS) and nitrogen species (RNS) during phagocytosis to aid in microbial killing. Nitric oxide (NO) is a highly reactive labile gas made by NO synthases (NOSs) from the guanidino nitrogen of L-arginine and molecular oxygen. It serves numerous biological roles related to metamorphosis, feeding, nervous system and vascular signaling, along with its fundamental role in host defense (7, 9). Genomic analysis of marine invertebrate NOSs indicate that the cofactor binding sites for FAD, NADPH, heme, tetrahydrobiopterin and calmodulin found in mammalian NOSs are all well conserved in these lower organisms (9). Moreover, some marine species, such as the sea urchin *Arbacia punctulata*, encode multiple *NOS* genes that display striking homology to

both constitutive (NOS1, NOS3) and inducible (NOS2) NOSs in mammals (47), reaffirming the long ancestry of this system. The *Anopheles* mosquito NOS displays the highest homology with mammalian inducible NOSs, complete with a lipopolysaccharide (LPS) and inflammatory cytokine response elements in its promoter (48). *Anopheles* NOS is induced by the JAK/STAT pathway in the midgut during infection with *Plasmodium falciparum* and acts to inhibit oocyst development and promote immunity (41).

Perhaps the most primordial NO defense network in eukaryotes appears with the emergence of the sponges some 600 Mya where dendritic-like cells in the parenchyma generate NO under a variety of stress conditions (49). As a reflection of the system's antiquity, haemocytes from the living fossil horseshoe crab *Limulus polyphemus*, an ancestor to both mammalian platelets and macrophages, deploy NO to regulate their aggregation (platelet function) and antimicrobial properties (macrophage function) (9). Similarly, haemocytes of the molluscs *Mytilus edulis* and *Viviparus ater* produce NO during the later stages of phagocytosis and confer cytotoxicity by inducing bacterial clumping (50, 51). Again, a role for NOS in antibacterial defense is not limited to immune-like cells, as peripheral tissue in renal tubules in *Drosophila* and *Anopheles* can autonomously respond to microbial challenge by inducing NOS (8). In addition to being directly antimicrobial, NO in turn increases systemic production of the AMP dipterin which is required for whole organism survival following bacterial challenge (52).

Besides RNS, reactive oxygen species (ROS) are also generated from reduction of molecular oxygen to superoxide ($O_2^{\cdot-}$) by the NADPH oxidase (NOX2) complex in a reaction known as the respiratory burst. A variety of highly reactive oxygen species are then formed that are directly injurious to microbes (53). In contrast to NOS which can be produced by immune and non-immune cells, NOX2 generation of ROS is tightly linked with phagocytosis, and is produced by immune-like cells across phylogeny; these span haemocytes from the cockroach *Blaberus discoidalis* (54) or the mussel *Mytilus galloprovincialis* (55) to the dendritic-like cells of the marine sponge *Sycon sp.* (56). In mammals, NOX2 is a multicomponent complex consisting of membrane bound subunits (p22^{phox} and gp91^{phox}; [cytochrome_{b558}]), cytosolic subunits (p40^{phox}, p47^{phox}, p67^{phox}) and members of the Ras superfamily of GTP-binding proteins (Rac1 or Rac2). During phagocytosis or cell activation, the cytosolic components are phosphorylated and relocate to the phagocytic cup, assembling into an active holoenzyme that uses molecular oxygen on the luminal side of the phagosome to generate ROS for killing engulfed prey (36, 57).

Homologous enzymes can be found in most eukaryotes, but to date no evidence of their utilization is found among prokaryotes. Even so, the presence of a *bis*-heme binding motif in gp91^{phox} suggests possible evolutionary links with prokaryotic *b* type cytochromes (58). gp91^{phox} appears the most ancient subunit, being present before the divergence of choanoflagellates, the closest living relatives to animals. However, gp91^{phox} seems to have been lost in some exuviating invertebrates (*Ecdysozoa*) during evolution of the protostomes, and is missing from the haemocytes of *Drosophila* and *C. elegans* (36). Similarly, the other membrane-associated subunit p22^{phox} can be found in *Choanoflagellata*, *Mollusca*, and *Cnidaria*, but not in *Annelida*, *Ecdysozoa*, and *Echinodermata*, suggesting ROS generation by *Drosophila* and *C. elegans* differs from the mechanism used by other metazoans (59).

Surprisingly, the intracellular killing mechanisms used by the haemocytes of these invertebrate model organisms remain largely undescribed.

Other sources of ROS besides NOX2 are evident in non-immune cells across phylogeny. In *Drosophila*, dual oxidases (DUOXs) harbor an NADPH domain and an N-terminal peroxidase domain to generate $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2). DUOXs are located on the plasma membranes of epithelial cells lining the digestive tract and produce extracellular H_2O_2 in response to bacteria (60) or their metabolites such as uracil (61). DUOXs serve a critical function in maintaining gut-microbe homeostasis as DUOX RNAi-expressing flies succumb to foodborne bacterial infections (62). This mucosal expression pattern is conserved among mammals with DUOX1 and DUOX2 in the human gastrointestinal and respiratory tracts, respectively (63, 64). However, unlike their noted impact on *Drosophila* and *C. elegans* immunity, the importance of DUOX in mammalian immunity remains largely uncharacterized. Invertebrates thus use ROS, NOS, phagocytosis and AMPs like many other taxa to combat infection. In addition, they have evolved their own defense modules, namely, lamelocyte-driven encapsulation and melanization that are unique to the invertebrate group (65). For the purpose of comparison with mammalian macrophages and other myeloid cells, however, these divergent mechanisms are not discussed here.

Jawless and jawed fishes

As the oldest vertebrates, fish are best known for the traceable evolution of the adaptive immune system. The development of lymphoid follicles, immunoglobulin and MHC molecules, and the B- and T-cell subsets can each be ascribed in a linear fashion to the appearance of the jawless (agnathans), cartilaginous, and bony fish respectively. This new adaptive arm complemented the more ancient innate effector mechanisms described above. Indeed, many of the primitive phagocytic and antimicrobial responses of invertebrates and amoebae are conserved in fish (and mammals) as well. In addition, however, an inducible defense circuitry specific to vertebrates has also emerged, possibly to limit self-injury by regulating the production of harmful effector molecules mobilized during repeated microbial encounters over a longer lifespan.

The family of antimicrobial peptides termed cathelicidins can be traced to hagfish, which diverged from the gnathostomes 500 Mya (66). Cathelicidins differ from the more ancient defensin-like AMPs, as they are synthesized as precursor proteins containing a conserved signal peptide and cathelin-like spacer domain followed by a highly variable antimicrobial domain (67). In hagfish, three cathelicidin proteins are synthesized by groups of myeloid cells within the loose connective tissue of the gut wall, perhaps a precursor to gut-associated lymphoid tissue (GALT), and defend against intestinal bacteria that accompany ingestion of dead and decaying food sources (66). Similarities emerge with human cathelicidin LL-37 that is not only found within granules of macrophages and neutrophils but also in gut epithelia (68), implying a broader role in host defense than previously thought. In a manner similar to fish cathelicidins, LL-37 is cleaved into its active form in response to inflammatory stimuli and released by exocytosis. Electrostatic interactions between the positively charged region of these cathelicidins and negatively charged bacterial membrane

promotes bacteriolysis (68). Humans lacking a functional LL-37 develop Chediak-Higaski syndrome and are highly predisposed to bacterial infections (69).

Gene duplication events after the agnathan-gnathosome split also gave rise to two of the three NOS isoforms currently found in mammals, namely neuronal NOS (nNOS; NOS1) and inducible NOS (iNOS; NOS2) (59). In contrast to invertebrate NOS, which bears closest homology to vertebrate NOS1 and can be constitutive or inducible depending on the species (70), vertebrate NOS2 is robustly induced in immune cells by microbial components like LPS or immunoregulatory cytokines like interferon (IFN)- γ . This generates copious amounts of NO during pathogen challenge and fish NOS2 has been described as essential for resisting challenge to the trout pathogen *Y. ruckeri* (71), and the salmonid pathogens *A. salmonidica* and *R. salmoninarum* (72, 73).

Nutritive defense mechanisms are also subject to inducible control. Teleost NRAMP1, which is constitutively expressed in amoebae, is responsive to LPS and IFN- γ transcriptional regulation akin to mammalian NRAMP1 (74). Like the mammalian isoform expressed in macrophages, fish NRAMP1 is involved in regulating chemokine secretion, iNOS activation, MHC class II upregulation and the respiratory burst (75). These novel roles supplement its antecedent function as a cation antiporter that depletes Fe^{2+} from the pathogen-containing vacuole (75, 76). Other teleost proteins regulate Fe^{2+} homeostasis for immunity. In mammals, the immune-inducible hormone hepcidin binds to the macrophage iron exporter ferroportin-1 and drastically decreases both extracellular and intracellular iron levels (77). Teleosts express several hepcidin orthologs depending on species, and consistent with their antimicrobial role, they are robustly induced during pathogen challenge (78). Interestingly, whereas mammalian hepcidin is mainly produced by the liver, macrophages themselves are the primary source of teleost hepcidin (79), hinting at a divergence in the cell-autonomous defense mechanisms of mammalian and fish hepcidin orthologues.

Marsupial Mammals

The marsupial and placental mammals diverged 130 Mya and thus offer clues to the phylogenetic origins of mammal specific effector mechanisms. One such mechanism is intracellular starvation by IFN- γ -induced degradation of the amino acid tryptophan through the kynurenin pathway. IFN- γ induces robust expression of the enzyme, indoleamine 2,3 dioxygenase-1 (IDO1), which depletes cytosolic tryptophan stores and restricts the growth of tryptophan auxotrophic microbes like *Toxoplasma gondii* and *Chlamydia trachomatis* (80). IDO has also been implicated in preventing the spread of viral pathogens; namely herpes simplex virus, human cytomegalovirus, and measles virus in both immune and non-immune cells (81–83). Moreover in myeloid cells, the kynurenine catabolites generated during tryptophan degradation can exert direct antibacterial activity, as evidenced by their toxicity towards intracellular *Listeria monocytogenes* (84).

IDO orthologues exist in molluscs, amphibians and bony fish, however, biochemical characterization revealed that the K_m value for these enzymes was 500–1000 fold lower than mammalian IDO's, suggesting L-tryptophan is not the cognate substrate for these ancient IDO orthologs (74). In fact, these genes have been renamed proto-IDO's and their biochemical and physiological roles are unknown. Interestingly, tandem copies of IDO are

present on the chromosome of both the marsupial opossum and humans, whereas only single copies of the pro-IDOs are found in the genome of lower vertebrates and molluscs (85). This implies that the mammalian IDOs arose from a proto-IDO after a gene duplication event that occurred before the divergence of marsupial and placental mammals. In addition to IFN- γ , IDO is robustly induced by a variety of activating stimuli in macrophages, including lipopolysaccharide (LPS), tumor necrosis factor (TNF), and IFN- α/β (80, 86). The responsiveness of IDO to these stimuli varies considerably in immune and non-immune tissues, and in some organs like the lung IDO can even be constitutively expressed (80).

SPECIALIZED ADAPTATIONS OF MAMMALIAN MACROPHAGES: COMPARISON WITH ANTIMICROBIAL MACHINERIES IN NON-MYELOID CELLS

Cell-autonomous immunity is a process embodied by the classical macrophage. These cells harbor a vast armamentarium that restrict and in many cases eliminate the microbial pathogens they encounter (Fig. 2). Robust production of cytotoxic gasses (eg. $O_2^{\cdot-}$, H_2O_2 , $\cdot NO$), antimicrobial peptides, lysosomal hydrolases and nutritive immunity all ensure classical macrophages play key roles in pathogen restriction. The evolutionary record suggests these pathways have largely been inherited from the defense repertoires of early vertebrates, invertebrates, amoeba and, in some cases, prokaryotic bacterial or archaeal systems. A common ancestry implies that cell-types other than macrophages also benefit from genetic inheritance of their own. Indeed, antimicrobial properties are already apparent in the somatic cells of lower organisms. Many of these defense pathways, however, are placed under inducible control in mammals by inflammatory stimuli such as IFN- γ , a powerful transactivating signal that induces hundreds of host defense genes (6). Herein, we focus on major effector mechanisms employed by mammalian macrophages against intracellular pathogens and contrast it to what is known about these mechanisms in non-immune cells.

NOXs and NOSs: Radical Gasses

Diatomic radical gasses like $O_2^{\cdot-}$ and $\cdot NO$ arose very early during eukaryotic evolution as effector mechanisms and are still an essential component of mammalian host defense. In this respect, humans that lack superoxide-generating activity suffer from recurrent life threatening infections (87, 88) and mice with combined gp91^{phox} and Nos2 deficiencies are highly susceptible to infection by their own commensal flora (89), underscoring the importance of gas-based protection for microbial control. In mammals, $O_2^{\cdot-}$ is generated by mammalian NADPH oxidase (NOX) complexes 1–5 as well as by DUOX1/2. Macrophages prototypically employ NOX2, the phagocyte oxidase. Upon assembling together with active GTP-bound RAC1, this complex pumps an electron through two sequential cytochrome units and onto a terminal oxygen acceptor. The resultant superoxide molecule is able to covalently link free amines, cysteines, and DNA, especially when converted into other antimicrobial metabolites (eg: $\cdot OH$ and H_2O_2) (90). Interestingly, $O_2^{\cdot-}$ and $\cdot OH$ inefficiently permeate the bacterial membrane suggesting that membrane-permeable peroxide may be a more efficient antimicrobial. Lastly, H_2O_2 can be co-opted by myeloperoxidase in

neutrophils or lactoperoxidase in goblet cells to produce antimicrobial hypochlorite and thiocyanate, respectively (57, 90).

DUOX1 and DUOX2 are large enzymes generating $O_2^{\cdot-}$ that is rapidly converted to H_2O_2 on the apical side of epithelial cells. A favored hypothesis is that the combination of DUOX and lactoperoxidase enzymes generate thiocyanate in the extracellular space; it is unclear, however, if these enzymes play intracellular roles in bacterial restriction. In mammalian epithelial cells, NOX1 and DUOX systems appear sufficient to help combat infection despite the absence of a NOX2 machinery. NOX1 and DUOX systems are each regulated by distinct organizer and activator protein subunits with certain non-immune cell culture models (eg. HeLa cells) used for reconstitution assays as they lack DUOX1 and the activator component of DUOX2 (DUOXA2) (91, 92). Hence cell-type specificity may dictate whether oxidant or nitrosative mechanisms predominate.

In mammals, inducible nitric oxide (iNOS/NOS2) is robustly elicited by IFNs and contributes to host defense by reduction of the terminal amine of L-arginine. A 5-electron oxidation is employed where e^- are shuttled from NADPH through two tetra-biopterin (BH_4) groups before haem transfer coupling oxygen to arginine yielding nitric oxide gas and L-citrulline (7, 93). Nitrosamines and nitrosothiols are other derivatives arising from $\cdot NO$ production by iNOS to target intraphagosomal bacteria (74, 94). $\cdot NO$ can likewise react with ROS to yield the more potent congener, peroxynitrite [$ONOO^-$] (95). Reactive nitrogen species covalently link free amines and thiols and *S*-nitrosylate DNA to cripple their microbial targets. Interestingly, species divergence for NOS2 is seen in mammals; rodent phagocytes produce large amounts of $\cdot NO$ during pathogenic challenge but in human macrophages $\cdot NO$ release is greatly diminished, perhaps as a consequence of limited BH_4 cofactor availability (96).

Lysosomal repertoire

In 1955 Christian de Duve isolated a highly degradative organelle now known as the lysosome (97). These acidic organelles are the major bacterial killing machines of single cell amoebae, harboring many acid hydrolases to digest macromolecules and deliver nutrients to the cytosol. Echoing the early studies of Elie Metchnikoff who fed litmus paper to macrophages, later studies using acid-sensitive dyes revealed that lysosomes actively maintain an acidic pH of ~ 4.5 through the electrogenic action of $V-H^+$ ATPases (98). Interestingly, not all lysosomal compartments equilibrate to the same pH and in some myeloid cells this is coupled to an ability for specialized functions. Dendritic cells, for example, maintain a more alkaline pH ($\sim pH 5.5$) to limit proteolytic digestion and aid MHC-II loading (99, 100) whereas macrophages can acidify lysosomes to pH ~ 4.5 for microbial killing (101, 102). Not surprisingly, macrophages use this acidic environment to activate hydrolases capable of disassembling most naturally occurring macromolecules; at least 50 hydrolytic enzymes are deployed for this purpose (103, 104). Fusion of the lysosome with the pathogen-containing phagosomes is a critical step for microbial killing and many pathogenic bacteria like virulent *Mycobacteria* spp. employ secreted effectors to prevent this step (105). IFN- γ activation helps overcome this blockage to induce phagosome-lysosomal fusion and concomitant control of *Mycobacteria*, *Salmonella* and *Legionella* which

preferentially survive within immature phagosomes (101, 106, 107). Proteomic studies indicate IFN- γ drives extensive remodelling of the phagosome, upregulating lipidases, maturation factors, and the MHC class I peptide-loading complex (108). Besides phagocytosed bacteria, the lysosome also acts as a major killing device for invasive cytosolic bacteria. Here bacteria are flagged for lysosomal delivery by the autophagic pathway as discussed below.

Autophagy

Autophagy was first coined in 1966 to describe cellular digestion of endogenous cytosolic components (97). Our current mechanistic understanding of autophagy stems from yeast genetic studies performed by Tsukada and Oshimi which uncovered 15 of the 31 ATG proteins required for this process (109, 110). Similarly, Klionsky et al. uncovered a cytoplasm to vesicle protein transport (Cvt) pathway (111). Although functionally distinct, the robust molecular characterization of these two systems largely shaped our view of autophagic sequestration of cytosolic materials. Autophagy is the process of cellular “self-eating” and aptly named given the autophagic process physically sequesters cytosolic contents. Currently, three independent forms of autophagy have been elucidated: chaperone-mediated autophagy, microautophagy, and macroautophagy, each of which rely on distinct molecular machineries and are phenotypically separable. Macroautophagy is characterized by sequestration of cytosolic contents often by a double bilayer isolation membrane (autophagosome) followed by fusion with lysosomes for degradation of their contents. The molecular details of macroautophagy have been extensively reviewed elsewhere (112–114), so they will not be covered in detail here.

Cues that drive autophagosome formation are intimately tied to recognition of cellular homeostatic imbalances. These sensory components are coupled to diverse families of ubiquitin (Ub) ligases that generally respond to lack of nutrients, protein aggregates, or foreign macromolecules in the cytosol (115). In each case, particular subsets of E3 Ub ligases conjugate ubiquitin-like modifiers harboring C-terminal LRLRGG motifs to lysine residues in target proteins. These modifications can be either mono or poly-ubiquitination (generally K11, K48, or K63). Recent studies indicate specific ubiquitination machinery tag intracellular organelles, or in some cases intracellular bacteria (known as xenophagy), for lysosomal delivery and degradation. These modes of selective autophagy are triggered by exposure of “hidden” glycan chains to the cytosol, phosphorylation of mitochondria-associated Ub molecules, or ubiquitination of the peroxisomal protein, PEX5 (116–119). Upon Ub tagging, one of five Ub-light chain 3 (LC3) bridging proteins (TAX1BP1, NBR1, p62/SQSTM1, NDP52, OPTN) mobilize autophagosomal isolation membranes to the intended cargo (120–123). Surprisingly, adapter proteins are mobilized independently of one another and typically associate with specific forms of autophagy via distinct interactions with ATG8 homologs (124–126). Currently it is unknown whether their predominant role is to simply facilitate acquisition of ATG8-homolog positive isolation membranes or help complete autophagosome closure (127). In the case of bacteria it is also unclear which of several triggers - nutrient deprivation, vacuolar damage or bacterial products - predominates to mobilize this autophagic cascade (128–130). Whatever the signal, both ubiquitination and Ub-LC3 bridging proteins appear critical for targeting intracellular bacteria to the

phagophore and subsequent delivery of bacteria-containing LC3⁺ autophagosomes to the lysosome for degradation (129, 131).

Autophagy-related homologues exist in all eukaryotic organisms examined to date (113) and confer immunity in lower as well as higher organisms. Autophagy genes are needed, for example, to restrict *Salmonella* Typhimurium in the amoeba *D. discoideum* and nematode *C. elegans* (132), as well as *Listeria monocytogenes* in *Drosophila* (133). In mammals, control of *Salmonella* Typhimurium and *Listeria monocytogenes* similarly enlists autophagy proteins to control bacterial proliferation, suggesting pre-adaptation from earlier species (116, 120, 122, 125, 134). In addition, activating signals like IFN- γ or CD40L engagement stimulate autophagic protection of macrophages against *Mycobacterium tuberculosis* (135) and non-immune cells against the protozoan parasite, *Toxoplasma gondii*, respectively (136). Dorsal root ganglionic neurons also enlist autophagy in an IFN-independent pathway to control neurotropic viral infection (137). Thus autophagic defense is a widely co-opted strategy to prevent colonization and, in some cases, dissemination of invasive pathogens among different cell types and species (138). A wider list of targeted pathogens appears in an accompanying autophagy-related chapter by Grinstein and colleagues of this volume.

Nutritional Immunity

All homeostatic events occur at physiological salt and ion concentrations. It is therefore not surprising that shifts in ionic balance result in permissive or restrictive niches for intracellular pathogens. Both Cu²⁺ and Zn²⁺ overload and Fe²⁺/Fe³⁺ sequestration are now accepted as important macrophage defense mechanisms (6). Cu²⁺ and Zn²⁺ accumulate at high concentrations in macrophage lysosomes during infection (139–142). While no specific mechanism has been identified, the P-type ATPase ATP7A can import Cu²⁺ into the phagosome where it has the potential to form toxic hydroxyl radicals (140). This together with the sequestration of Fe²⁺/Fe³⁺ by NRAMP1 indicates the availability of specific trace metals within the phagosome is a powerful antibacterial strategy. These trace elements can also have indirect microbicidal effects, as Cu²⁺, Zn²⁺ and Fe²⁺/Fe³⁺ likely incorporate into the semi-selective binding pockets of iron binding proteins and activate metalloproteases within the lysosome (143). Additionally, Fe²⁺ cations convert H₂O₂ into the more powerful OH[•] radical via Fenton chemistry, thereby augmenting ROS generation.

Whereas trace metal manipulation appears most evident in immune phagocytes, tryptophan depletion by IDO1 is more broadly deployed, in part reflecting its emergence with marsupials early during mammalian evolution. In human epithelial cells and fibroblasts, IFN- γ -induced IDO1 expression has been shown to deplete intracellular tryptophan stores inhibiting the growth of tryptophan auxotrophic organisms such as *Chlamydia trachomatis* (144) and *Toxoplasma gondii* (145) as well as auxotrophic mutants of *M. tuberculosis* (146). In mice, combined IDO1 and IDO2-deficiency renders animals highly susceptible to *Toxoplasma* challenge, although more resistant to infection with *Leishmania* (147). Hence, pathogen-specific resistance of IDOs coincides with tryptophan dependence of the infecting parasite.

Recent evidence points to the importance of microbial carbon (C2) catabolism as a target for cell-autonomous immunity. Virulent bacteria and fungi resort to a carbon-salvaging process

termed anaplerosis to survive nutrient shortage in the face of the host immune pressure. LPS and IFNs induce the mitochondrial protein, immunoresponsive gene-1 (IRG1; also termed aconitate decarboxylase 1 [ACOD1]), that contains a MmgE/PrpD-like methylcitrate dehydratase (MCD) domain and decarboxylates the TCA intermediate *cis*-aconitase needed for isocitrate synthesis (148, 149). This diverts carbons away from the anaplerotic glyoxylate shunt that is essential for the persistence of several pathogens including *M. tuberculosis*, *S. typhimurium*, *Leptospira*, and fungi such as *Candida* and *Magnaporthe* (149–151). In addition to a described effector function, ICL1 also appears to regulate mitochondrial respiration and inflammatory cytokine production by inhibiting succinate dehydrogenase-mediated oxidation of succinate (152). Thus carbon and amino acids are heavily contested between host and microbe to influence infection.

Antimicrobial Peptides

Mammalian antimicrobial peptides (AMPs) arose from primitive gene duplication events resulting in structurally distinct protein families: α -Defensins, β -Defensins, θ -Defensins, Cathelicidins, Histatins, and Anionic antimicrobial peptides (153). In humans, α - and β -defensins predominate in phagocytes and epithelial secretions, whereas θ -defensins and cathelicidins are genomically sparse. Of these protein families, human β -defensins are constitutively expressed in epithelium whereas α -defensin expression predominates in lymphoid cells (153). In other species there has been cell-specific adaptations, such as mouse neutrophils which lack all α -defensins (154), or marsupials that possess a greatly expanded cathelicidin repertoire, the likely consequence of giving birth to underdeveloped and immunologically naïve young (155). Further cell-specific adaptations are seen in macrophages where bacterial killing is enhanced by the mobilization of preformed or acid-responsive AMPs to the phagosome during phagocytosis (156). This achieves a higher local concentration of AMPs than is observed during extracellular secretion by non-immune epithelial cells.

Different families of mammalian AMPs adopt unique structures. Nonetheless, the molecular mechanisms that bestow their bacterial restriction are thought to be mechanistically similar (157). AMPs are small (<100 AA) peptides containing high surface density and use positively charged residues as a means to target anionic, glycosylated components in microbial outer membranes. Ultimately this promotes permeabilization of the bacterial membrane or viral envelope. Different structural aspects of AMPs suggest three models of action: the carpet, toroidal-pore, and barrel-stave models. The carpet model posits that accumulation of anti-microbial peptides on negatively charged membranes facilitates bending and loss of membrane integrity (158). This is the likely mechanism of action for AMPs that lack significant amounts of hydrophobic regions. In contrast, the toroidal-pore model proposes AMPs insert themselves into lipid bilayers to biophysically cluster lipid tails (159). This ultimately results in membrane involution and pore formation. Lastly, the barrel-stave model consists of peptide bridging the lipid bilayer to form a proteinaceous pore that is reminiscent to the action of bacterial cholesterol-dependent cytolysins (160). Even though the biological significance and evolutionary expansion suggests importance for cationic AMPs, it is curious that charge dependency necessitates their functionality. On this point,

many bacteria have found ways to modify their outer membrane to be less anionic, thereby avoiding actions of cationic AMPs (161, 162).

In addition to AMPs, other enzymes capable of dismantling bacterial structural components like peptidoglycan have long been utilized as a means to lyse bacteria *in vitro* and may also promote bacterial restriction *in vivo*. The most famous is of these enzymes is lysozyme, which can be traced to its digestive function in the amoeba contractile vacuole. The term “lysozyme” was first coined by Alexander Flemming in 1922 to describe a substance that exhibits remarkable bacteriolytic effects in tears, nasal secretions, and hen egg-whites (163). Today the term lysozyme is more refined to describe enzymes that catalytically break down bacterial peptidoglycan via the hydrolysis of the glycosidic bond linking *N*-acetylmuramic acid and *N*-acetylglucosamine polymers. Interestingly, the lysozyme family is heavily expanded in simple eukaryotes, but has been limited to four members in humans (LYZC, LYZL1, LYZL2, LYZL4), many of which are predominantly expressed in the testes. Specific lysozyme family members may help generate hydrolyzed peptides reported to subsume antimicrobial roles in mammalian macrophage lysosomes, such as cleaved ubiquitin that can restrict *L. monocytogenes*, *S. typhimurium* and *M. tuberculosis in vitro* to confer cellular defense (164, 165).

Immune GTPases

Among the most potent cell-autonomous defense proteins operating in vertebrates are an emerging superfamily of immune GTPases (166, 167). These IFN-inducible proteins orchestrate antimicrobial activities against bacteria, viruses and protozoa. In many cases, they do so by directly targeting the pathogen-containing vacuole (PCV), as first seen for *Mycobacterium tuberculosis* and *Toxoplasma gondii* in IFN- γ -activated macrophages and fibroblasts, respectively (101, 168). IFN-inducible GTPases fall into four subgroups based on paralogy and molecular mass: 21–47kDa Immunity-Related GTPases (IRGs), 65–73kDa Guanylate Binding Proteins (GBPs), 72–82kDa Myxoma (MX) resistance proteins and ~200–285kDa GVINs (GTPase, Very Large Inducible) (166). The last group has not been assigned host defense functions, however, the other subfamilies exhibit pathogen-specific activities. Their evolutionary origins and distinct antimicrobial profiles are briefly discussed below.

IRGS—The IRG proteins have been identified in early protochordates such as amphioxys or lancelets (*Brachistomata floridae* and *B. japonicum*) suggesting origins before the radiation of chordate subphyla (169). A mammalian IRG ortholog (IRGD/IRG47) was first identified in pre-B cell lines treated with IFN- γ (170) and five additional IRGs (IRGM1-3, IRGB6, and IRGA6) discovered within a decade (171–174). Bioinformatic approaches then uncovered 14–16 additional IRGs in the euchromatic mouse genome, bringing the complete family to 21–23 genes (169, 175, 176). This expanded *Irg* repertoire in mice appears atypical among vertebrates, however, because humans possess only 4 *IRG* genes with rats, dogs, zebrafish and pufferfish harboring 7,9,11 and 2 members, respectively (169, 175, 176). Thus gene loss and acquisition show IRGs are a fast evolving family of defense proteins at the interface of host-pathogen interactions. Indeed, recent evidence in outbred wild mice suggests an expanded polymorphic IRG repertoire may arise due to parasite-host-prey

relationships where IRGs help mice combat asexual *Toxoplasma* transmission as part of the parasite life cycle completed within a natural predator, cats (177).

The first functional evidence revealed IRGs confer some of their cell-autonomous defense activities by targeting the pathogen-containing compartment (PCV). Studies in IFN- γ activated macrophages infected with *M. tuberculosis* found IRGM1 (formerly lipopolysaccharide response gene of 47kDa; Lrg-47) targeted mycobacterial phagosomes to facilitate fusion of this compartment with microbicidal lysosomes (101, 178). The molecular basis of this targeting arose from recognizing host-derived lipid species including membrane phosphoinositides and diphosphatidylglycerol on the phagocytic cup and PCVs shortly after bacterial uptake (178). This fitted the hypothesis that IRGs and other IFN-induced GTPases may recognize "altered self" signals rather than PAMPs (179). A similar outcome was subsequently observed with the human IRGM ortholog in IFN- γ activated monocytes where it promoted *M. bovis* BCG phagosomal maturation via the autophagic pathway following recognition of diphosphatidylglycerol (180). IRGs are likewise recruited to PCVs harboring *T. gondii* and *C. trachomatis* in murine fibroblasts which may occur through "missing self" recognition (168, 181). In the case of *T. gondii*, multiple IRGs hierarchically associate with PCVs, directly or indirectly helping to disrupt this organelle (168). To counter such activity virulent *Toxoplasma* strains have evolved mechanisms to inactivate IRG effectors (eg. IRGB6) through ROP18-mediated phosphorylation (182).

GBPs—A second family of immune GTPases, the 65–73kDa guanylate binding proteins (GBPs), have recently emerged as potent cell-intrinsic defense factors against intracellular bacteria, viruses and protozoa (167, 183). Comprehensive Hidden Markov Modeling (HMM) screens across 91 taxa revealed GBPs have ancestral orthologs as far back as deuterostomes, plants, amoebae and algae (184). Close inspection of the fossil record found that teleost GBPs harbored caspase activation and recruitment domains (CARDs) like those in human NLRP1 and ASC, suggesting mammalian GBPs may also regulate inflammasome responses, except here the relevant domains reside on separate GBP and inflammasome proteins (183, 184). This proved to be the case: IFN-induced GBP5 and GBP2 promote NLRP3 and AIM2 inflammasome responses to microbial triggers while other GBP family members also direct inflammasome responses to Gram-negative bacteria via the caspase-11 pathway (185–189). In *Danio rerio*, zGBP4 is responsible for inflammasome-dependent PGE₂ synthesis by neutrophils for protection against *Salmonella* (190), reinforcing this relationship across taxa (183).

Prior to the connections with inflammasome machinery emerged, a number of studies had reported the ability of GBPs to restrict bacterial (*Mycobacteria*, *Listeria*, *Salmonella*, *Chlamydia*), viral (HCV, Influenza) or protozoan (*Toxoplasma*) replication in IFN-activated macrophages, hepatocytes, fibroblasts and epithelia (167, 191–195). Ascription of these new roles benefitted from the identification of complete *GBP* gene clusters in both human and mouse genomes (167, 175, 196, 197). It also enabled family-wide loss-of-function screens to uncover member-specific activities along with susceptibility profiles in the first Gbp1-deficient mice (167). This has now been extended to bacterial and protozoan vulnerability in Gbp2-, Gbp5- and Gbp^{chr.3}-deficient animals (184, 189, 195, 196, 198, 199).

At the protein level, human GBPs share 40.0–98.4% amino acid identity and possess a bidomain architecture composed of similarly sized N-terminal GD and CTHD, the latter of which harbors 12–13 amphipathic α -helices involved in tail-to-tail dimer contacts (184, 200). Both the GD and CTHD contribute to homotypic self-assembly akin to the ~90kDa dynamin-like GTPases with which they share structural similarities (184, 201–204). GBPs bind GTP, GDP or GMP with equimolar affinity and exhibit high intrinsic rates of GTPase and GDPase activity (k_{cat} ~100–150 min⁻¹) (201, 205) due to an internal GAP domain, obviating the need for external GTPase-activating proteins (GAPs) to accelerate GTP or GDP hydrolysis and oligomerization (206). Other accessory proteins, however, may be required; a Rab GDP dissociation inhibitor, RabGDI α , was recently implicated in turning off Gbp2 activity during *T. gondii* infection (207). Whether RabGDI α -mediated suppression impacts inflammasome kinetics or selectivity awaits testing.

Macromolecular assembly of the GBPs facilitate interactions with native protein partners and, for some members, association with lipid membranes. GBP7 engages NADPH oxidase subunits [gp91^{phox}, p67^{phox}] to help assemble Nox2 on PCVs (167). Genetic GBP1, GBP2 or GBP5 mutants unable to undergo nucleotide-dependent tetramerization also fail to engage antimicrobial effectors and localize to the Golgi, autolysosomes, PCVs or target escaped bacteria or HIV-1 inside host cells (167, 198, 208–211). Conformational changes induced by transition-state tetramerization is also needed for human GBP1 to bind liposomes in cell-free assays (212). This membrane targeting may be further facilitated by isoprenylation at a C-terminal CaaX motif in In GBPs 1, 2 and 5; here, CVIL or CTIS mutants likewise abolish relocation to organelles and PVCs (167, 198, 210, 213). Thus, higher-order assembly and/or lipid anchorage appear important for GBPs to target membrane-bound as well as cytosolically-escaped pathogens.

Interestingly, many of these biochemical properties are retained across large evolutionary distances (169, 214). This is due to high levels of intra-familial protein conservation in the G and Middle domains that facilitate GTP hydrolysis and mechano-transduction, respectively (215). It ensures that “alternative mechanical coupling” of antimicrobial machinery to membrane surfaces are a conserved feature of many GBPs within most vertebrate species. Where the functional differences emerge are in the C-terminal domain; variation in this region often helps dictate the location at which GBPs interfere with infection inside host cells (215, 216).

MXs—A third group of IFN-induced dynamin-like GTPases, the Mx proteins, are potent antiviral restriction factors (214). Originally identified as an inherited trait in influenza A-susceptible mouse strains harboring deletions or nonsense mutations in the murine *Mx1* (*Myxoma resistance gene 1*) locus, the MX proteins are major antiviral proteins operating against flu and influenza-like togaviruses, plus bunyaviruses, rhabdoviruses, and Thogoto, Coxsackie, and Hepatitis B viruses (6, 217). Their importance is underscored by the human MxA (MX1) transgene to rescue mice lacking IFN type I (IFN α/β) receptors from influenza infection (217). This protein has undergone strong purifying selection in higher species such as primates where a surface exposed L4 loop is critical for species-specific activity against Influenza A and Thogo viruses (218). MxA assembles into large oligomeric complexes that adopt ring-like structures to block viral nucleoprotein formation (219).

In contrast, MxB (MX2) appears to be more specific for lentiviruses such as HIV-1 where most of the structural determinants for activity are in the C-terminal tail. Here MX2 interacts with nuclear pores to prevent HIV from integrating into the host genome (220, 221). Multiple functional losses have occurred across evolution for MxB (MX2), either by pseudogenization or via gene conversion from *MxA* genes. Notably, both *MX* genes are nonfunctional in Odontoceti cetaceans (toothed whales, including dolphins and orcas) and this loss occurred soon after the divergence of *Odontocetes* and *Mystocetes* (baleen whales) ~33–37 Mya (222). Presumably compensation occurs elsewhere in the genome since examination of 56 other mammalian genomes have found both *MX* genes to be intact. Hence this potent family of IFN-induced antiviral proteins is a prominent feature of most mammalian hosts.

Retrospect & Prospect

This chapter has focused on the evolution of the cell-autonomous effector mechanisms that bestow potent antimicrobial functions not only on specialized immune cells such as macrophages, but also guard the sanctity of non-immune lineages. This ancient system exhibits many tissue-specific phenotypes and could be viewed as offensive when deployed by phagocytic cells to detect and destroy microbes within the endolysosomal system (ROS, NO, AMPs, xenophagy), or defensive when mobilized in non-immune cells to restrict invasive and cytosolic intracellular microbes (e.g IDOs, IRGs, GBPs).

Gene duplication has been a driving force in the evolution of these new effector mechanisms, allowing one copy to accumulate mutations subject to Darwinian selection while the other maintains its original function. Gene conversion has been another route, especially within the MX family. Several of these effector genes, such as the GBPs, MXs and IDO1, have evolved rapidly, leading to a remarkable degree of functional plasticity even within closely related species. Hence selective pressure provided by pathogens has been a dominant factor in the acquisition of new effector mechanisms across species. It has also extended the boundaries of cell-autonomous defense beyond macrophages and other myeloid derivatives to encompass cell types outside the classical immune system.

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Fig 1. Evolution of antimicrobial effector mechanisms

Depicted is a phylogenetic tree of the Unikonta (Amoebozoa and Opisthokonta) and a summary of accompanying cell-autonomous effector mechanisms common to each major group. Scale indicates divergent nodal distance across NCBI taxa. Phylogram generated in Dendroscope 3.

Fig 2. Canonical effector mechanisms in myeloid cells

A) Enzymatically-derived metabolites are potent antimicrobials. Macrophages deploy nutritive mechanisms to reduce accessible nutrients and inhibit microbial growth; including natural resistance-associated macrophage protein one (NRAMP1, SLC11A1) which pumps iron out of the phagosome, and IFN- γ induced indoleamine 2,3 dioxygenase 1 (IDO1), which degrades intracellular tryptophan into kynurenine metabolites which in themselves can be toxic to microbes. Superoxide generation by NADPH oxidase (NOX2) and possibly DUOX1/2 generate reactive oxygen species on the luminal side of the phagosome which is directly antimicrobial. Nitric oxide (NO) generation by nitric oxide synthase (NOS) is also cytotoxic. Acidification by lysosomal V-ATPases, or import of copper/zinc ions also function to create a bactericidal environment within the phagosome. **B)** Antimicrobial peptides (AMPs) are generated within the Golgi and trafficked to the pathogen-containing endosome. Here, they are processed into their active form to induce bacterial lysis. **C)** Intracellular pathogens, either vacuolar or cytosolic, can become targeted by the autophagic machinery via host adaptor proteins which recognize signals of microbial infection. Proposed mechanisms include adaptors like p62/SQSTM1 which bind to ubiquitinated microbes or vacuoles, lectins like galectin 8 (LGALS8) which bind glycans exposed on damaged vacuoles, and TRIM21, which binds antibodies on the surface incoming bacteria and viruses. These adaptors promote phagophore formation around their cargo by binding to autophagosome adaptor LC3. Microbes are then sequestered within the autophagosome, a process that requires the ATG proteins, and fuse with the lysosome for degradation.