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Autophagy in Immunity Against *Mycobacterium tuberculosis*: a Model System to Dissect Immunological Roles of Autophagy

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Abstract

The recognition of autophagy as an immune mechanism has been affirmed in recent years. One of the model systems that has helped in the development of our current understanding of how autophagy and more traditional immunity systems cooperate in defense against intracellular pathogens is macrophage infection with *Mycobacterium tuberculosis*. *M. tuberculosis* is a highly significant human pathogen that latently infects billions of people and causes active disease in millions of patients worldwide. The ability of the tubercle bacillus to persist in human populations rests upon its macrophage parasitism. One of the initial reports on the ability of autophagy to act as a cell-autonomous innate immunity mechanism capable of eliminating intracellular bacteria was on *M. tuberculosis*. This model system has further contributed to the recognition of multiple connections between conventional immune regulators and autophagy. In this chapter, we will review how these studies have helped to establish the following principles: (1) autophagy functions as an innate defense mechanism against intracellular microbes; (2) autophagy is under the control of pattern recognition receptors (PRR) such as Toll-like receptors (TLR), and it acts as one of the immunological output effectors of PRR and TLR signaling; (3) autophagy is one of the effector functions associated with the immunity-regulated GTPases, which were initially characterized as molecules involved in cell-autonomous defense, but whose mechanism of function was unknown until recently; (4) autophagy is an immune effector of Th1/Th2 T cell response polarization—autophagy is activated by Th1 cytokines (which act in defense against intracellular pathogens) and is inhibited by Th2 cytokines (which make cells accessible to intracellular pathogens). Collectively, the studies employing the *M. tuberculosis* autophagy model system have contributed to the development of a more comprehensive view of autophagy as an immunological process. This work and related studies by others have led us to propose a model of how autophagy, an ancient innate immunity defense, became integrated over the course of evolution with other immune mechanisms of ever-increasing complexity.

1 Introduction: Autophagy as an Antimicrobial Defense Mechanism Against Bacteria, Protozoan Parasites and Viruses

The role of autophagy as a cell-autonomous antimicrobial defense has been suspected for a long time, but remained difficult to define until a recent burst of studies capitalizing on the genetic definition of autophagic machinery (Deretic 2005; Levine and Deretic 2007; Schmid and Munz 2007). These studies (Andrade et al. 2006; Birmingham et al. 2006; Checroun et al. 2006; Cullinane et al. 2008; Gutierrez et al. 2004; Liang et al. 1998; Ling et al. 2006; Liu et

al. 2005; Nakagawa et al. 2004; Ogawa et al. 2005; Orvedahl et al. 2007; Py et al. 2007; Singh et al. 2006; Talloczy et al. 2002; Yano et al. 2008) have demonstrated that autophagy can function as a cell's defense against bacteria, protozoa and viral pathogens. At a first approximation, autophagy can eliminate intracellular pathogens in a process akin to the sequestration and degradation of large macromolecular aggregates or surplus and dysfunctional intracellular organelles. This is in keeping with one of the primary functions of autophagy as a cytoplasmic clean-up process. A subset of the recent studies have demonstrated a role of autophagy in the elimination of microorganisms such as *M. tuberculosis* that reside within phagosomes (Gutierrez et al. 2004), intracellular pathogens that escape from the phagosome into the cytosol such as *Shigella* (Ogawa et al. 2005), and extracellular pathogens when they erode into the interior of the host cell, as demonstrated for Group A *Streptococci* (Nakagawa et al. 2004). A further testament to the cell-protection role of autophagy is provided by the evolutionary adaptations of pathogens that have developed countermeasures to defend themselves against autophagy (Jackson et al. 2005). For example, HSV-1 interferes with autophagy using a specific gene product ICP34.5 (Orvedahl et al. 2007). *Shigella* normally evades autophagy but falls pray upon the loss of one of its intracellular motility regulators (Ogawa et al. 2005), while *Listeria* inhibits autophagic maturation using its pore-forming toxins to counter luminal acidification (Birmingham et al. 2008).

Once the primary function of autophagy as a cell-autonomous defense against invading microbes had been established, the next important step was to examine whether links existed between the well-established innate and adaptive immune systems and autophagy. This was important to establish, since autophagy has been mostly viewed as a system that is involved in feeding cells during starvation and acts as a cell death/survival pathway. The role of autophagy in the control of intracellular *M. tuberculosis* has grown into a model system for making such connections. For example, the first publication showing that autophagy can eliminate *M. tuberculosis* also demonstrated that the cardinal Th1 cytokine IFN- γ can induce autophagy to eliminate an intracellular microbe (Gutierrez et al. 2004). This paved the way for a demonstration that autophagy is a previously unappreciated effector of Th1/Th2 polarization (Harris et al. 2007). It turned out that, in contrast to the protective function of IFN- γ via autophagy, the Th2 cytokines IL-4 and IL-13 inhibited autophagy and counteracted IFN- γ -induced autophagy, thus sparing intracellular mycobacteria (Harris et al. 2007). The opposing roles of Th1 and Th2 cytokines in dictating the macrophage's ability to control intracellular bacteria such as *M. tuberculosis* can now be attributed, at least in part, to autophagy-activating effects of Th1 cytokines and autophagy-repressing effects of Th2 cytokines.

Additional important connections between conventional immune systems and autophagy have followed with the use of the *M. tuberculosis* macrophage system, including the following examples. (1) *Autophagy as an effector of Toll-like receptors (TLRs), pattern recognition receptors (PRRs), and pathogen-associated molecular pattern (PAMP) signaling.* Two different groups have shown that activation of innate immunity using TLR ligands can stimulate the autophagic elimination of *M. tuberculosis* (Delgado et al. 2008; Xu et al. 2007). These and additional studies have shown that, in general, stimulating pattern recognition PRRs with PAMP can activate autophagy (Delgado et al. 2008; Sanjuan et al. 2007), and that this matters in vivo (Virgin 2008; Yano et al. 2008). (2) *Immunity-related GTPases (IRGs) and autophagy.* IRGs are now recognized as being regulators of cell-autonomous defense systems downstream of IFN- γ activation (Martens and Howard 2006; Taylor et al. 2004), but their mechanism of action—how they carry out their antimicrobial function—was still to be elucidated (Howard 2008). Since one of the murine IRGs (Irgm1, also known as LRG47) protected against *M. tuberculosis* (MacMicking et al. 2003), and IFN- γ induced both Irgm1 expression and autophagy, we tested whether expression of Irgm1 alone can induce autophagy (Gutierrez et al. 2004). This turned out to be the case (Gutierrez et al. 2004). A similar relationship, albeit with a somewhat altered form vis-à-vis IFN- γ , also held up when the sole

human IRG protein, IRGM, was tested. Thus, the model system for the autophagy-based control of intracellular *M. tuberculosis* has helped to establish several key connections between conventional regulatory immunity processes and autophagy.

2 *M. tuberculosis* Parasitizes Host Macrophages

M. tuberculosis asymptomatically infects over a billion people and causes millions of new active disease cases annually, with a 25–30% mortality rate worldwide (Dye et al. 1999). Of particular importance is the strong link between the incidence of active tuberculosis and AIDS (Goldfeld et al. 2008; Nunn et al. 2005). HIV and *M. tuberculosis* are intimately associated on the global health stage, and one-third to two-thirds of all AIDS patients around the world are coinfecting with *M. tuberculosis* (Reid et al. 2006). In HIV-infected individuals, overt tuberculosis can occur before the CD4⁺ counts drop to levels that allow other less potent pathogens to survive and other symptoms of AIDS to show (Reid et al. 2006), and thus tuberculosis can often serve as a sentinel disease for underlying HIV infection. Worldwide, the treatment of HIV has impacted on tuberculosis control, whereas tuberculosis is a frequent cause of death in those with AIDS (Nahid and Daley 2006). Tuberculosis and AIDS, most alarmingly in combination, have been recognized as global health emergencies (<http://www.who.int/en/>).

One of the best characterized virulence determinants of *M. tuberculosis* and a key feature of its pathogenesis is the ability of the tubercle bacillus to infect and survive in macrophages by blocking the maturation of its phagosome into a degradative organelle called the phagolysosome (Armstrong and Hart 1971). This paradigm is often referred to as the inhibition of phagosome–lysosome fusion, phagosomal maturation block, or inhibition of phagolysosome biogenesis (Armstrong and Hart 1971). By preventing phagosomal maturation, *M. tuberculosis* avoids the bactericidal (Pieters 2008) and, with some exceptions (Majlessi et al. 2007), antigen-processing environment of the phagolysosome (Ramachandra et al. 2005; Torres et al. 2006) immediately upon phagocytosis, and possibly for prolonged periods of time. The suppression of phagosome maturation by *M. tuberculosis* is a critical process within the infectious cycle that allows the pathogen to establish a foothold. The confinement of *M. tuberculosis* within the phagosome, which is typical of its survival in macrophages, may extend to at least some parts of the protective granuloma. The status quo is maintained in these dynamic structures for extended periods of time, including during latency (Manabe and Bishai 2000; Russell 2007). Latency is the “preferred” state of *M. tuberculosis*, which asymptomatically persists in billions of people (Manabe and Bishai 2000). Most infected individuals are not ill and remain asymptomatic so long as a large number of immunological mediators needed for control, in particular IFN- γ and TNF- α (Flynn and Chan 2001) and T cells, continue to cooperate effectively. The latter include MHC II-restricted CD4⁺ T cells, MHC I-restricted CD8⁺ cells (Ottenhoff et al. 2008) and “unconventional” T cells (CD1a-c-restricted T cells, $\gamma\delta$ -restricted T cells, invariant TCR CD1d-restricted NKT cells, variant/diverse TCR CD1d-restricted NKT cells, Treg cells, Th17 cells, and nonclassical HLA-E restricted CD8⁺ T cells) (Behar and Boom 2008; Lewinsohn et al. 2000; Moody et al. 2004). This complex immunological control can break down with age, nutritional/environmental changes, HIV infection, or upon immunosuppressive therapy (Saunders and Britton 2007).

3 Autophagy Eliminates Intracellular *M. tuberculosis*

There are very few known macrophage microbicidal mechanisms that are capable of killing *M. tuberculosis*. Conventional antibacterial effectors such as reactive oxygen and nitrogen intermediates, acidification of the phagosome, and degradation of microbes in the phagolysosome do not readily inhibit *M. tuberculosis*, a property that has been known for decades (Armstrong and Hart 1975). This has been reaffirmed by modern molecular analyses,

as the known *M. tuberculosis* determinants of persistence in macrophages include: (1) specific inhibitors of phagosome-lysosome fusion (Vergne et al. 2004); (2) resistance to acidic conditions of the lysosome (Vandal et al. 2008); (3) disruption of recruitment of iNOS to the vicinity of the phagosome (Davis et al. 2007), and (4) protection against oxidants by the components of the lipid-rich envelope (Yuan et al. 1995).

In contrast to the notorious resistance of *M. tuberculosis* to other microbicidal effectors, our studies have shown that autophagy can efficiently eliminate intracellular *M. tuberculosis* (Gutierrez et al. 2004). These initial observations have been confirmed by several groups in different contexts, including a report showing that autolysosomes contain ubiquitin fragments that act as mycobactericidal peptides (Alonso et al. 2007), a study linking TLR stimulation and autophagy (Xu et al. 2007), a screen for novel autophagy inducers (Floto et al. 2007), and a recent study (Biswas et al. 2008) showing that the previously reported ATP stimulation of P2X7-receptor leading to the elimination of intracellular mycobacteria (Lammas et al. 1997) is achieved through autophagy (Biswas et al. 2008).

4 Unique Properties of Autolysosomes in Microbial Killing

The autophagy-*M. tuberculosis* model system has also provided insight into some special properties that are possibly unique to autophagic killing of intracellular microbes. There are two points that need to be made before describing published experimental findings. Firstly, while one may assume that having any lysosomal environment is microbicidal enough to eliminate any microbe, this is not always the case. For example, when *M. tuberculosis* is forced (by antibody opsonization) into a standard phagolysosome it still does not get killed. This has been known since 1975 (Armstrong and Hart 1975). Secondly, autophagy captures normal cytosolic components and processes/digests them in autolysosomes, thus creating a different lysosomal composition compared to the conventional lysosome. Taking these two points together, and the reports that autophagy can kill mycobacteria (Xu et al. 2007; Floto et al. 2007; Biswas et al. 2008; Lammas et al. 1997), it is possible that novel microbicidal components are present in the autolysosome.

Initial progress in this direction has been made using *M. tuberculosis* in a study in which Alonso et al. (Alonso et al. 2007) showed that ubiquitin fragments generated by ubiquitin digestion in autophagosomes endow lysosomes with higher mycobactericidal capacities. This is in keeping with the earlier reports that ubiquitin fragments generally possess antimicrobial properties (Kieffer et al. 2003), although how they were delivered to pathogens was not known. The experimental evidence indicates that autophagy, after capturing cytosolic ubiquitin or ubiquitinated proteins or their aggregates, proteolytically generates ubiquitin fragments during maturation into autolysosomes, and that these fragments are delivered (directly or indirectly) to intracellular *M. tuberculosis* to enhance its killing. Whether ubiquitin fragments are produced upon the induction of autophagy beforehand or are produced in the same compartment in which *M. tuberculosis* resides in cells activated for autophagy is not known at present. Furthermore, additional cytosolic components may contribute to the special microbicidal properties of autolysosomes. For instance, cytoplasmic proteins such as ubiquicidin (a unique ribosomal protein, S30, with homology to ubiquitin) and the ribosomal polypeptides S19 and L30 all display antimicrobial activity (Howell et al. 2003). Since ribosomes are common autophagic substrates (Kraft et al. 2008), some of these polypeptides may be present in autophagosomes. Intriguingly, microbicidal ribosomal peptides have been isolated from colonic epithelial cells based on their antimicrobial activity against enteric bacteria, and given the recently uncovered links between autophagy and inflammatory bowel disease (specifically Crohn's disease) (Burton et al. 2007; Hampe et al. 2007; Massey and Parkes 2007; Parkes et al. 2007; Rioux et al. 2007), these potential links and other unique aspects of autolysosomes warrant further investigations.

5 Autophagy is an Effector of Th1/Th2 Polarization

It is now known that autophagy is regulated by immunologically relevant cytokines and ligands (Andrade et al. 2006; Arico et al. 2001; Djavaheri-Mergny et al. 2006; Gutierrez et al. 2004; Harris et al. 2007; Inbal et al. 2002; Li et al. 2006; Paludan et al. 2005; Petiot et al. 2000; Pyo et al. 2005; Schlottmann et al. 2008). This knowledge has its early roots in nonimmunological studies where cytokines were used simply as convenient agonists or antagonists to induce or repress autophagy (Arico et al. 2001; Inbal et al. 2002; Petiot et al. 2000). With this as a starting point, we have used the autophagy-*M. tuberculosis* model system to show that key Th1/Th2-polarization cytokines, IFN- γ , IL-4 and IL-13, affect autophagy in immunologically relevant contexts (Fig. 1) (Gutierrez et al. 2004; Harris et al. 2007; Singh et al. 2006). In addition to these cytokines, TNF- α has been shown to activate autophagy under conditions where NF- κ B is inhibited (Djavaheri-Mergny et al. 2006). Other cell-mediated immunity regulatory systems can induce autophagy, such as CD40L-CD40 stimulation in the context of protection against the parasite *Toxoplasma gondii* (Andrade et al. 2006), apparently in association with TNF α secreted downstream of CD40-TRAF6 stimulation (Sabauste et al. 2007). The key Th1 cytokine IFN- γ , which is strongly associated with protective immunity against *M. tuberculosis* (Fortin et al. 2007), stimulates autophagy (Gutierrez et al. 2004; Inbal et al. 2002; Pyo et al. 2005). How IFN- γ signaling induces autophagy is yet to be fully defined. In the mouse, one pathway includes STAT-1-dependent expression of IRG (Gutierrez et al. 2004; Singh et al. 2006). In murine cells, IFN- γ induces autophagy in an Irgm1 (also known as LRG47)-dependent manner. However, the human equivalent, IRGM, is required for autophagy but its expression is not controlled by IFN- γ , thus indicating that additional signaling pathways are involved.

In contrast to Th1 cytokines, which induce autophagy, the Th2 cytokines IL-4 and IL-13 are antagonists of autophagy (Harris et al. 2007). This is in part based on the activation of the Akt-Tor cascade by IL-4 and IL-13, and in part on the STAT-6 pathway (Harris et al. 2007). Treatment of macrophages with IL-4 and IL-13 inhibits starvation- or IFN- γ -induced autophagic delivery of mycobacteria into degradative compartments and counteracts mycobacterial killing in infected macrophages stimulated for autophagy (Harris et al. 2007).

IL-4 and IL-13 signal through a shared receptor, IL-4R α , which complexes with the γ -common chain in the case of IL-4 or with IL-13R α 1 in the case of IL-13 as a ligand (Nelms et al. 1999). IL-13 can also signal through a high-affinity receptor, IL-13R α 2. Once the IL-4 and IL-13 receptors are engaged, this results in not only the activation of the STAT-6 pathway (well appreciated in immunological studies) but also signaling via the insulin receptor substrate (IRS)-1 and 2 (Nelms et al. 1999). The signaling via IRS stimulates the Akt pathway, which provides the basis for IL-4 and IL-13 inhibiting autophagy induced by starvation (Harris et al. 2007). However, a different signaling pathway, independent of Akt and dependent on STAT-6, is required to suppress IFN- γ -induced autophagy. The inhibitory action of IL-4 and IL-13 translates into the inhibition of autophagic control of intracellular *M. tuberculosis* (Harris et al. 2007). Collectively, the induction of autophagy by IFN- γ , the autophagic control of *M. tuberculosis* following activation with Th1 cytokines, the inhibition by IL-4 and IL-13 of autophagy, and the suppression of autophagic killing of *M. tuberculosis* by Th2 cytokines indicate that autophagy is an effector of Th1/Th2 polarization. This in turn can help explain why Th1 cytokines are protective against and Th2 cytokines are permissive to intracellular pathogens. Significantly, the overriding suppressive effects of IL-4 and IL-13 on the induction of autophagy by IFN- γ can explain why Th1/Th2 polarization does not need to be sharply defined (and indeed this rarely happens) in infection sites. It turns out that presence of Th2 cytokines may override IFN- γ when cytokine responses are mixed.

6 Immunity-Related GTPases (IRGs) Regulate Autophagy in Antimicrobial Defense and Inflammation

The work on *M. tuberculosis* led to the initial connection between IRGs and autophagy in murine cells (Gutierrez et al. 2004), and has been recently expanded to the control of *M. tuberculosis* in human cells (Singh et al. 2006). IFN- γ is a major correlate of immunity against tuberculosis, but the exact nature of IFN- γ antimycobacterial action remained an elusive issue, as neither reactive oxygen nor reactive nitrogen intermediates could explain its potent antimycobacterial action (MacMicking et al. 2003). It has been demonstrated that IFN- γ acts through an IRG family member, Irgm1 (LRG-47), to control *M. tuberculosis* in murine macrophages and in the mouse model of tuberculosis (MacMicking et al. 2003), and that autophagy is the result of Irgm1 action (Gutierrez et al. 2004). Thus, in the case of *M. tuberculosis* it appears that at least some dots have been connected, between IFN- γ , IRG, autophagy, and intracellular control of the tubercle bacilli.

The IRG factors (Fig. 2) were initially recognized as being mediators of efficient cell-autonomous defense (at the time of an unknown nature) against intracellular pathogens. The mode of action for these systems remained a mystery until autophagy was connected to at least one member (Irgm1/LRG-47) of the IRG family in the mouse (Gutierrez et al. 2004) and to the only member (IRGM) of this family in humans (Singh et al. 2006). The role of autophagy downstream of murine IRG has also been demonstrated in control of *T. gondii*, where another murine IRG protein, Irgm3 (also known as IGTP) has been implicated in autophagy induction and association with autophagosomes (Ling et al. 2006). The additional proposed and still contested roles for IRGs (Howard 2008) include the enhancement of phagosomal acidification (MacMicking et al. 2003), the disintegration of the parasitophorous vacuole membrane (Martens et al. 2005) and the aberrant function of hematopoietic stem cells during infection (Feng et al. 2008). Significantly, the majority of the above mechanisms are compatible with autophagy, can be potentially explained by autophagy, or may involve autophagy as a component.

The IRG family (Bekpen et al. 2005) in the mouse is represented by a total of 23 *Irg* genes (Fig. 2). There are 19 interferon-controlled complete *Irg* genes. *Irgm1*, *Irgm2*, *Irgm3*, *Irgb1*, *Irgb3*, *Irgb5*, *Irgb6*, *Irgb8*, *Irgb9*, and *Irgb10* are on mouse chromosome 11. The *Irgb7* ψ within the *Irgb* cluster is a pseudogene. The *Irg* cluster on the mouse chromosome 18 has *Irga1*, *Irga2*, *Irga3*, *Irga4*, *Irga6*, *Irga7*, and *Irga8* genes, and *Irga5* ψ as a pseudogene. The expression of the murine *Irg* genes depends on activation with interferon via a combination of IRES and GAS promoter elements, with the exception of *Irgb5* and *Irgb9*, for which only IRES sites have been recognized in bioinformatics approaches. The two *Irg* paralogs on mouse chromosome 7 represent an interferon-independent gene (*Irgc*) or an incomplete, quasi-GTPase (*Irgq*) gene. Based on this, *Irgc* and *Irgq* have been a priori (although experimental evidence is lacking) excluded as potential immune regulators or effectors (Bekpen et al. 2005). Figure 2 also features the pregenomic era names of the murine *Irgs*, from the research period when several of them were individually characterized.

The prolific nature of the *Irg* loci in the mouse genome starkly contrasts with the dearth of *IRG* genes in the human genome. In humans, there are only three *IRG* paralogs, with *IRGM* thus far being the only *IRG* gene considered and functionally characterized in human cells (Singh et al. 2006). *IRGC* and *IRGQ* on human chromosome 19, which are syntenic with the mouse *Irgc* and *Irgq*, have not been (by analogy to the murine genes) considered thus far in immunity. The sole immunologically implicated human *IRG* gene, *IRGM*, on human chromosome 5 is surrounded by chromosomal regions syntenic with the murine *Irgm* and *Irga* chromosomal loci, suggesting its genetic correspondence to the immunologically defined murine *IRG*. Moreover, human *IRGM* has been functionally characterized as playing a role in

immune processes (Chaturvedi et al. 2008; Singh et al. 2006). Although IRGM represents a much-shortened (N-terminally and C-terminally truncated) version relative to the murine Irgm proteins, it exceeds the size of Ras and Rab proteins. Curiously, human IRGM is not regulated by IFN- γ , and is instead constitutively expressed from the long terminal repeat (LTR) of a human endogenous retrovirus repetitive element, ERV9. However, IRGM is still required for full autophagy activation in cells stimulated with IFN- γ , starvation, or rapamycin (Fig. 1). The exact mechanisms by which IRGM in human cells and Irgm1 (LRG-47) in murine cells promote autophagy are not known and are presently being investigated.

7 Autophagy is an Effector of Pattern Recognition Receptor Signaling

The autophagy-*M. tuberculosis* system has also helped to connect immunologically relevant autophagy with innate immunity receptor signaling. The innate immunity receptors and downstream effectors are responsible for early detection and initial elimination of invading microbes plus the modulation of adaptive immunity that subsequently develops (Ishii et al. 2008; Medzhitov 2007). The innate immunity receptors (Fig. 3), collectively referred to as PRRs, encompass three major classes: TLRs, retinoic acid-inducible gene I (RIG-I)-like helicase receptors (RLRs), and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) (Takeuchi and Akira 2008). PRRs recognize PAMP and induce a number of proinflammatory cytokines as a well-known, conventional output. A new addition to the repertoire of PRR stimulation outputs is the recently described induction of autophagy downstream of TLR stimulation (Delgado et al. 2008; Lee et al. 2007; Sanjuan et al. 2007; Xu et al. 2007) (Fig. 3). Two out of the four initial reports used *M. tuberculosis* killing and mycobacterial phagosome maturation as a physiological output to demonstrate that autophagy induced downstream of TLR is physiologically and immunologically relevant (Delgado et al. 2008; Xu et al. 2007).

TLR1, TLR2, TLR4, TLR5, and TLR6 partition primarily to the cell surface and recognize bacterial components, whereas TLR3, TLR7, TLR8, and TLR9 are primarily located in the endosomal compartments and recognize viral products (Lee and Kim 2007). TLRs recruit a different combination of four TIR domain-containing adaptor molecules: myeloid differentiation primary response protein 88 (MyD88), employed by all TLRs except TLR3; TIR domain-containing adaptor protein (TIRAP) or MyD88 adaptor-like (MAL), used by TLR2 and TLR4 as a bridge to recruit MyD88; TIR domain-containing adaptor-inducing interferon- β (TRIF) or TIR domain-containing adaptor molecule 1 (TICAM-1), employed by TLR3 and TLR4; and TRIF-related adaptor molecule (TRAM) or TICAM-2, employed only by TLR4 for interactions with TRIF (Kawai et al. 2004; Lee et al. 2007; O'Neill and Bowie 2007). A group of TLRs signal exclusively through MyD88 (TLR1, TLR2, TLR5, TLR6, TLR7, TLR8 and TLR9). TLR3 signals exclusively via TRIF. TLR4 signals with both MyD88 and TRIF. The duality in the context of TLR4 has been underscored by the discovery that TLR4 acts in a sequential manner, with the MyD88 pathway engaged on the plasma membrane and TRIF engaged upon endocytosis in the early endosome (Kagan et al. 2008). The majority of these signaling cascades activate NF- κ B and AP-1, leading to the production of inflammatory cytokines and chemokines, which in turn recruit and activate innate immune cells such as monocytes, neutrophils, and natural killer cells (Lee and Kim 2007). TLR3, TLR4, TLR7, TLR8 and TLR9 furthermore activate IRF3 or IRF7, leading to the production of IFN- α and IFN- β (type I IFN) (Lee and Kim 2007). Type I IFN can induce an antiviral state in most cells (Lee and Kim 2007). In addition to the above well-appreciated PRR outputs, it has recently been demonstrated that the stimulation of a number of TLRs with their cognate ligands activates autophagy as a defense mechanism that is capable of directly eliminating intracellular pathogens (Delgado et al. 2008; Sanjuan et al. 2007; Xu et al. 2007).

The TLR4 agonist LPS/endotoxin induces autophagy (Delgado et al. 2008; Sanjuan et al. 2007; Xu et al. 2007). An increase in double-membrane vacuoles monitored by electron microscopy (EM) was reported in RAW cells after LPS treatment (Xu et al. 2007). The punctate distribution of LC3, a sign of autophagosome formation, was also observed by immunofluorescence in human alveolar macrophages upon LPS stimulation (Xu et al. 2007). Induction of autophagy with LPS was reported by Xu et al. to be dependent on TLR4, TRIF, RIP1, and p38 MAPK, but to be independent of MyD88 (Xu et al. 2007). The same group reported that LPS treatment induced localization of *M. tuberculosis* in autophagosomes. TLR4 was also found by others to induce autophagy (Delgado et al. 2008; Sanjuan et al. 2007).

Two different ligands for mouse TLR7 induce autophagy in RAW, J774 macrophage-like cells, and (to a lesser extent) in primary murine bone marrow-derived macrophages (Delgado et al. 2008). Single-stranded (ss) RNA induced LC3 puncta formation, LC3-I-to-LC3-II conversion, and formation of late-stage autophagosomal profiles (autolysosomes) detected by EM (Delgado et al. 2008). LC3-II conversion was detected as early as 30 min following ssRNA addition, in the presence of Bafilomycin A1 (to preserve LC3-II by inhibiting autophagic flux) (Delgado et al. 2008). Imiquimod, an artificial TLR7 agonist, induced LC3 puncta formation (Delgado et al. 2008; Sanjuan et al. 2007) and increased the proteolysis of long-lived proteins in RAW macrophages (Delgado et al. 2008). Both TLR7 ligands increased GFP-LC3 puncta in murine macrophages from transgenic GFP-LC3 mice (Delgado et al. 2008). The autophagy induced by TLR7 ligands depends on Beclin 1, TLR7 and MyD88, as shown by siRNA knockdown experiments (Delgado et al. 2008). Autophagy elicited by TLR7 agonists can induce the killing of heterologous targets (intracellular *M. tuberculosis*) (Delgado et al. 2008), in a similar manner to autophagy induced by starvation, rapamycin, or overexpression of LRG47 (Irgm1) (Alonso et al. 2007; Gutierrez et al. 2004; Singh et al. 2006). The TLR7-induced killing of *M. tuberculosis* depended on MyD88, Beclin 1 and Atg5 (Delgado et al. 2008). It is important to note that *M. tuberculosis* is not known to stimulate TLR7, and yet induction of autophagy by an artificially added TLR7 agonist resulted in the elimination of intracellular *M. tuberculosis*, indicating that PRR-induced autophagy may not discriminate among microbial targets, and that this could be exploited in the future for therapeutic purposes.

Importantly, PRR-induced autophagy has been detected under conditions where cells were infected with a virus corresponding to a natural infection. Infection of HeLa cells with HIV, a TLR7/TLR8-activating virus, stimulated autophagy (Delgado et al. 2008). However, in pDCs there was no detectable autophagic increase upon infection with vesicular stomatitis virus (Lee et al. 2007). This may be due to an already high baseline level of autophagy for dendritic cells, as reported by Schmid et al. (2007). Thus, the induction of autophagy may be cell-type dependent. Nevertheless, autophagy even plays a role in TLR signaling in pDCs, as it serves to deliver cytosolic viral ligands to endosomally localized TLR7 (Fig. 3) (Lee et al. 2007), as described in detail in the chapter by Tal and Iwasaki in this volume.

There are mixed reports regarding TLR9 and CpG in the induction of autophagy (Delgado et al. 2008; Sanjuan et al. 2007), and more work is needed to address these discrepancies. Moreover, some TLRs require combinatorial stimulation, as TLR2 agonists (Pam3CSK4 or by Pam2CSK4) alone did not induce autophagy (Delgado et al. 2008), but zymosan engaged TLR2/TLR6 and Dectin-1, inducing autophagic markers (Delgado et al. 2008; Sanjuan et al. 2007). These issues and downstream signaling pathways leading to autophagy induction remain to be studied in detail. Another TLR ligand, dsRNA, can induce autophagy (Delgado et al. 2008), which is indicative of TLR3 engagement. However, an exclusive role for TLR3 was not demonstrated in these experiments. Since poly(I:C) can also activate MDA-5 (Takeuchi and Akira 2008), perhaps an RLR was engaged in those experiments, although MDA-5 has not been studied in the context of autophagy (Fig. 3).

Other PRRs have been shown to play a role in inducing autophagy. For example, Yano et al. (2008) have shown in *Drosophila* that a cytosolic PRR, PGRP-LE, which recognizes diaminopimelic acid-type peptidoglycan, induces autophagy which then protects the fruit fly from *Listeria monocytogenes* infection. At present it is not known whether NLRs play an analogous role in autophagy induction.

In addition to the activation of autophagy downstream of PRR stimulation, autophagy proteins seem to affect PRR signaling. The Atg5–Atg12 conjugate, which is a key regulator of autophagy, directly associates with one of the RLRs, RIG-I, and its downstream partner IPS-1 through the CARD domains. This association has been reported to negatively regulate the signaling mediated by IPS-1 and to suppress type I IFN production (Jounai et al. 2007). The biological meaning of Atg5–Atg12 negative regulation of RIG-I–IPS-1 signaling was interpreted by Jounai et al. as viral interference via autophagy with type I IFN production. It is perhaps interesting to also consider an alternative possibility that autophagic proteins may inhibit proinflammatory PRR signaling at a stage after induction in order to prevent potentially deleterious excessive stimulation of proinflammatory cytokines.

8 Conclusions and a Model

Figure 4 collates the presently known layers of conventional immunity regulators superimposed on the role of autophagy in immunity. We believe that autophagy may have been one of the very earliest eukaryotic cell defenses against pathogens. For example, phagocytosis or uptake of bacteria by eukaryotic cells (amoebae feeding on bacteria still occurs today) may sometimes, depending on the prey, result in bacterial escape from the phagosome, as in the case of *Rickettsia* spp., *Shigella*, and *Listeria*. Autophagy may help cells survive such events. Incidentally, a *Rickettsia*-like α -proteobacterium is believed to be the mitochondrial ancestor, and present-day mitochondria remain one of the classical substrates for autophagy (mitophagy) (Lemasters 2005; Lyamzaev et al. 2008; Sandoval et al. 2008; Schweers et al. 2007; Twig et al. 2008). During subsequent evolution, layers of immune regulation have been added to control autophagy, as we have covered extensively in this chapter. These layers involve PRR (specifically TLR and perhaps others), a step that links early recognition of microbial products to the induction of autophagy, which can eliminate intracellular pathogens such as *M. tuberculosis* (Delgado et al. 2008; Sanjuan et al. 2007; Xu et al. 2007; Yano et al. 2008). In vertebrates, IRG (p47 GTPases) (Martens and Howard 2006), along with other proposed functions (Howard 2008), became coupled to autophagy as a defense against highly evolved intracellular pathogens such as *M. tuberculosis* and *Toxoplasma* (Gutierrez et al. 2004; Ling et al. 2006; Singh et al. 2006). In its most evolutionarily advanced stage, cytokine networks have also gained control over autophagy in order to optimize the autophagic response to pathogens. This is reflected in autophagy being upregulated by Th1 cytokines and downregulated by Th2 cytokines, in keeping with the protective role of Th1 cytokines against intracellular bacteria and protozoan parasites, and the permissive role of Th2 cytokines towards intracellular pathogens. Th2 cytokines are protective against metazoan parasites (e.g., helminthes), but interfere with the protective Th1 responses. Hence, autophagy appears to be one of the antimicrobial effectors that can explain the immunological consequences of the Th1/Th2 polarization of T cell responses (Gutierrez et al. 2004; Harris et al. 2007).

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Fig. 1.

Th1/Th2 polarization and autophagy regulation: Th1 cytokines activate and Th2 cytokines inhibit autophagy. Mouse cells: IFN- γ activates expression of immunity-related GTPase Irgm1 (LRG-47) to induce autophagy. Human cells: the IRG factor IRGM is expressed independently of IFN- γ , but is required for IFN- γ -induced autophagy. IL-4 and IL-13 inhibit autophagy. Inhibition of starvation-induced autophagy by IL-4 and IL-13 depends on Akt/PKB; inhibition of IFN- γ autophagy by IL-4 and IL-13 depends on Stat-6. Th1 cytokines (in particular IFN- γ and TNF- α) are critical for protection against tuberculosis. Th2 cytokines have been shown to be permissive in mycobacterial diseases

Fig. 2.

Immunity-related GTPases (IRGs): genomic organization and roles in autophagy. The overall genomic organization of IRG genes. The mouse and humans are at the opposite ends of the spectrum in terms of the number of IRG genes. The murine *Irg* loci with dual names (underneath the chromosomes) have been implicated in defense against intracellular pathogens. *Red arrows* indicate those IRG that have been implicated in the autophagic control of intracellular pathogens, *Mycobacterium tuberculosis* and *Toxoplasma gondii*. Modified with permission from Singh et al. (2006)

Fig. 3.

Autophagy is a downstream effector of PRR signaling. *PAMP*, pathogen associated molecular patterns (microbial products, e.g., LPS/endotoxin, peptidoglycan constituents, viral replication intermediates etc.); *PRR*, pattern recognition receptors (*TLR*, Toll-like receptors; *RLR*, RIG-I-like receptors; *NLR*, Nod-like receptors); *PGRP-LE*, a *Drosophila* cytosolic PRR; *Dectin*, fungal cell wall receptor). Autophagy downstream of PRR can result in direct microbial elimination (e.g., killing of *M. tuberculosis*) (Delgado et al. 2008; Xu et al. 2007), and increases MHC II-restricted presentation of microbial cytosolic antigens (e.g., EBNA1 protein of EBV) (Paludan et al. 2005). Autophagy has also been shown to deliver TLR ligands to endosomal TLR (e.g., VSV nucleic acids to TLR7), and to play a role in downregulating PRR signaling (e.g., Atg5–Atg12 complex inhibits RIG-I-IPS-1 signaling) (Jounai et al. 2007). *Parentheses* indicate PRR or processes where the indicated function has not been firmly established

Fig. 4.

Autophagy and immune regulatory systems: an evolutionary timeline. Autophagy may be one of the very earliest eukaryotic cell-autonomous defenses against pathogens. Feeding by protozoa (e.g., amoebae) on bacteria via phagocytosis has been preserved to the present day. In metazoans, phagocytic uptake of bacteria occurs by the cells of the reticulo-endothelial system as an innate immune defense. Even mammalian cells that do not readily phagocytose microbes can be induced by bacterial pathogens for phagocytosis. Sometimes, bacteria escape from the phagosome, as in the case of *Rickettsia* spp., *Shigellae* spp., and *Listeria* spp., and they kill the host cell. A *Rickettsia*-like α -protobacterium is believed to be the mitochondrial ancestor, and present-day mitochondria remain one of the classical substrates for autophagy (mitophagy) (Lemasters 2005; Lyamzaev et al. 2008; Sandoval et al. 2008; Schweers et al. 2007; Twig et al. 2008). During evolution, immune systems with increasing levels of complexity have been integrated with autophagy as an integral part of comprehensive innate and adaptive immune defense networks. (i) PRR (specifically TLR and perhaps others) link the early recognition of microbial products to the induction of autophagy, which can eliminate intracellular pathogens such as *M. tuberculosis* (Delgado et al. 2008; Sanjuan et al. 2007; Xu et al. 2007; Yano et al. 2008). (ii) IRG (p47 GTPases) (Martens and Howard 2006) were coupled in vertebrates to autophagy as a defense against highly evolved intracellular pathogens such as *M. tuberculosis* and *Toxoplasma* (Gutierrez et al. 2004; Ling et al. 2006; Singh et al. 2006). (iii) Cytokine networks and Th1/Th2 polarization also control autophagy in order to optimize autophagic response. Autophagy is upregulated by Th1 cytokines and downregulated by Th2 cytokines. This partially explains the protective role of Th1 cytokines and the permissive role of Th2 cytokines vis-à-vis intracellular pathogens, with mycobacterial infections being a prime example (Gutierrez et al. 2004; Harris et al. 2007)