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Immune regulation of the host autophagic response and the elimination of Mycobacterium tuberculosis

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IMMUNE REGULATION OF THE HOST AUTOPHAGIC RESPONSE AND THE ELIMINATION OF *MYCOBACTERIUM TUBERCULOSIS*

BY

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B.S., Biochemistry, California State University, Los Angeles
2002

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

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DEDICATION

I dedicate this dissertation to the loving memory of Edwin A. Baca, Lena Garcia, Bernice (Boots) Whatley, and Dr. Anthony Andreoli.

I could not have done this without your love, encouragement, and support. You helped me realize that dreams matter and that winners keep rising from their falls. I wish you were here to see what you accomplished with me. Thank you and I love you.

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ABSTRACT

Autophagy is an immune effector response against a variety of intracellular pathogens, including *Mycobacterium tuberculosis*. Mycobacteria survive in macrophages by blocking maturation of the phagosome. Induction of autophagy by the T helper 1 (Th1) cytokine IFN-γ, or amino acid starvation, enables macrophages to overcome the phagosome maturation block and impedes mycobacterial survival. This study investigates the role of T helper 2 (Th2) cytokines Interleukin 4 (IL-4) and Interleukin 13 (IL-13) in autophagy in macrophages and their ability to eliminate intracellular mycobacteria. The Th2 cytokines abrogated autophagy and autophagy-induced transfer of mycobacteria into lysosomal compartments, thus enhancing mycobacterial survival within infected macrophages. In murine and human macrophages, inhibition of starvation-induced autophagy by IL-4 and IL-13 was dependent on Akt signaling, whereas the inhibition of IFN-γ-induced autophagy was Akt independent and signal transducer and activator of transcription 6 (STAT6) dependent. These findings establish a mechanism through which Th1-Th2 polarization differentially influences the immune control of intracellular pathogens.

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1. INTRODUCTION

1.1. Overview of tuberculosis

1.1.1. Epidemiology

The facultative intracellular pathogen *Mycobacterium tuberculosis* (MTB) is the etiological agent of tuberculosis (TB), a disease of considerable social and economic impact globally since it is the principal cause of death from any single bacterial infection. MTB infects more than a billion people worldwide and, according to the World Health Organization (WHO), 8-10 million new cases arise annually. Of the newly infected, 2-3 million die each year [1]. The primary route of TB infection is through the inhalation of aerosolized droplets containing the MTB bacteria that escape from the lungs of individuals with the active disease. After inhalation, the bacteria are able to penetrate the alveoli of the respiratory tract of the uninfected individual [2]. Ultimately, the bacteria reach the distal regions of the lung, where in most cases, the infection is contained after formation of a granuloma, the culmination of a T-helper 1 (Th1)-biased adaptive immune response. This results in a latent infection without any detectable symptoms; however, about 10% of infected individuals remain susceptible to developing the active disease, which results in irreversible lung destruction [3]. The latent infection can be reactivated when the host immune response is compromised, as in malnutrition, steroid therapy, and old age; or, in parallel diseases such as diabetes, leukemia, and HIV [4]. Understanding the processes involved in granuloma formation and containment of the bacteria is essential in the development of new treatments and vaccine strategies against tuberculosis.

The rise of multiple drug-resistant (MDR) strains of TB has compounded the deadly synergistic effect of co-infection of TB in HIV infected individuals. Drug-sensitive TB has been treated with combination therapy for over half of century, as a course of therapy that uses only single drugs can result in the rapid development of resistance and treatment failure [5]. In addition, when treatment compliance is compromised, as is repeatedly the case, newly resistant strains can easily emerge [6]. The standard treatment course for TB involves the use of first-line drugs for six months. The use of second-line drugs may be necessary to help prevent drug resistance during therapy [7]. Although the individual is considered cured after six months of multiple drug treatment, a 2-3% probability of relapse still exists. The standard treatment for latent TB is six to nine months of single drug therapy [7]. These anti-tuberculosis treatments, along with the use of directly observed therapy, where trained personnel monitor the patient taking each dose of anti-tuberculosis medication, have lead to superior treatment outcomes and a decline in the overall spread and incidence of TB and MDR TB [8-10]. However, treatment of TB has led to an increase in deaths arising from liver injury associated with the use of some of the anti-tuberculosis drugs [11]. Furthermore, reports also indicate that in places with high occurrence of HIV extensively drug-resistant strains of TB have emerged; these TB strains are resistant to nearly all second-line drugs used to treat TB [12, 13]. As a result, the synergism between TB and HIV and the appearance of drug-resistant varieties of TB, which unaffected by an increasing list of anti-TB agents, represent a critical health concern and a significant global emergency. Currently, there are no

effective vaccines against TB; therefore, understanding the process involved in the molecular and cellular machinery used by both pathogen and host is urgently needed as a basis for development of more effective vaccines and treatments to prevail in the fight against tuberculosis.

1.1.2. Pathogenesis

Mycobacterium tuberculosis infection begins when the bacilli reach the pulmonary alveoli, invade, and subsequently replicate within the endosomal compartments of the alveolar macrophage [14, 15]. Under natural circumstances, the alveolar macrophage ingests the mycobacterial bacilli, which then are confined in phagosomes [16]. T lymphocytes, B lymphocytes, and fibroblasts participate to form a granuloma which surrounds the infected macrophage. The granuloma functions to prevent dissemination of mycobacteria, as well as to provide a local environment for communication between cells of the immune system. Within the granuloma, T lymphocytes secrete cytokines, including IFN-γ, which activate the infected macrophage to destroy the bacteria within [16]. However, tuberculosis lingers in the human populations due to the ability of mycobacteria to evade innate host killing mechanisms, which allows it to survive in the alveolar phagocytic cells of the lung [17]. Therefore, *Mycobacterium tuberculosis* persists as a thriving intracellular pathogen.

1.2. Cellular innate immune system and tuberculosis

1.2.1. Macrophages

Macrophages are crucial effector cells of the innate microbicidal machinery. They are able to ingest *Mycobacterium tuberculosis*, an intracellular pathogen, and confine it to a phagosomal compartment. Subsequently, if the macrophage is activated, the mycobacteria-containing phagosomes fuse with lysosomes, in a process known as phagosome maturation, for the killing of the internalized pathogens (Figure 1.1). The elimination of mycobacteria via phagosome maturation occurs through multi-tiered mechanisms, including reactive oxygen intermediates, reactive nitrogen intermediates, acidification of the phagosome, and proteolysis of bacteria in the phagolysosome. In contrast, if the alveolar macrophage is not activated, the internalized bacilli alter the phagosomes to preclude normal phagosome maturation to phagolysosomes and avoid terminal endocytic/lysosomal organelles. This action allows the bacilli to survive and multiply within the phagosomes by escaping direct bactericidal mechanisms of the host cell and eluding efficient antigen presentation [18] (Figure 1.1). Some of the ways MTB persists in the infected macrophage include: 1) inhibition of phagosome-lysosome fusion (phagosome maturation arrest) [19, 20], 2) disruption of actin-dependent recruitment of iNOS to the vicinity of the phagosome [21], 3) expression of detoxifying and anti-oxidant agents [22-24], and 4) protection by the components of the lipid-rich envelope [25]. Phagosome maturation arrest is seen as a major pathogenic determinant of MTB [18] and is the focus of our investigation.

1.2.2. Dendritic Cells

Dendritic cells (DCs) journey through peripheral tissues and are essential for the induction of cellular immunity against intracellular pathogens, including mycobacteria [26]. DCs have been demonstrated to be the major antigen presenting cells (APC) for the initiation of primary T cell responses, and as the initial source of IL-12 during microbial infections [27-29]. The interaction of DCs with naïve T cells also leads to the production of IFN-γ [30], a vital cytokine in the control of mycobacterial infections, initiating a cross-talk between T cells and other cells of the immune response, notably macrophages. Human or murine myeloid DCs infected with *Mycobacterium tuberculosis* or *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) induce a synchronized program of cell maturation and an elevation of IL-12 levels [31, 32]. In addition, the transfer of BCG-infected DCs into mice results in a rapid IFN-γ rise by T cells in response to mycobacterial antigens [31]. This rapid IFN-γ response is also seen using *M. tuberculosis*-infected DCs [33]. Therefore, DCs increase the ability of immune cells to control mycobacterial replication.

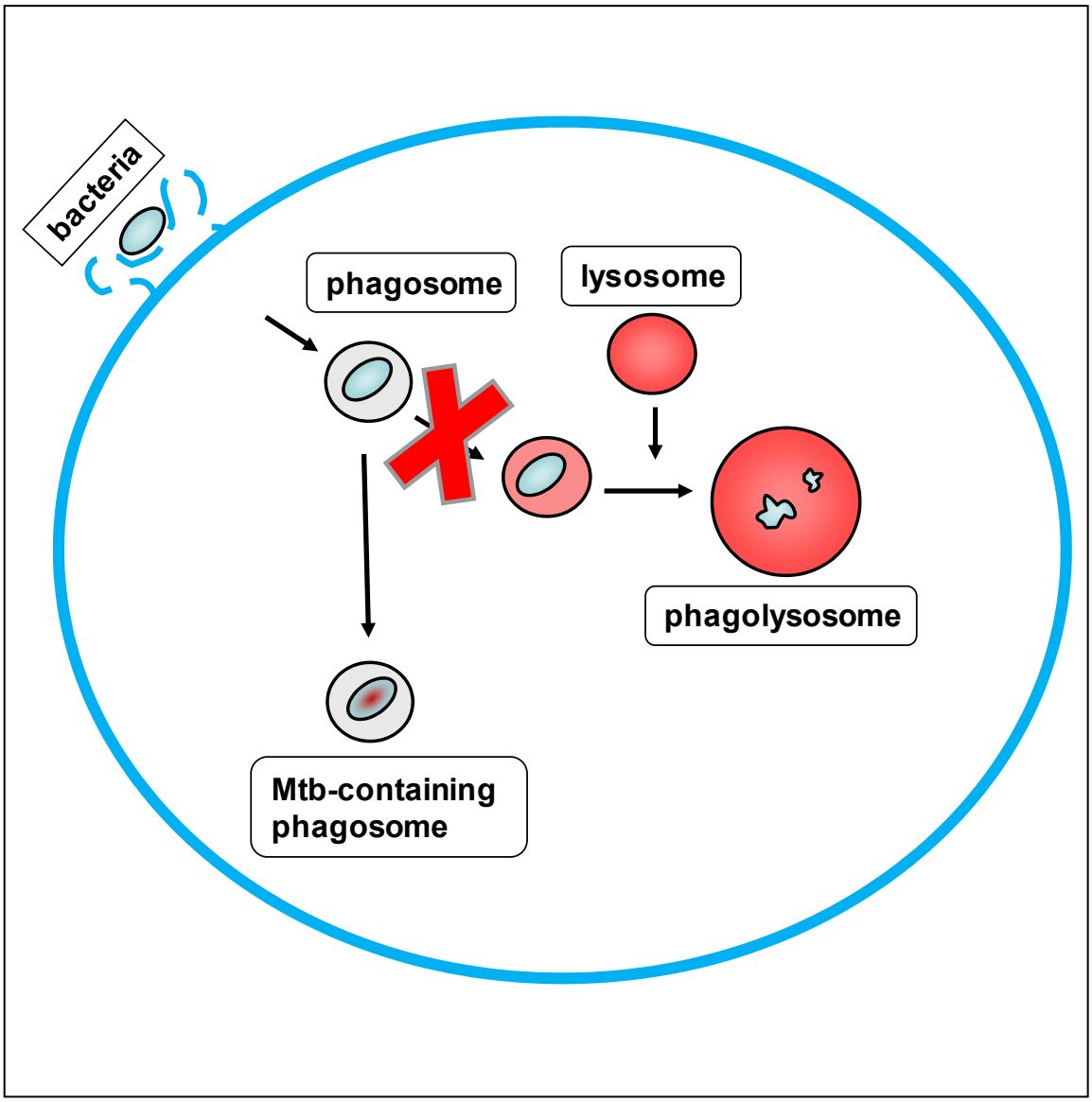

Figure 1.1. Phagocytosis and Phagosome Maturation Arrest in Macrophages.
During phagocytosis, mycobacteria are trapped in a phagosome. The phagosome undergos conversion, known as phagosomal maturation, accomplished through fusion with lysosome, forming phagolysosome for degradation. Effective phagocytosis therefore requires mycobacterial ingestion and phagosomal maturation. *Mycobacterium tuberculosis* (MTB) survival is due to its ability to manipulate and arrest phagosome maturation. Here mycobacteria thrive and persist.

1.3. Role of cytokines in the immune response to mycobacteria

1.3.1. Th1 cell-mediated response

Th1 cells, a subset of CD4 T cells characterized by the production of IFN-γ, play an important role in the development of resistance to intracellular pathogens [34, 35], primarily through the production of macrophage-activating cytokines. The Th1 cytokine IFN-γ has been identified as a key cytokine controlling mycobacterial infections and is produced by both CD4 and CD8 T cells [36, 37] in infected individuals as well as by NK cells [38, 39]. To date, IFN-γ knockout (GKO) mice are among the most susceptible to challenge with virulent MTB [40, 41], and individuals defective in genes for IFN-γ or the IFN-γ receptor are prone to serious mycobacterial infections [42]. Reports also indicate that professional APC, along with T cells and NK cells, are a source of IFN-γ [43-45]. IL-12 and microbial stimulation, alone or in combination, are the most effective inducers of IFN-γ production by APCs and T cells [46]. In IFN-γ-activated macrophages, the phagolysosome maturation block induced by *M. tuberculosis* is released [47]. The production of IFN-γ by APCs has been demonstrated mainly *in vitro* with the use of cultured DCs [48]; however, recent studies using intact intracellular pathogens have now provided additional evidence for the production of APC-derived IFN-γ by *in vivo* murine model systems [49, 50]. These data demonstrate the vital role of the Th1 cytokine IFN-γ in immunity against mycobacteria.

Once mycobacteria are deposited in the lungs, they have a 3-day lag period when they do not grow; subsequently, they grow with an average of a 28 h doubling time and reach approximately 5 logs by 20 days. The cessation of

bacterial growth correlates with the arrival of IFN-γ-producing antigen specific major histocompatibility complex (MHC) class II-restricted T cells [51]. Genetic defects in the receptors for IL-12 and IFN-γ increase susceptibility to progressive mycobacterial disease [52-54], while treatment with TNF-α-specific monoclonal antibodies, used in the treatment of inflammatory disorders such as rheumatoid arthritis and Crohn's disease, can result in latent tuberculosis becoming an active infection [55, 56]. Mice genetically deficient in IFN-γ (GKO mice) are able to form granulomas, however, they fail to produce nitric oxide and, thus, are unable to restrict intracellular growth of the bacilli [41]. Similarly, IL-12 increases protection against mycobacterial infection in susceptible BALB/c mice, but it is unable to do the same in GKO mice. These data demonstrate that the protective effects of IL-12 are dependent on IFN-γ [57]. In humans, dendritic cells also promote IFN-γ release from natural killer (NK) T cells, a major source of early IFN-γ in tuberculosis pleurisy [58]. Furthermore, lymphocytes in the lungs of patients with pulmonary tuberculosis typically have a Th1 phenotype, secreting IFN-γ IL-12 and TNF-α [59, 60]. Therefore, the role of Th1 cytokines in the immunity against tuberculosis is essential.

1.3.2. Th2 cell-mediated response

Th2 cells, subset of CD4 T cells characterized by the production of IL-4 and IL-13, play an important role in the protection against extracellular bacteria [61]. In contrast to the beneficial effects of Th1 cytokines, there is currently much speculation about the role of Th2 cytokines in the response to *Mycobacterium tuberculosis*. A Th1 response develops rapidly following exposure to *M. tuberculosis* [62]. However, studies show that a Th2 response also develops,

abating the efficacy of Th1 cytokines and promoting immunopathology [63, 64]. Conventional views of the role of IL-4 in TB indicate that it has a useful anti-inflammatory role, or that it is merely a late consequence of progressive disease [65]. However, some studies indicate that the IL-4 response in tuberculosis is not just a by-product of infection, but that it is actively involved in reducing protection and increasing immunopathology. Studies have shown that pre-existing Th2 activity against some components of *M. tuberculosis* prior to infection leads to increased immunopathology [66, 67]. Other studies show that the absence of IL-4 in *IL-4*$^{-/-}$ gene knock-out mice leads to diminished bacterial growth [68]. In addition, it has been shown that IL-4 has the ability to downregulate macrophage activation [69]. Furthermore, the hypervirulent strains of *M. tuberculosis* promote secretion of IL-4 and IL-13 by infected human monocytes [70]. These characteristics suggest IL-4 is an early potential player in the events that determine whether the infection becomes latent or progressive. The findings also raise important questions regarding the role of cytokines, especially in the context of Th1 versus Th2, in regulating the immune response to pathogens. Therefore, it is possible that Th2 cytokines are detrimental in the response against *Mycobacterium tuberculosis*.

1.4. Phagosome maturation in macrophages

Macrophages are key effector cells in the innate defense system that seek and subsequently eliminate bacterial pathogens. Elimination of invading microorganisms is primarily by phagocytosis. Phagocytosis begins by the clustering of surface receptors and their interaction with their associated ligand. A variety of phagocytic receptors can recognize innate components of the pathogen surface, or pathogen-associated molecular patterns [71-74]. Other phagocytic receptors recognize an exogenous, host-derived ligand, or opsonin, that adheres to the pathogen surface. Members of the integrin family, CR3 and CR4, are complement receptors that recognize primarily complement-coated pathogens. Fcγ receptors (FcγRs), bind to IgG-opsonized pathogens. Once formed, the phagosome steadily matures, by way of intracellular signals, and sequentially acquires microbicidal and degradative properties [75-77] by a process referred to as phagosome maturation [78]. The early phagosome is able to fuse with early endosome (pH 6.1) to form an intermediate phagosome, intermediate phagosome fuses with late endosome (pH 5.5–6.0), and late phagosomes with lysosomes to produce a phagolysosome [79-82] (Figure 1.1). Consequently, these fusion events transform the phagosomal lumen into a highly acidic and oxidizing environment, filled with a variety of hydrolytic enzymes that can degrade its content [83, 84]. Newly synthesized MHC class II molecules pass through the acidified vesicles and bind peptide fragments of the antigen, transporting them to the cell surface for presentation to T cells [85]. Specific markers identify the stage of development during phagosome maturation

(Table 1). Therefore, the phagocytic compartments of the macrophages are a significant component in the innate response against invading pathogens.

Organelle	Markers
Sorting endosome; early phagosome	EEA-1, Rab5, PI(3)P, syntaxin-13, transferrin receptor, VAMP3
Late endosome; late phagosome	Rab7, Rab9, mannose-6-phosphate receptor, syntaxin-7, LAMPs, LBPA
Lysosome; phagolysosome	LAMPs, mature cathepsin D, CD63

Table 1. Molecular Markers of Phagosome Maturation.
EEA-1 (Early endosomal antigen 1); PI(3)P (Phosphatidylinositol-*3*-phosphate); VAMP3 (Vesicle-associated membrane protein 3 (cellubrevin)); LAMPs (Lysosomal-associated membrane proteins); LBPA (Lyso-bisphosphatidic acid); CD63 (cluster of differentiation63, LAMP3).

1.4.1. Mycobacteria inhibit phagosome maturation

A significant factor in the success of *M. tuberculosis* as an intracellular pathogen is its ability to survive in macrophages by disrupting phagolysosome biogenesis. The major route of entry for *M. tuberculosis* into macrophages is still a matter for discussion, but most likely involves a number of surface receptors, including complement receptors and C-type lectins, such as the mannose receptor [86-88]. Upon entry into the macrophage, the nature of the *M. tuberculosis* phagosome is well understood, and can be characterized by incomplete luminal acidification and the absence of mature lysosomal hydrolases [18]. The block in phagolysosome biogenesis occurs between the maturation stages controlled by the GTP-binding proteins Rab5 (early endosomal) and Rab7 (late endosomal) [89]. The mycobacterial phagosome maintains access to early endosomal contents, including transferrin-bound iron, through Rab5-mediated processes, but does not acquire late endosomal or lysosomal components [89-91]. Many Rab5 effectors are recruited to the mycobacterial phagosome, while early endosomal autoantigen 1(EEA1) is displaced from it [92]. EEA1 interacts directly with Rab5 [93] and binds to phosphatidylinositol-3-phosphate (PI3P). PI3P is generated on organellar membranes by hVPS34, the only type III phosphatidylinositol-3-kinase (PI3K) [93, 94]. Although retained on phagosomes containing dead mycobacteria, PI3P is continuously eliminated from those containing live bacilli [95]. This indicates that the phagosomes retaining PI3P continue toward maturation and elimination of mycobacteria. Therefore, preventing the development of the phagolysosome is crucial for the survival of *Mycobacterium tuberculosis*.

Several mycobacterial factors have been shown to regulate different aspects of the phagolysosome biogenesis block and affect pathogenesis. The MTB-derived glycophospholipids phosphatidylinositol mannoside (PIM) and lipoarabinomannan (LAM) are able to insert into host endomembranes and traffic within infected cells [96]. Whereas LAM blocks calcium- and PI3K-dependent phagosome maturation [92, 97, 98], PIM stimulates fusion of early endosomes with the mycobacterial phagosome, permitting acquisition of nutrients [20]. MTB also secretes an acid phosphatase, sapM, which removes any PI3P that may be generated past the LAM block [95]. It is likely that additional mycobacterial factors target host cell effectors to influence, either directly or indirectly, the intracellular and extracellular environment [99, 100]. One example is the inhibition of MHC class II antigen processing that results from the interaction between mycobacterial factors and the Toll-like receptor (TLR) family [101]. Furthermore, recent reports indicate that the *Mycobacterium tuberculosis zmp1* gene products suppress inflammasome activation and IL-1β production, increasing mycobacterial virulence and survival in murine macrophages [102]. Therefore, *Mycobacterium tuberculosis* virulence factors are essential to host manipulation and survival of mycobacteria.

1.4.2. IFN-γ promotes phagosome maturation

In addition to its value in the innate response, IFN-γ is also important for phagosome maturation. Numerous studies have shown that priming macrophages and dendritic cells with IFN-γ prior to infection increases intracellular killing of mycobacteria [103, 104]. This killing appears to be directed by both nitric oxide synthase 2 (NOS2)-dependent and–independent

mechanisms. In mice, IFN-γ-induced expression of NOS2 restricts *M. tuberculosis* replication via the generation of NO. However, $NOS2^{-/-}$ mice infected with *M. tuberculosis* survive significantly longer than mice lacking IFN-γ, IFN-γ R1 or STAT1, through which IFN-γ signals, indicating the existence of IFN-γ-dependent, NOS2-independent immunity against *M. tuberculosis* [105]. One effector in this response is the GTPase Irgm1 (previously called LRG-47), which has specific anti-mycobacterial actions [105]. However, the precise role of Irgm1 in the response to mycobacterial infection is not yet clear. The success of mycobacteria is accomplished by a phagosome maturation block while still allowing fusion with transferrin-containing endosomes, consequently maintaining a habitable environment that promotes survival [104, 106]. However, IFN-γ activates macrophages to overcome this phagosome maturation block [47, 107]. Following activation with IFN-γ, Irgm1 is recruited to the mycobacterial phagosome, but in $Irgm1^{-/-}$ mice phagosome maturation is impaired [105]. The role of Irgm1 on the phagosome remains unknown. This demonstrates IFN-γ is a pleiotropic cytokine that is critical for innate immunity against mycobacteria.

1.5. Autophagy and the innate immune response to mycobacteria

Autophagy is a fundamental homeostatic mechanism for cell survival that maintains a balance between synthesis, degradation, and recycling of cellular products. In autophagy, cells sequester discrete portions of the cytoplasm and deliver them to lysosomes for degradation [108, 109]. This process removes damaged or surplus organelles such as leaky mitochondria and excess peroxisomes and, by degrading long-lived cytoplasmic macromolecules during periods of starvation, promotes cell survival [110]. During autophagy, an isolation membrane, the phagophore, surrounds portions of the cytoplasm to form a double-membrane organelle referred to as an autophagosome. The autophagosome then fuses with late endosomes or lysosomes, forming an autophagolysosome, where the captured cytoplasmic material is degraded [108, 111] (Figure 1.2). Autophagy has also been shown to play a role in innate immunity against intracellular pathogens, including Epstein-Barr Virus [85], *Shigella flexneri* [112], *Salmonella typhimurium* [113], *Toxoplasma gondii* [114], and *Mycobacterium tuberculosis* [115]. In addition, autophagy has been implicated in adaptive immunity, by promoting endogenous antigen processing and presentation on MHC class II [85, 116-118]. Therefore, autophagy is an important player in cellular homeostasis and in the protection against intracellular pathogens.

As a multistep process, autophagy begins following induction with sequestration of a damaged organelle or a portion of cytoplasm by a nascent autophagosomal structure called the isolation membrane (phagophore) (Figure 1.2). The phagophore is enlarged during the elongation stage by the addition of

new membrane of undefined origin, but suspected to come from the endoplasmic reticulum [119] or a combination of sources including Golgi and endosome [120, 121]. The isolation membrane eventually closes to form an autophagosome. The autophagosome is differentiated from the conventional phagosome by the presence of a double membrane (two lipid bilayers) and intra-lumenal cytosolic content including membranes originating from the confined organelles. A subsequent fusion of autophagosomes with lysosomes, referred to as flux or maturation, results in a degradative compartment known as the autolysosome (Figure 1.3).

Atg8, also known as LC3, has been a fundamental tool to study autophagy, especially as it is recruited to the autophagosome in the initiation step (Figure 1.2). Phagophore enlargement and wrapping around its target in the elongation stage require two specialized ubiquitination-like protein conjugation systems, including E1 activation and E2 conjugation enzymes but apparently not needing an E3 ligase. Atg12 is initially conjugated to Atg7, which acts as an E1-activating enzyme, and then gets transferred to the E2-like conjugating enzyme Atg10. This intermediate presents Atg12 to be conjugated to Atg5 (in mouse) [110]. The Atg5-12 conjugate, stabilized in a noncovalent complex with Atg16, triggers oligomerization on the outside membrane of the growing phagophore. The Atg5-12/16 complex stimulates a second conjugation system, whereby LC3 undergoes conversion from its free C-terminus state (LC3-I) to its C-terminally lapidated form (LC3-II) covalently modified by phosphatidylethanolamine. The lipidated LC3-II localizes to the membrane on both sides of a growing

phagophore. Upon autophagosome closure, sealing the typical double-membrane organelle, Atg5-12/16 and LC3 (delipidated by Atg4) dissociate from the outer autophagosomal membrane and get recycled. The LC3 associated with the lumenal membrane remains trapped in the autophagosome but is degraded during maturation, which involves fusion with late endosomal and lysosomal organelles (Figure 1.2). LC3 lipidation and LC3-II association with autophagosomal membranes provide the basis for two frequently employed assays of autophagy [122]: (i) autophagosome formation can be scored by immunofluorescence microscopy as a transition of LC3 from its diffused cytosolic to a membrane-associated, punctate intracellular distribution and (ii) the LC3-I to LC3-II conversion can be monitored by immunoblotting.

Autophagy is dependent on the generation of PI3P by the type III PI3K, hVPS34, during initiation and maturation of the autophagosome. PI3P is also generated on phagosomes destined for fusion with the lysosome [95] This led our group to investigate whether the induction of autophagy in *M. tuberculosis*-infected macrophages could reverse the mycobacteria-induced phagosome maturation block. In a model system using *M. tuberculosis* variant bovis BCG and the murine macrophage cell line RAW 264.7, induction of autophagy by starvation or with rapamycin increased acidification of mycobacterial phagosome and promoted maturation of the phagolysosome [115]. These effects could be inhibited by the addition of 3-methyladenine (3-MA) and wortmannin, both inhibitors of PI3Ks commonly used to inhibit autophasome formation. Following induction of autophagy, BCG can be seen by electron microscopy in vacuoles

containing partially degraded internal membranes, a characteristic of maturing autophagosomes [123-125]. In addition, BCG showed increased colocalization with microtubule-associated protein 1 light chain 3 (MAP-LC3, or LC3) and beclin, both associated with autophagy [115]. Moreover, the survival of both BCG and virulent *M. tuberculosis* in macrophages (RAW 264.7 cells and primary murine and human macrophages) was inhibited after induction of autophagy with rapamycin or by starvation [115]. Thus, autophagy promoted the elimination of *M. tuberculosis* by diverting the mycobacterial phagosome to an autophagic vacuole, which in turn, fused with the lysosomes, overcoming the *M. tuberculosis*-induced phagolysosome biogenesis block. Therefore, this suggests that autophagic killing of *Mycobacterium tuberculosis* may be essential in innate defense.

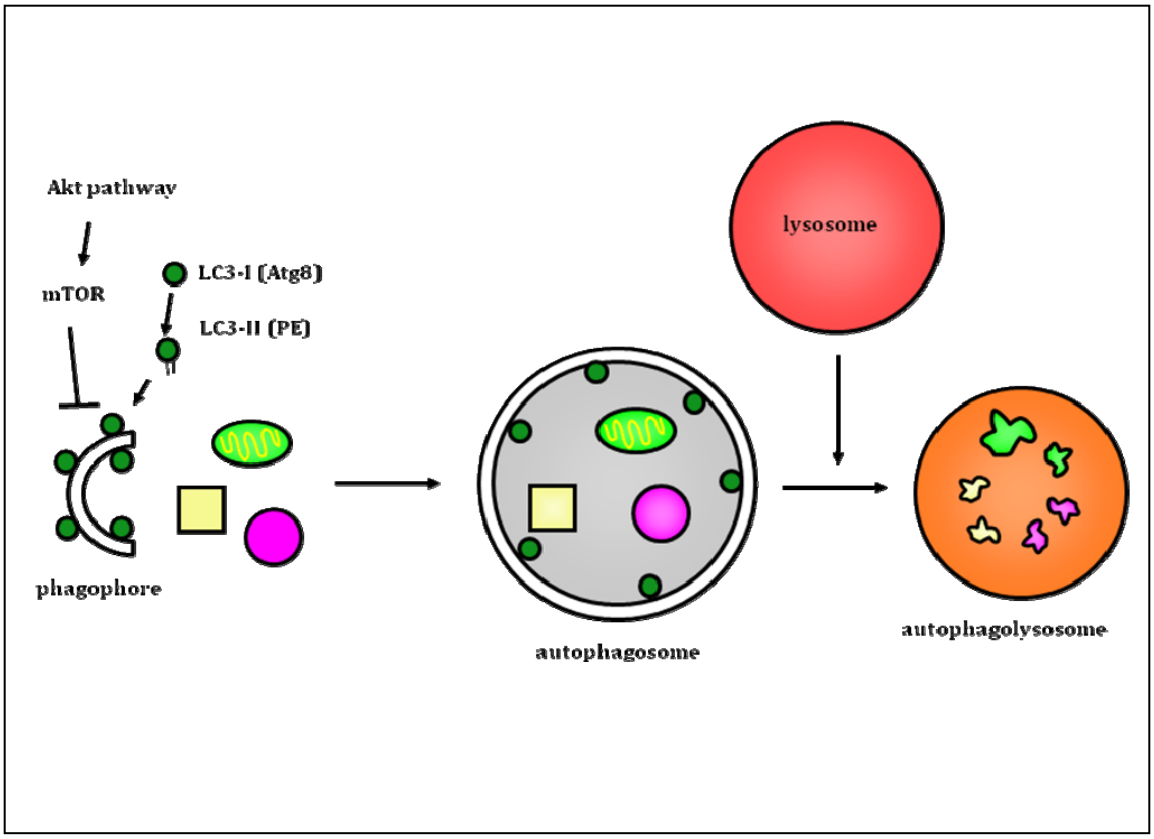

Figure 1.2: Stages of Autophagy.
Autophagy follows a series of steps regulated by autophagy-related (Atg) proteins. The first step involves the formation of an isolation membrane, or phagophore, which envelops the target until it fuses with itself to form an autophagosome, with a double membrane. The autophagosome fuses with lysosome to become an autolysosome. Elongation is dependent on the Atg12 conjugation system, in which Atg12 forms a complex with Atg5 and Atg16, which in turn triggers lipidation of LC3 I to form the membrane-bound LC3-II (PE). The Atg5-Atg12-Atg16 complex and LC3-II are recycled, while LC3-II on the luminal membrane remains and is degraded in the autolysosome.

Autophagy can be modulated by pharmacological agents, cytokines, and other immunological signals [126-128]. Rapamycin is an agent that induces autophagy through inhibition of TOR, a conserved Ser and Thr kinase that regulates cell growth and metabolism in response to growth factors, energy inputs, and nutritional demands [129]. TOR integrates various inputs; its activation stimulates anabolic processes and biomass production, whereas its inhibition enhances catabolic processes, including autophagy. Another example of this system in action is demonstrated by amino acid starvation, which also leads to inhibition of TOR and induction of autophagy. Conversely, TOR can be activated by growth factors via the Akt (also known as PKB) pathway, resulting in the inhibition of autophagy [129]. In some cases, withdrawal of growth factors from cells is sufficient for induction of autophagy, even in the presence of adequate nutrients [130]. However, even in the presence of adequate nutrients the cells die more rapidly when autophagy is blocked, suggesting that autophagy is a survival mechanism in these cells [130]. Positive regulators of autophagy include the Th1 cytokines IFN-γ and TNF-α [115, 123, 131]. These diverse immunological signals demonstrate the complexity of the autophagic process.

1.5.1. Th1 cell-mediated response and autophagy

The protective role of IFN-γ against mycobacteria has been associated with autophagy [115, 128], indicating that this process is an important effector mechanism of the Th1 response [115, 123, 131]. The mechanism by which Th1 cell-mediated/IFN-γ-response induces autophagy in macrophages is not yet fully understood. However, it is clear that Irgm1 is involved in this process. Transfection of murine macrophages with Irgm1 promotes the formation of

autophagosomes, while siRNA knockdown of Irgm1 inhibits IFN-γ-induced autophagosome formation [115, 128]. The human ortholog of Irgm1, IRGM also participates in autophagy. Although IRGM is not induced by IFN-γ, siRNA knockdown of IRGM in human macrophages inhibits autophagosome formation and maturation of BCG-containing phagosomes, thereby promoting mycobacterial survival [128]. The extent to which autophagy contributes to IFN-γ-induced phagosome maturation in macrophages infected with mycobacteria still remains unknown. However, siRNA knockdown of Beclin 1, a key regulator of autophagosome formation, completely abrogates the effect of IFN-γ on phagosome-lysosome fusion in infected murine RAW264.7 macrophages [132], indicating that autophagy may be responsible for the IFN-γ-induced phagosome maturation in these cells. These data suggest autophagy plays a vital role in the Th1 response to eliminate mycobacteria.

TNF-α is an important factor in the protective immune response to *M. tuberculosis*, as it is essential for the formation and maintenance of the granuloma [133]. Moreover, TNF-α induces apoptosis of mycobacteria-infected macrophages, depriving the mycobacteria of their niche cell [134] and packaging mycobacterial antigens in apoptotic vesicles for uptake and presentation by dendritic cells [135, 136]. Numerous studies also indicate that TNF-α may be important in the modulation of autophagy. Autophagic elimination of *Toxoplasma gondii* occurs in infected macrophages following ligation of CD40, coupled with exogenous or autocrine TNF-α signaling [114, 126]. In addition, autophagy has been observed in T lymphoblastic leukemic cell lines at an early stage of TNF-α-

induced cell death [137]. Expression of the autophagy genes LC3 and Beclin 1 is induced in human atherosclerotic vascular smooth cells following treatment with TNF-α. This effect is dependent on the activation of the Jun kinase (JNK) pathway [138]. Furthermore, TNF-α might also promote autophagy by inhibiting activation of the Akt pathway [138], as siRNA-mediated knockdown of Akt induces autophagy in murine macrophages [132]. Activation of the Akt pathway inhibits autophagy through the activation of mTOR. In MCF-7 human breast cancer cells, TNF-α-induced autophagy is dependent on ERK1/2 signaling since inhibition of ERK signaling results in greater sensitivity to TNF-α-induced cell death [139]. In Ewing sarcoma cells, TNF-α-induced autophagy is inhibited by activation of NF-kB and is dependent on the production of reactive oxygen species [127]. *Mycobacterium tuberculosis* can inhibit TNF-α-induced apoptosis by activating NF-kB and enhancing the production of soluble TNF receptor 2 [140]. These may also represent strategies to inhibit TNF-α-induced autophagy.

1.5.2. Th2 cell-mediated response and autophagy

While IFN-γ and TNF-α stimulate autophagy, resulting in the killing of *Mycobacterium tuberculosis*, the cytokines IL-4 and IL-13, associated with Th2 responses, are considered less protective in mycobacterial disease [70]. In the human colonic epithelial cell line HT-29 (responsive to IFNγ and TNFα), IL-13 is a potent inhibitor of starvation-induced autophagy [141, 142]. IL-13 stimulates tyrosine phosphorylation of an adaptor molecule, insulin receptor substrate, that in turn facilitates coupling of the receptor to type I PI3K (p85p110) (Figure 1.3). Both IL-4 and IL-13 signal through IL-4Rα, which forms a heterodimer with gamma common (γc) chain (for the IL-4 receptor) or the IL-13Rα1 (for the IL-13 receptor) [143]. Ligation of these receptor complexes results in signaling via the insulin receptor substrate (IRS)-1/2 and STAT6 pathways [143]. While STAT6 is involved in IL-4 and IL-13-induced gene expression, IRS-1/2 signaling activates the type I phosphatidylinositol 3-kinase (PI3K). Type I PI3K (unlike type III PI3K hVPS34) acts as an inhibitory PI3K in autophagy, since it leads to the activation of Akt and TOR, downregulators of autophagy [141, 142, 144] (Figure 1.3). However, it should be noted that these cytokines could influence the response to *M. tuberculosis* (and other pathogens) through other mechanisms, such as inhibition of the inducible nitrogen oxide (iNOS) expression [145] or indirectly through inhibition of Th1 responses [69, 99]. The predominantly Th1 environment within the lungs of patients with active tuberculosis should promote intracellular killing of mycobacteria; however, some reports indicate there is a higher Th2 response in anergic patients and those with more severe pulmonary

tuberculosis [146, 147]. As such, IL-4 and IL-13, released by macrophages infected with virulent strains of *Mycobacterium tuberculosis* [70, 148, 149] could act in an autocrine manner to inhibit the autophagic process. These data suggest that autophagy, as well as being a potentially important anti-mycobacterial response in macrophages, may be modulated by Th1 and Th2 cytokines.

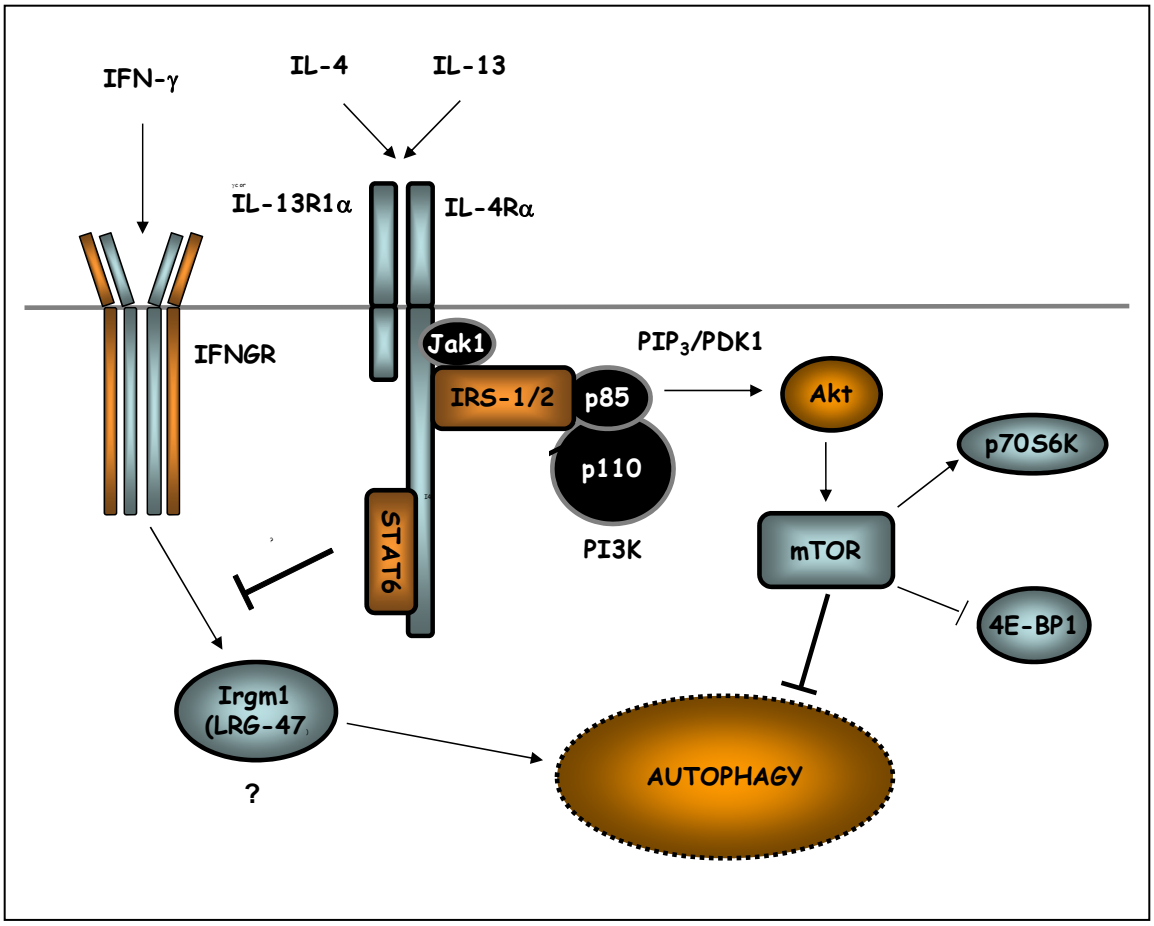

Figure 1.3. Opposing Effects of IFN-γ and IL-4/IL-13 in Autophagy Induction Via Akt Pathway.

Following ligation of the IL-4/IL-13 receptor, the insulin receptor substrate (IRS 1/2) interacts with the phosphorylated IL-4R motif of the IL-4Rα via Jak1. Type I PI3K and PDK1 are recruited to the complex, leading to the formation of phosphoinositides (including PIP3), which results in Akt phosphorylation. Through phosphorylation of the tuberous sclerosis protein 1 and 2 complex (TSC1/2), Akt activates mammalian target of rapamycin (mTOR), which inhibits autophagy. Inhibition of mTOR, such as by rapamycin, induces autophagy. IFN-γ is a positive regulator of autophagy. These opposing roles of IFN-γ and IL-4/IL-13 suggest that Th1/Th2 balance could affect the ability of macrophages to eliminate *Mycobacterium tuberculosis* by autophagy.

1.6. Hypothesis

Macrophages represent the first line of defense against *Mycobacterium tuberculosis*. The Th1 cytokine IFN-γ brings about the inhibition of mycobacterial growth in macrophages; in contrast, the Th2 cytokines, IL-4 and IL-13, facilitate the survival of mycobacteria. Autophagy plays an important role in innate immunity against mycobacteria. IL-4 and IL-13 signal through the Akt-TOR cascade, known to inhibit autophagy, and STAT-6 pathways. Excessive induction of IL-13 has been shown in macrophages infected with hypervirulent strains of *M. tuberculosis*; this induction of IL-13 is believed to modify the host cellular immune response.

I hypothesize that the Th2 cytokines, IL-4 and IL-13, inhibit the autophagic control of *Mycobacterium tuberculosis* in macrophages. This study investigates the roles of IL-4 and IL-13 in autophagy in macrophages, in the context of *Mycobacterium tuberculosis* infection.

2. MATERIALS AND METHODS

2.1. Materials

Unless otherwise stated, reagents were from Sigma (St. Louis, MO). Recombinant murine and human IL-4 and IL-13 were purchased from R & D Systems. Rabbit polyclonal antibody against CD63 was from Santa Cruz Biotechnology, rabbit polyclonal antibody against LC3 was from T. Ueno and E. Kominami. pEGFP-LC3 was from T. Yoshimori and GFP-LC3 transgenic mice were from N. Mizushima. Tdtomato-LC3 was from G. Bjorkoy.

2.2. Methods

2.2.1. Cells and bacterial cultures

Murine RAW264.7 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and L-glutamine (full-nutrient medium). The human monoblastic cell lines, U937 and THP-1, were maintained in RPMI- 1640 (Invitrogen) with 10% FBS, L-glutamine, and HEPES. Before use, U937 and THP-1 cells were differentiated with PMA (100 nM) for 24–72 h. Mycobacterium bovis BCG was grown in Middlebrook 7H9 broth with 0.5% Tween, 0.2% glycerol, and albumin-dextrosecatalase supplement (BD Diagnostics, Franklin Lakes, NJ, USA). Primary human monocyte-derived macrophages (MDMs) were isolated and cultured from buffy coats by density gradient centrifugation on Ficoll-Paque Plus (GE Healthcare). PBMCs were allowed to adhere to gelatin-coated plates for at least 1 h, washed so that nonadherent cells could be removed, and cultured in RPMI-1640 with 10% human human AB serum for 24 h. After this time, cells were lifted, counted, and plated on tissue-culture plastic or glass coverslips for 5–

7 days. Bone marrow-derived macrophages (BMMs) were isolated and cultured as described.

2.2.2. Flow cytometry

After blocking with seroblock (AbD Serotec), RAW264.7 cells were stained with rat anti-mouse CD124 (IL-4Ra)-PE (BD) for 30 min on ice. U937 and THP-1 cells were blocked with human serum (10%) and stained first with anti-human CD124 for 30 min on ice, then with anti-mouse IgG1-PE (BD) for 30 min on ice. The cells were analyzed on a BD FACScaliber, and data were processed with CellQuest software.

2.2.3. Induction of autophagy

Autophagy was induced either by amino acid starvation, in which cells were incubated for 2 hr in Earle's balanced salt solution (EBSS) or by treatment with IFN-γ (200 U/ml) for 24 h [115].

2.2.4. Macrophage transfection

RAW264.7 and U937 cells were transfected by nucleoporation as previously described [150]. In brief, cells were harvested after 2–3 days in culture and resuspended in 100 µl of the appropriate electroporation buffer (Amaxa Biosystems) with 5–10 µg plasmid DNA or 1.5 µg siGENOME SMARTpool siRNA or siCONTROL nontargeting siRNA (Dharmacon) and nucleofected with Amaxa Nucleofector apparatus. After electroporation, cells were cultured in full nutrient medium for 24 h before use.

2.2.5. Fluorescence confocal microscopy

Cells were cultured on glass coverslips, fixed in 2% paraformaldehyde for 20 min at room temperature, permeabilized, and blocked in PBS with 0.5%

Tween 20, 1% BSA, and 2% goat serum for 30 min at room temperature. Cells were incubated with primary antibody for 1 h and then with secondary antibody for an additional 1 h. Alternatively, cells were incubated with Lysotracker Red DND-99 (LT, Invitrogen) for 2 h prior to and during incubation with mycobacteria. For labeling of acidic, lipid-rich vacuoles, cells were incubated with monodansylcadaverine (MDC, 50 mM) for 15 min prior to fixing. Bone marrow macrophages expressing GFP-LC3 were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.2% saponin for 5 min, blocked for 30 min, and incubated with rabbit polyclonal antibodies against GFP (Abcam) overnight for visualization of the GFP-LC3 fusion protein (GFP fluorescence in BMMs isolated from these transgenic mice is sporadic, and GFP antibody is routinely used for visualization of GFP-LC3.). Slides were incubated with a secondary anti-mouse fluorescein isothiocyanate-conjugated antibody. Coverslips were mounted onto glass slides with Permafluor Aqueous mounting medium (Thermo Scientific, Waltham, MA, USA) and analyzed on a Zeiss LSM510 META laser-scanning confocal microscope.

2.2.6. Immunoblotting

Cells were washed in PBS and lysed 1 h in buffer containing 10 mM Tris HCl, 150mM NaCl, 0.5% deoxycholate, 2mM EDTA,2% NP-40, 1mM PMSF, and protease inhibitor cocktail (Roche Applied Science). A total of 50 µg of protein was loaded and separated on a 12% or 15% SDS polyacrylamide gel (BioRad) and transferred to nitrocellulose. The membrane was blocked in 5% milk or 3% BSA in PBS/Tween 20 (0.1%) and probed with primary antibodies overnight at 4^0C. After washing with PBS/Tween, the blot was probed with HRP-conjugated

secondary antibody for 1 h at room temperature. Staining was revealed with SuperSignal West Dura Extended Duration Substrate (Pierce). ImageJ (NIH) was used for gel analyses.

2.2.7. Phagolysosome maturation and mycobacterial-survival assays

For phagocytosis of mycobacteria, macrophages were incubated with Texas red-labeled BCG, GFP-BCG, or Mycobacterium tuberculosis strain H37Rv for 15–30 min, washed, and incubated for an additional 2 h, in the presence of starvation media, in the presence or absence of cytokines as indicated. After staining the fixed cells for the lysosomal marker CD63 (LAMP3), confocal microscopy was used so that the percentage of CD63-positive mycobacterial phagosomes from at least 100 cells could be recorded. For mycobacterial-survival assays, RAW cells or MDM were infected with BCG or H37Rv for 1 h, washed, and incubated for an additional 2 hr with starvation media and cytokines as indicated. Cells were washed with PBS and lysed with distilled water. Serial dilutions of lysates were made, and 5 ml aliquots were inoculated on Middlebrook 7H10 agar plates supplemented with oleic acid–albumin–dextrose–catalase (BD). Plates were sealed and incubated for 2 weeks at 37^{o}C, and colonies were counted from dilutions yielding 10–50 visible colonies. Data are expressed as colony-forming units per ml (cfu/ml).

2.2.8. Statistical analysis

Data are presented as means ± SEM (approximately three independent experiments); p values (Student's t test; two-tailed) are relative to the control, unless otherwise specified.

3. RESULTS

3.1. IL-4 and IL-13 inhibit starvation-induced autophagy

Induction of autophagy in the murine RAW264.7 and human U937 monocyte or macrophage cell lines was monitored with morphometric analysis after the formation of pEGFP-LC3-labeled [122] autophagosomes (≥ 1 μm). LC3 is the mammalian equivalent of yeast Atg8, a specific marker that translocates from the cytosol to autophagosomal membranes [122]. Amino acid starvation resulted in a significant increase in the number of pEGFP-LC3^{+} puncta per cell (Figures 3.1 and 3.2). Translocation of cytosolic LC3 to autophagosomal organelles was also detected by 4D live confocal microscopy [150-152] in macrophages transfected with tdTomato-LC3 [153] (Movie SM1). Amino acid-starvation-induced formation of LC3 puncta was abrogated with the addition of 3-MA (Figure 3.3), a classical inhibitor of autophagy [154]. Both IL-4 and IL-13 signal through IL-4Rα [143]. After ascertaining the expression of IL-4Rα by flow cytometry (Figure 3.4), IL-4 and IL-13 were added to macrophages induced for autophagy by amino acid starvation. Either one of the Th2 cytokines tested abrogated autophagy, as shown by inhibition of pEGFPLC3^{+} puncta formation in both RAW and U937 cells (Figures 3.1 and 3.2).

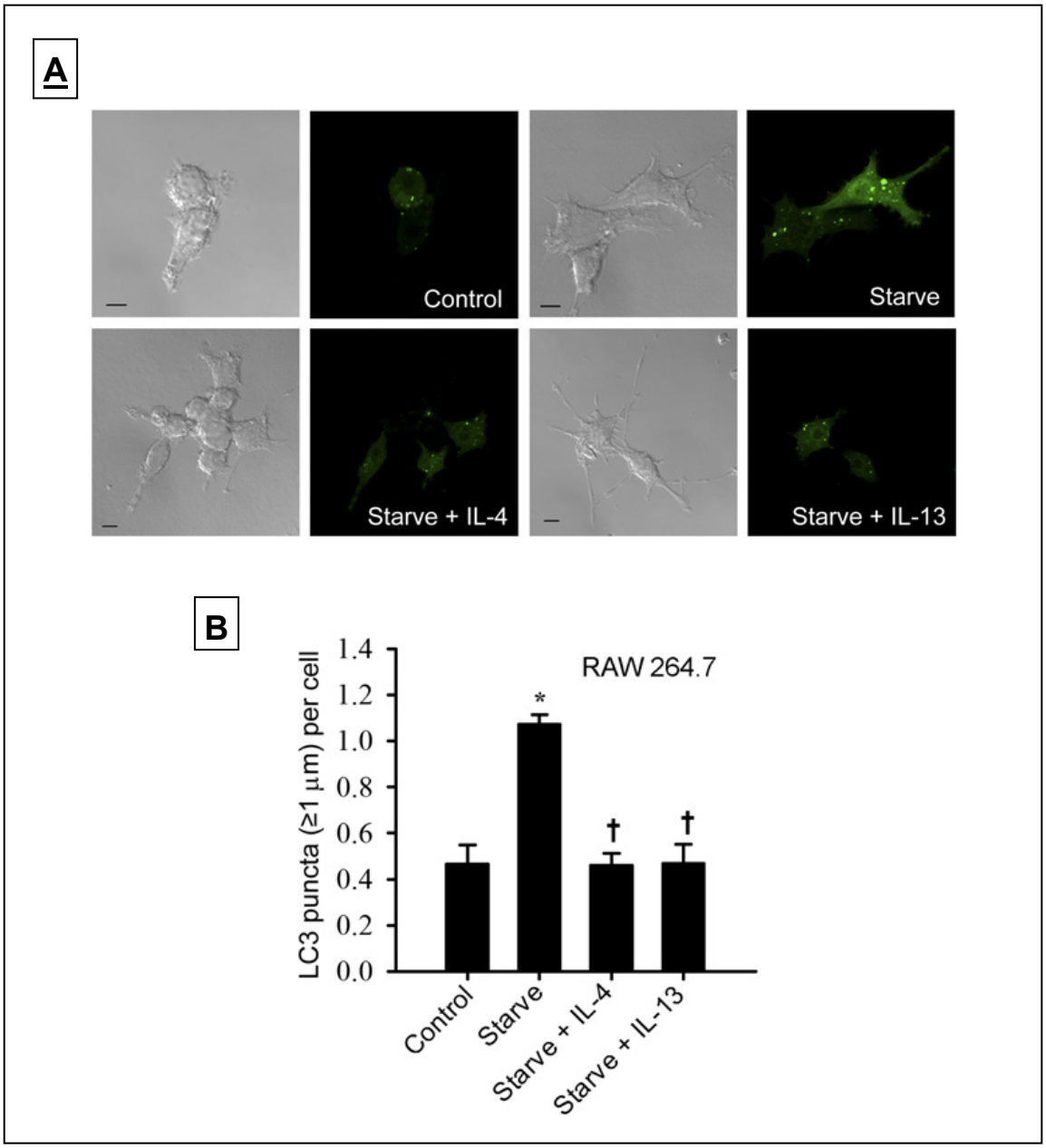

Figure 3.1: Inhibition of starvation-induced autophagy by IL-4 and IL-13 in murine macrophage cell line RAW264.7.
(**A**) Murine RAW264.7 cells were transiently transfected with pEGFP-LC3, amino acid- and serum-starved for 2 hr with or without IL-4 or IL-13 (30 ng/ml), and analyzed by confocal microscopy. (**B**) The number of large (≥1 μm) LC3 puncta per cell were quantified. Data are presented as means ± SEM; *p < 0.05 and †p ≥ 0.05; n = 3. Scale bars represent 5 μm. **(Courtesy of Dr. James Harris)**.

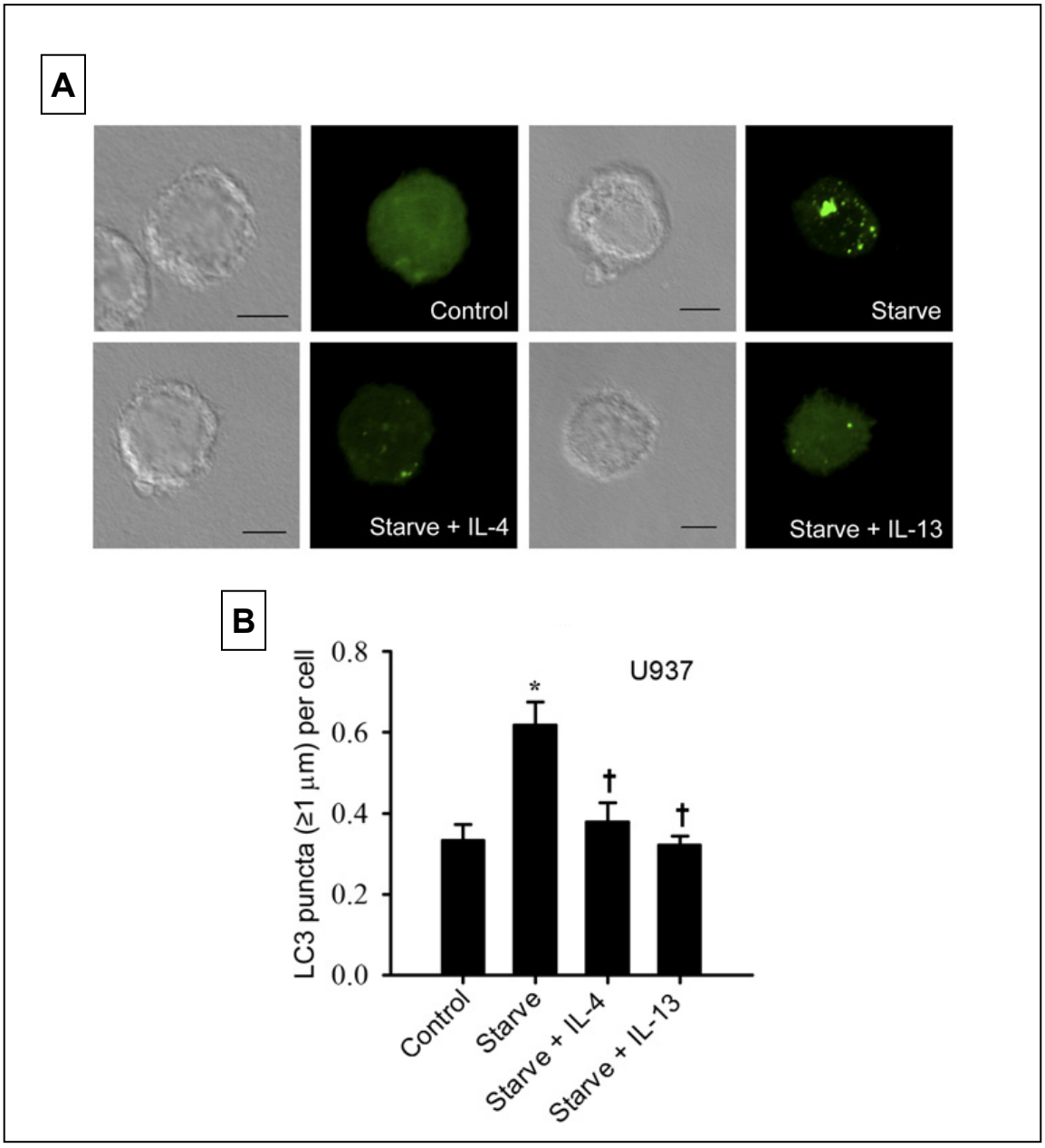

Figure 3.2: Inhibition of starvation induced autophagy by IL-4 and IL-13 in human macrophage-like cell line U937.
(**A**) Human U937 cells were transiently transfected with pEGFP-LC3, amino acid- and serum-starved for 2 hr with or without IL-4 or IL-13 (30 ng/ml), and analyzed by confocal microscopy. (**B**) The number of large (≥1 µm) LC3 puncta per cell were quantified. Data are presented as means ± SEM; *$p < 0.05$ and †$p \geq 0.05$; $n = 3$. Scale bars represent 5 µm.

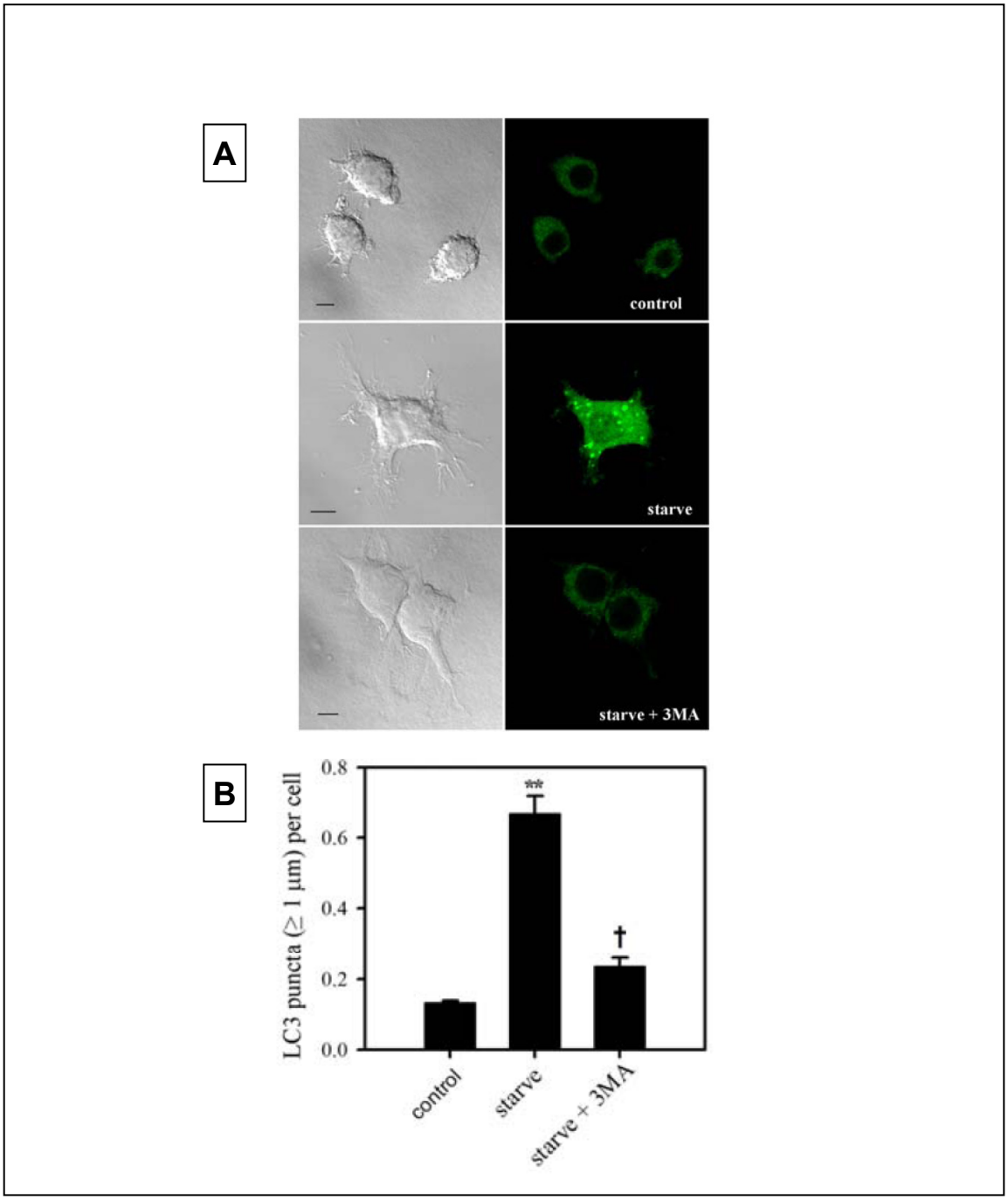

Figure 3.3. Induction of Autophagosome Formation by Starvation.
(**A**) Murine RAW264.7 cells were transiently transfected with pEGFP-LC3, amino acid- and serum-starved for 2 hr with or without 10 mM 3-methyladenine (3-MA) and analyzed by confocal microscopy. Treatment with 3-MA inhibits puncta formation. (**B**) The number of large (≥1 μm) LC3 puncta per cell were quantified. Data are presented as means ± SEM; ** $p < 0.01$, † $p \geq 0.05$. Scale bars represent 5 μm. **(Courtesy of Dr. James Harris)**.

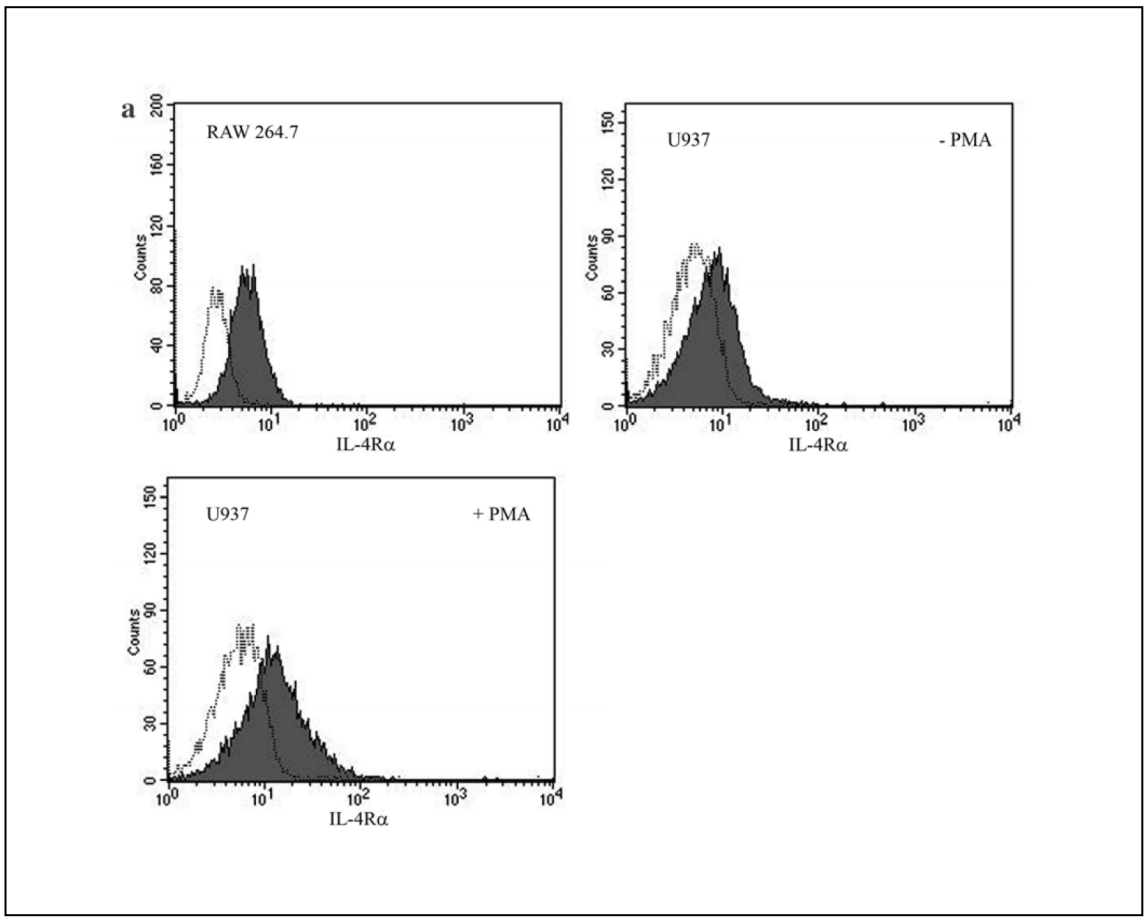

Figure 3.4. Flow cytometry analysis of IL-4rα surface expression.
Macrophage cell lines used in the study were tested for IL-4Rα surface expression. There was no positive staining with the antibody isotype control. RAW 264.7 cells frequently showed two populations of IL-4Rα expression (IL-4Rα^{high} and IL-4Rα^{low}) and were enriched for IL-4Rα^{high} by cell sorting prior to use, when necessary.

Similar results were obtained with primary bone marrow-derived macrophages (BMMs) from GFP-LC3 transgenic mice [155] (Figures 3.5A and 3.5B). A titration of IL-4 and IL-13 in RAW264.7 cells showed that at 0.3 ng/ml, both cytokines still exerted their effects, albeit IL-4 was losing some of its potency at the lowest concentration tested (Figure 3.6A).

The inhibitory effects of IL-4 and IL-13 on starvation-induced autophagy were independently confirmed by an assay that measures conversion of LC3-I (nonlipidated form with lower electrophoretic mobility) to LC3-II (LC3 form C-terminally lipidated by phosphatidylethanolamine, displaying higher electrophoretic mobility) with immunoblots [122]. As expected, induction of autophagy by starvation reduced both LC3-I and LC3-II band intensity in RAW264.7 cells (Figure 3.6B and Figure 3.7A, lanes 1 and 2) because of the autophagic consumption of LC3 [122]. At the same time, the intensity of the LC3-II band changed (increased) relative to the intensity of LC3-I band (Figure 3.6B and Figure 3.7A, lanes 1 and 2). When IL-4 and IL-13 were added to starved macrophages, two changes occurred: (1) The intensity of the LC3-I band increased (Figure 3.6B, lanes 3 and 4, relative to lane 2), indicative of a reduced LC3-I-to-LC3-II conversion, consistent with the inhibition of autophagy induction by IL-4 and IL-13. An increase in the LC3-I band was also observed in primary murine BMM (Figure 3.7B). (2) The LC3-II band intensity decreased in starved RAW264.7 macrophages treated with IL-4 and IL-13 (Figure 3.6B, lanes 3 and 4, compared to lane 2). This could be the result of reduced LC3-I-to-LC3-II

conversion, in keeping with an inhibition of autophagy induction. However, in BMM only an LC3-I increase but no LC3-II decrease was observed (Figure 3.7B).

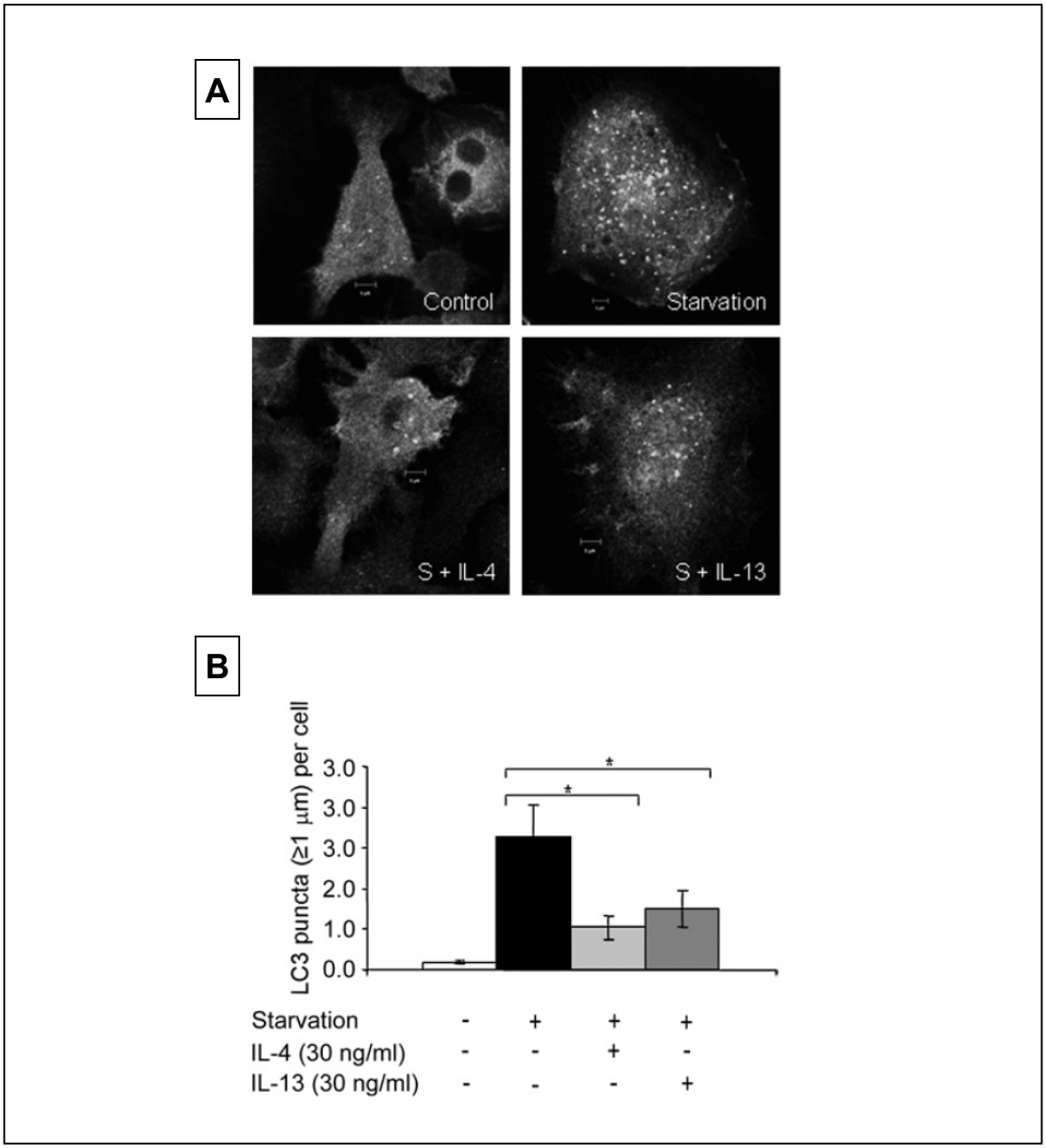

Figure 3.5. Inhibition of Starvation-Induced Autophagy by IL-4 and IL-13 in Murine Bone Marrow Macrophages.
(**A**) Bone marrow macrophages (BMMs) from GFP-LC3 transgenic mice were amino acid- and serum-starved (S) for 2 hr with or without IL-4 or IL-13 (30 ng/ml) and analyzed by confocal microscopy. (**B**) Quantification of large (>1 μm) GFP-LC3 puncta per cell (within the denominator; calculations included cells without any large puncta); n= 3. Data are represented as means **±** SEM; * $p <$ 0.01. **(Courtesy of Dr. Monica Delgado)**.

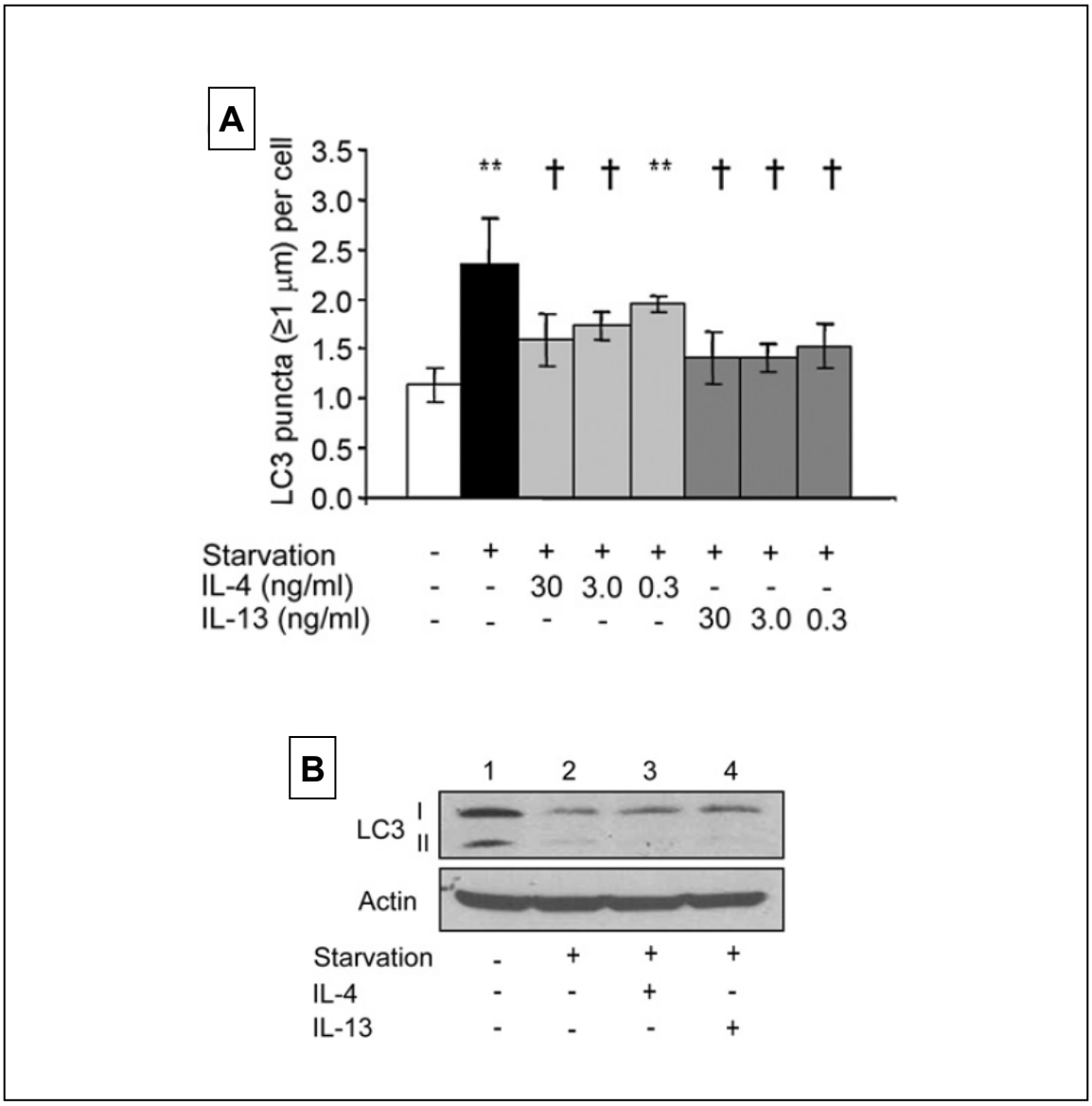

Figure 3.6. Inhibition of Starvation-Induced Autophagy by IL-4 and IL-13 in Murine RAW264.7 Macrophages.

(**A**) Quantification of large (≥1 μm) LC3 puncta in RAW264.7 cells transfected with GFP-LC3 and induced for autophagy by starvation in the presence of decreasing concentrations of IL-4 or Il-13; n = 3 (separate slides; shown is one out of two independent experiments). Data are presented as means ± SEM; **p < 0.01, and †p ≥ 0.05. (**B**) Immunnoblot analysis of LC3 lipidation state in RAW264.7 cells starved and incubated without (none) or with IL-4 or IL-13. Full; cells grown in full medium. Cells were amino acid- and serum-starved for 2 hr (lanes 2–4). Actin was used as the loading control. **(Courtesy of Dr. Monica Delgado)**

The differences in LC3-II band intensity between RAW264.7 and BMM may be the net result of differences in the rates of LC3-I-to-LC3-II conversion versus LC3-II depletion through degradation in autolysosomes. This was substantiated with lysosomal and autolysosomal protease inhibitors (Figure 3.7A, lanes 3 and 4, compared to lane 2), which, as expected, increased LC3-II amounts by blocking its degradation both in the presence or absence of IL-4. Consistent with the conclusion that Th2 cytokines inhibit LC3-I-to-LC3-II conversion, IL-4 presence increased LC3-I band intensity (Figure 3.7A, lane 4 compared to lane 3). Because the presence of IL-4 did not diminish LC3-II band levels relative to protease inhibitors alone (Figure 3.7A, lane 4 versus lane 3), as one might expect from lower LC3-I-to-LC3-II conversion, Th2 cytokines may have an additional effect on the fate of LC3-II by partially inhibiting its delivery to or its degradation in autolysosomes. This conclusion is consistent with the apparent preservation of the LC3-II band in BMM (Figure 3.7B). Of note is that IL-4 and IL-13 did not change expression of the murine LC3 gene MAP1-LC3b under starvation conditions (Figure 3.7C). In conclusion, IL-4 and IL-13 inhibit LC3-I-to-LC3-II conversion and initiation of autophagy but may have additional effects on the autophagic pathway; these effects become apparent when different cells are examined.

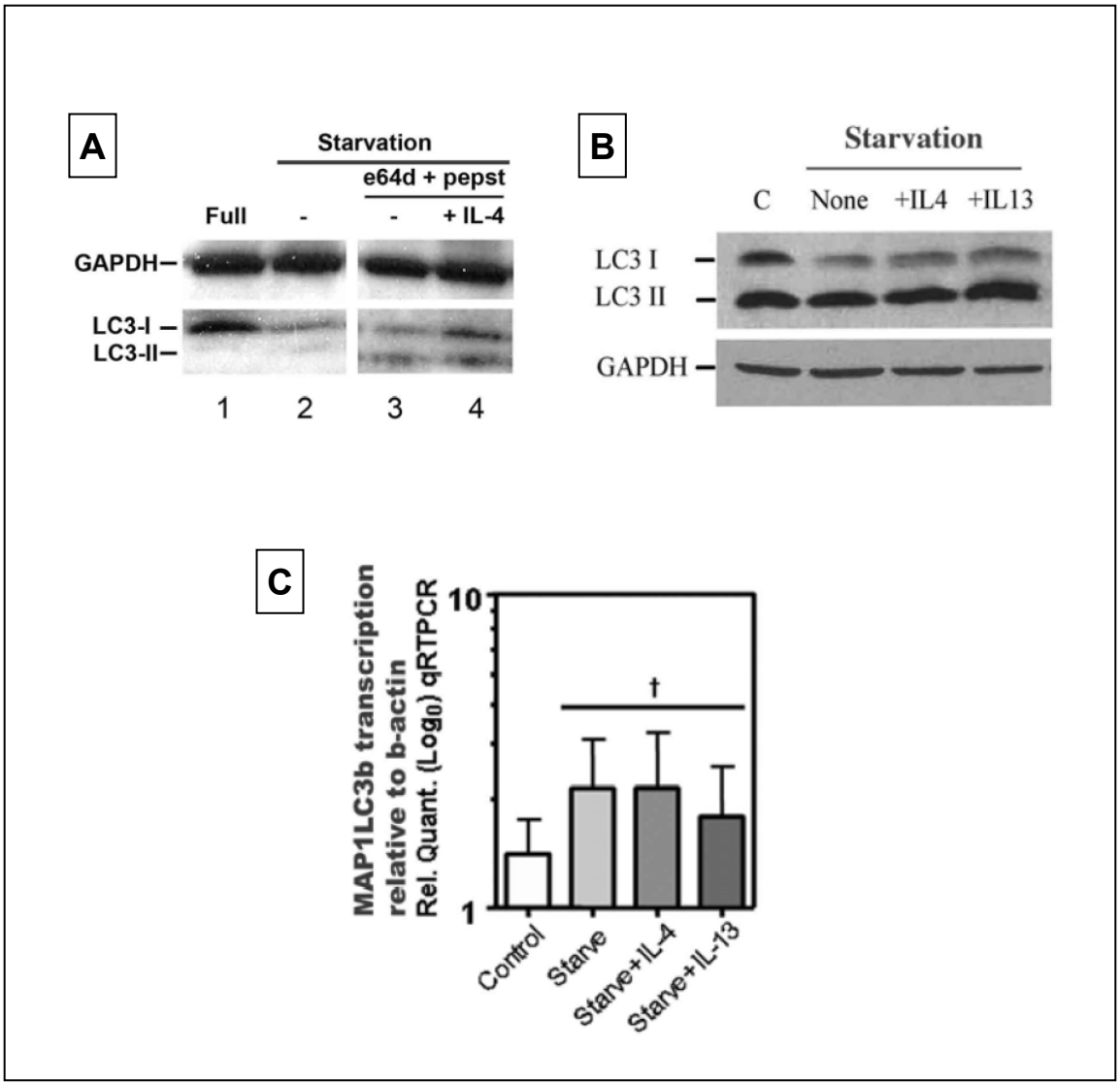

Figure 3.7. Inhibition of LC3 lipidation in Starvation-Induced Autophagy by IL-4 and IL-13.

(**A**) IL-4 inhibits LC3-I to LC3-II conversion and slows down starvation-induced LC3-II turnover in RAW264.7 macrophages. Western blot analysis of LC3 lipidation state in RAW264.7 cells, incubated in full medium (Full), or starved and incubated without (-) or with IL-4. Where indicated, lysosomal protease inhibitors e64d and pepstatin were added. GAPDH, loading control. (**B**) IL-4 and IL-13 inhibit LC3-I-to-LC3-II conversion in murine bone marrow derived macrophages. Macrophages were induced for autophagy by amino acid and serum starvation in the absence (None) or in the presence of IL-4 (+IL-4) or IL-13 (+IL-13). C, control incubated in full medium. GAPDH, loading control. (**C**) Quantitative (q) RT-PCR analysis of LC3b expression. Data are represented as means ± SEM; † $p \geq 0.05$.

3.2. IL-4 and IL-13 inhibit IFN-γ-induced formation of autophagosomes

Treatment of macrophages with IFN-γ promotes the formation of autophagosomes [115, 128]. We tested whether this effect could be inhibited by treating RAW and U937 cells with IFN-γ in combination with either IL-4 or IL-13. In both cell types, IL-4 and IL-13 significantly reduced the number of IFN-γ-induced pEGFP-LC3^{+} puncta per cell (Figure 3.8 and Figure 3.9). In addition, we found that IFN-γ treatment increased the percentage of RAW cells with large vacuoles that stained positively for monodansylcadaverine (MDC), another marker for autophagic vacuoles [156], and this effect was inhibited by IL-4 or IL-13 (Figures 3.10A and 3.10B). By using the MDC assay, we confirmed the effects of IL-4 and IL-13 in primary human, peripheral blood monocyte-derived macrophages (MDMs) and found, in titration experiments, a similar concentration-dependence pattern to that seen in murine macrophages (Figure 3.10C).

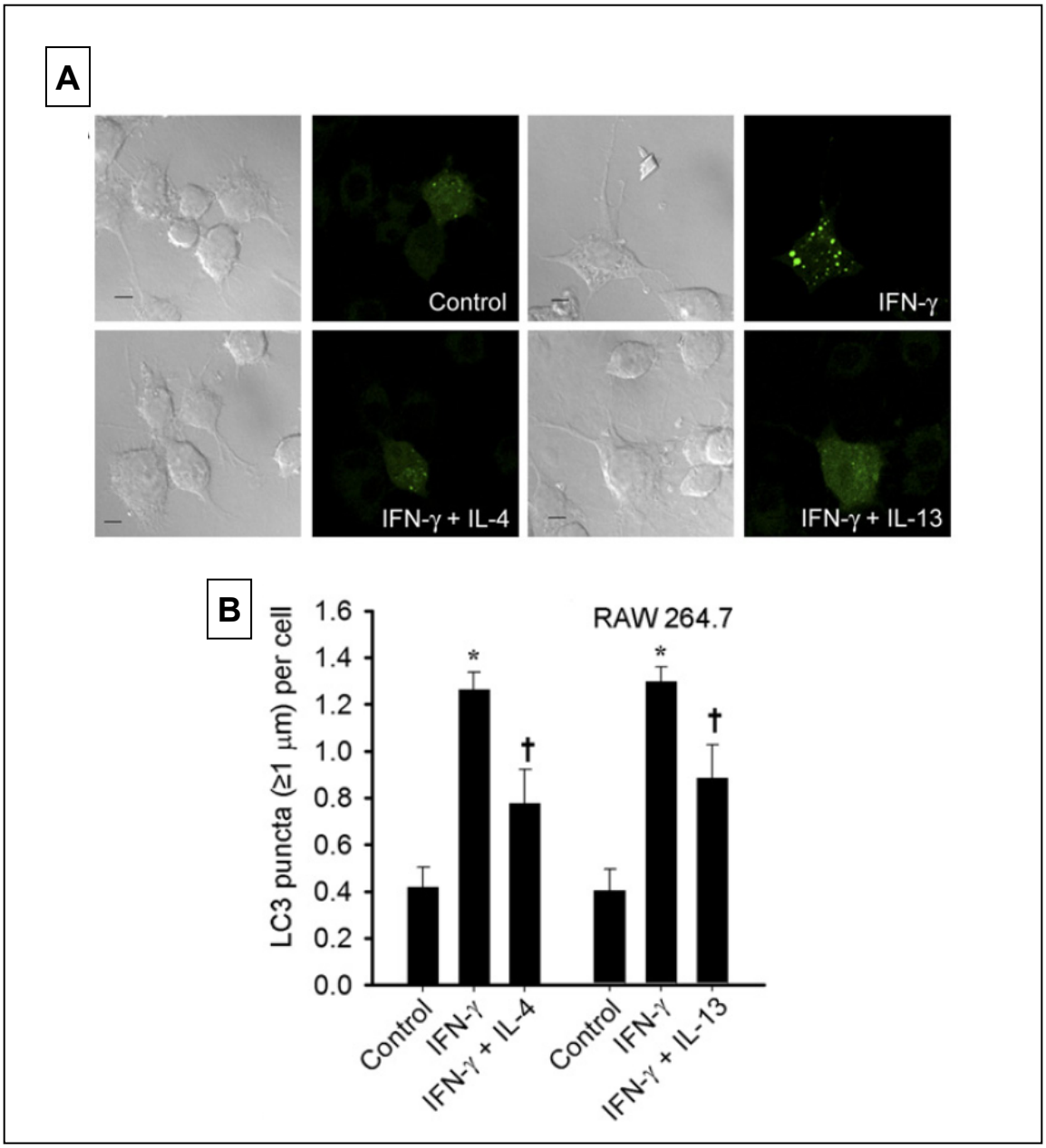

Figure 3.8. Inhibition of IFN-γ-Induced Autophagy by IL-4 and IL-13 in RAW264.7.

(**A**) Murine RAW 264.7 were transiently transfected with pEGFP-LC3 and treated with 200 U/ml IFN-γ or IFN-γ in combination with IL-4 or IL-13 (30 ng/ml) for 24 hr and analyzed by confocal microscopy. (**B**)The number of large (≥1 μm) LC3 puncta per cell were quantified. Data are presented as means ± SEM; * $p < 0.05$, ** $p < 0.01$, †$p \geq 0.05$. Scale bars represent 5 μm. **(Courtesy of Dr. James Harris)**.

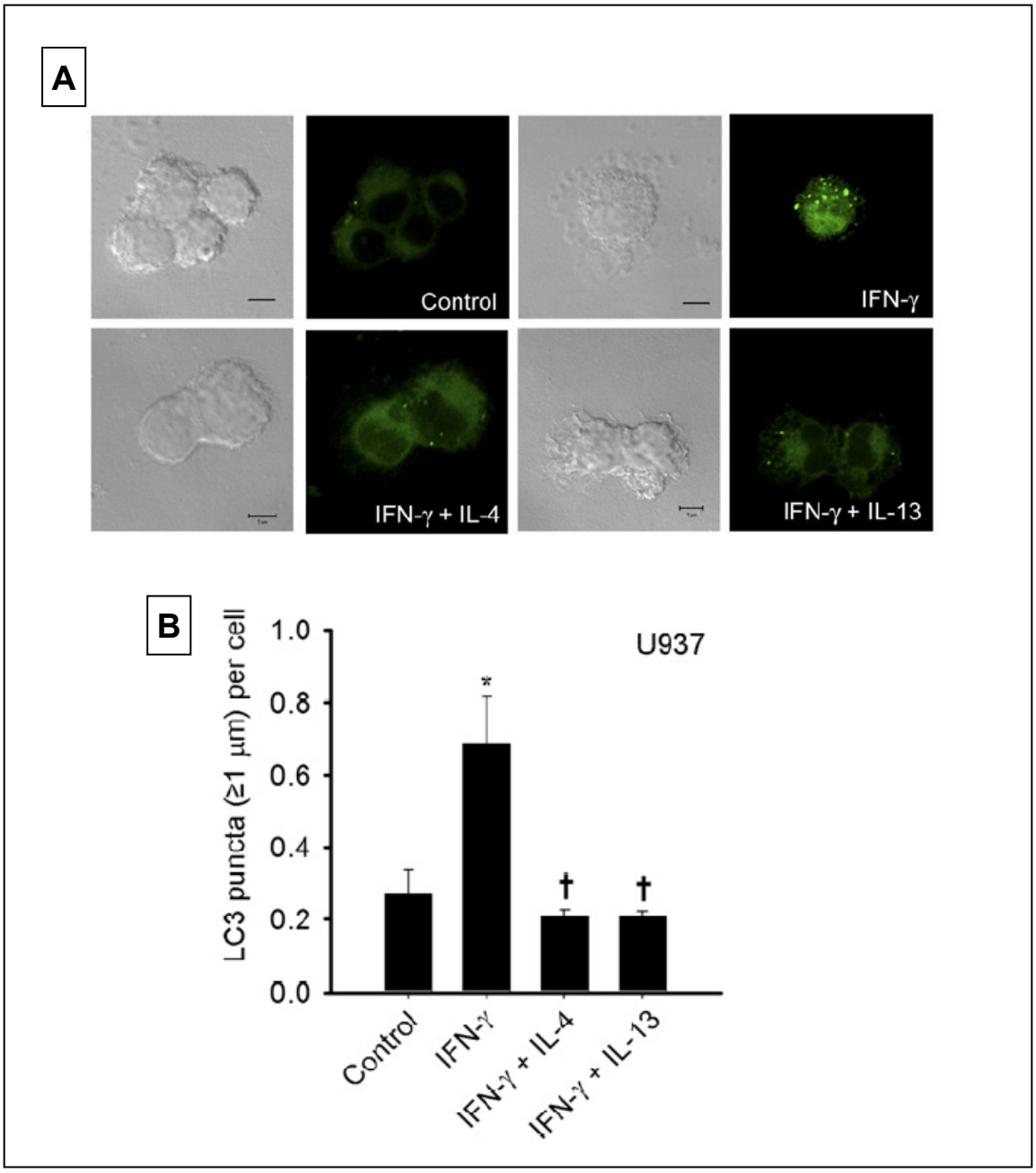

Figure 3.9. Inhibition of IFN-γ-Induced Autophagy by IL-4 and IL-13 in U937. (**A**) Human U937 cells were transiently transfected with pEGFP-LC3 and treated with 200 U/ml IFN-γ or IFN-γ in combination with IL-4 or IL-13 (30 ng/ml) for 24 hr and analyzed by confocal microscopy. (**B**) The number of large (≥1 μm) LC3 puncta per cell were quantified. Data are presented as means ± SEM; * $p < 0.05$, ** $p < 0.01$, †$p \geq 0.05$. Scale bars represent 5 μm.

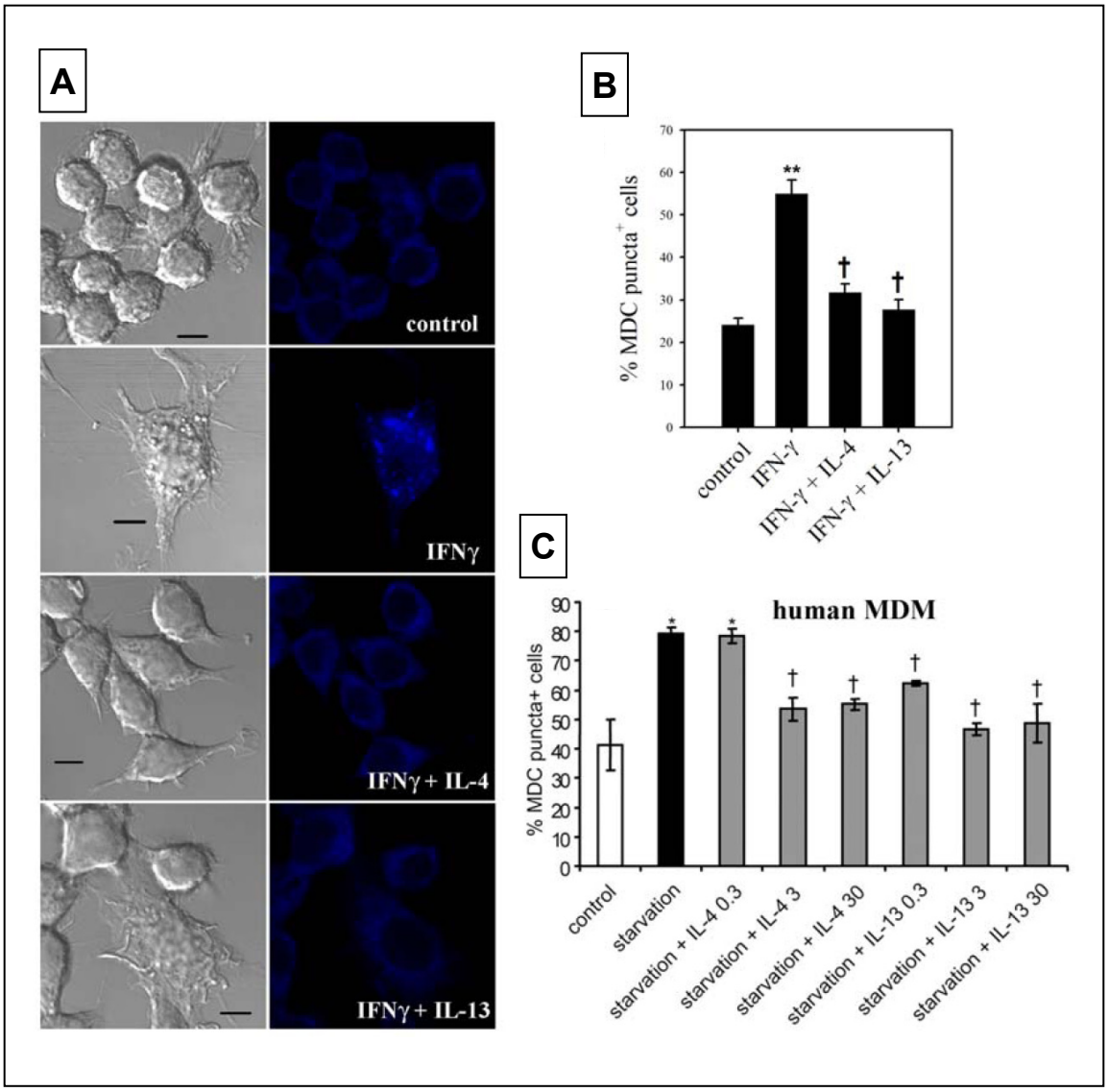

Figure 3.10. IL-4 and IL-13 Inhibit Autophagosome Formation in Murine and Human Macrophages Assessed by Monodansylcadaverine Staining.
(**A**) Murine RAW 264.7 were treated with IFN-γ (200 U/ml) with or without IL-4 or IL-13 (30 ng/ml) for 24 hr and stained with monodansylcadaverine (MDC) for 15 min. and were analyzed by confocal microscopy. (**B**) The percentage of cells with MDC^+ vacuoles were quantified. (**C**) Titration of IL-4 and IL-13 (concentration in ng/ml) effects on reversing starvation-induced autophagy in human primary, peripheral blood monocytes-derived macrophages (MDMs). Data are presented as means ± SEM; * $p < 0.05$, ** $p < 0.01$, †$p \geq 0.05$. Scale bars represent 5 μm. **(Courtesy of Dr. James Harris)**.

3.3. IL-4 and IL-13 inhibit autophagy-dependent BCG phagolysosome maturation

Mycobacterium tuberculosis normally resides in phagosomes that do not acquire phagolysosomal properties, such as lumenal acidification and the presence of lysosomal hydrolases [157]. Induction of autophagy has been shown to promote the transfer of mycobacteria into degradative autolysosomal organelles [115]. To investigate whether IL-4 and IL-13 counteract this effect, we examined the maturation of *M. tuberculosis* variant bovis BCG (BCG) phagosomes by monitoring the late endosomal or lysosomal marker CD63. Induction of autophagy by starvation or IFN-γ in BCG-infected RAW cells significantly increased colocalization of GFP-BCG with CD63 (Figures 3.11A and 3.12A). The effect of starvation-induced autophagy on BCG phagosome maturation was inhibited by the addition of either IL-4 or IL-13 (Figures 3.11A and 3.12A). Both IL-4 and IL-13 also inhibited IFN-γ-induced phagosome maturation in BCG-infected cells (Figure 3.12). Starvation increased phagosome maturation in U937 cells, an effect that was inhibited with either IL-4 or IL-13 (Figure 3.13). We also tested PMA-differentiated human THP-1 cells, by using the acidotropic dye LysoTracker Red (LT) to visualize lysosomal compartments. As with RAW cells, phagosome maturation was increased by either starvation (Figure 3.14A and 3.14B) or IFN-γ (Figures 3.14A and 3.14C); this effect was inhibited by treatment with either IL-4 or IL-13 (Figures 3.14).

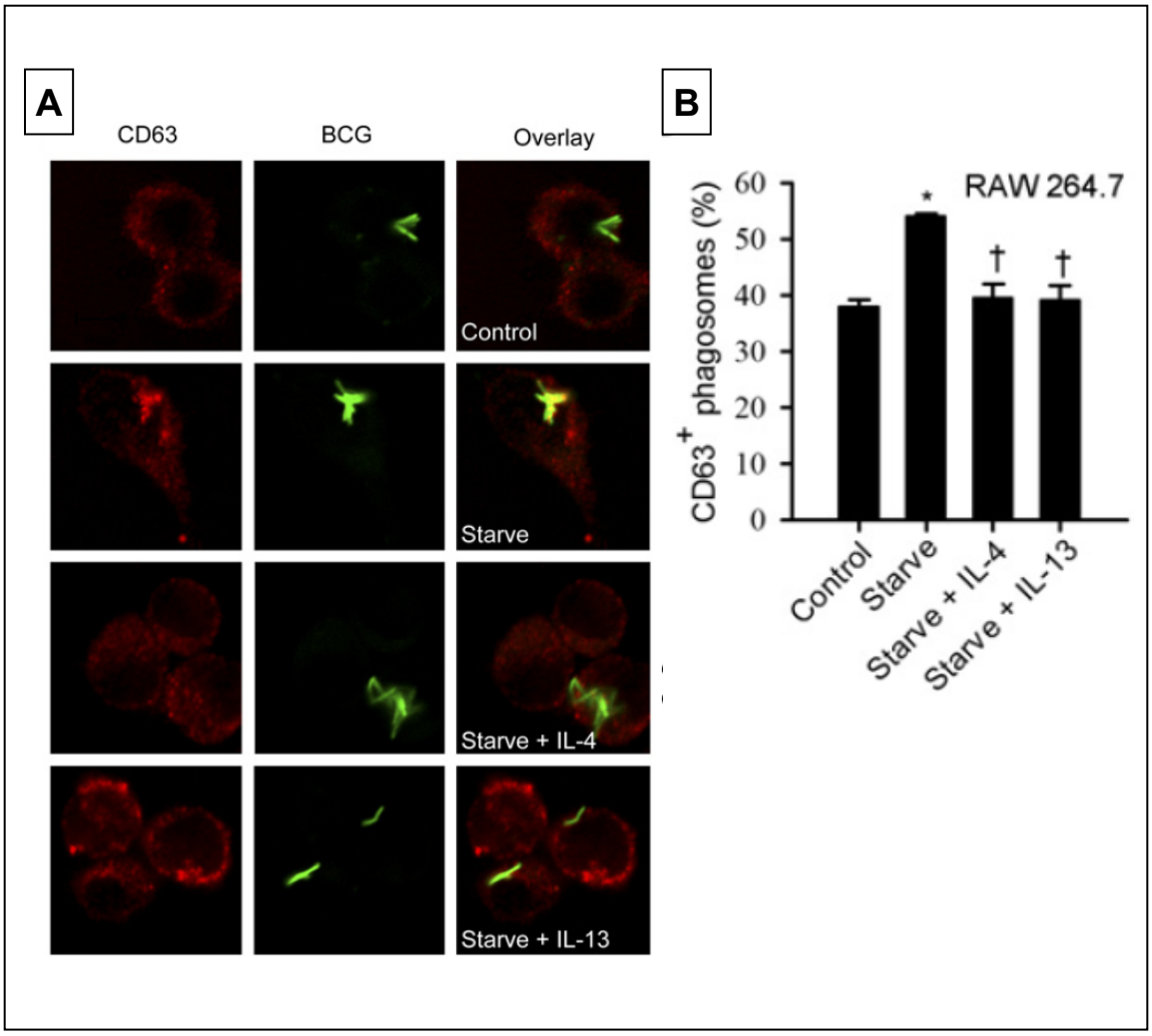

Figure 3.11. Inhibition of Starvation-Induced BCG Phagosome Maturation by IL-4 and IL-13.

(**A**) Confocal images of murine RAW 264.7 macrophages infected with GFP-BCG for 2 hr. Cells were amino acid- and serum-starved for 2 hr (during infection) with or without IL-4 or IL-13 (30 ng/ml). After infection, cells were fixed and stained for CD63. (**B**) The percent of $CD63^+$ was quantified. Data are presented as means ± SEM; * $p < 0.05$ and † $p \geq 0.05$; n = 3.

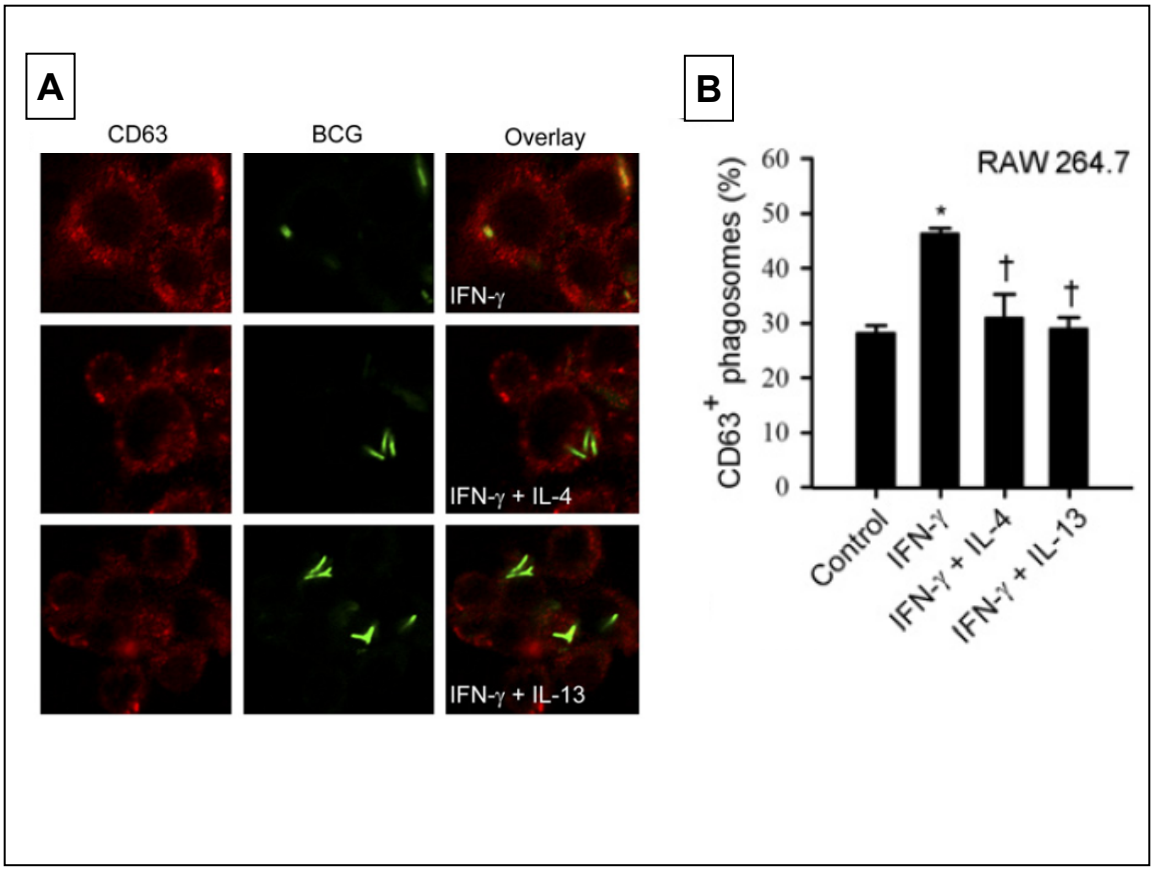

Figure 3.12. Inhibition of IFN-γ-Induced BCG Phagosome Maturation by IL-4 and IL-13.
(**A**) Confocal images of murine RAW 264.7 macrophages infected with GFP-BCG for 2 hr. Cells were treated with IFN-γ (200 U/ml) for 24 hr prior to infection, with or without IL-4 or IL-13 (30 ng/ml). After infection, cells were fixed and stained for CD63. (**B**) The percent of $CD63^+$ was quantified. Data are presented as means ± SEM; * $p < 0.05$ and †$p \geq 0.05$; n = 3. **(Courtesy of Dr. James Harris)**.

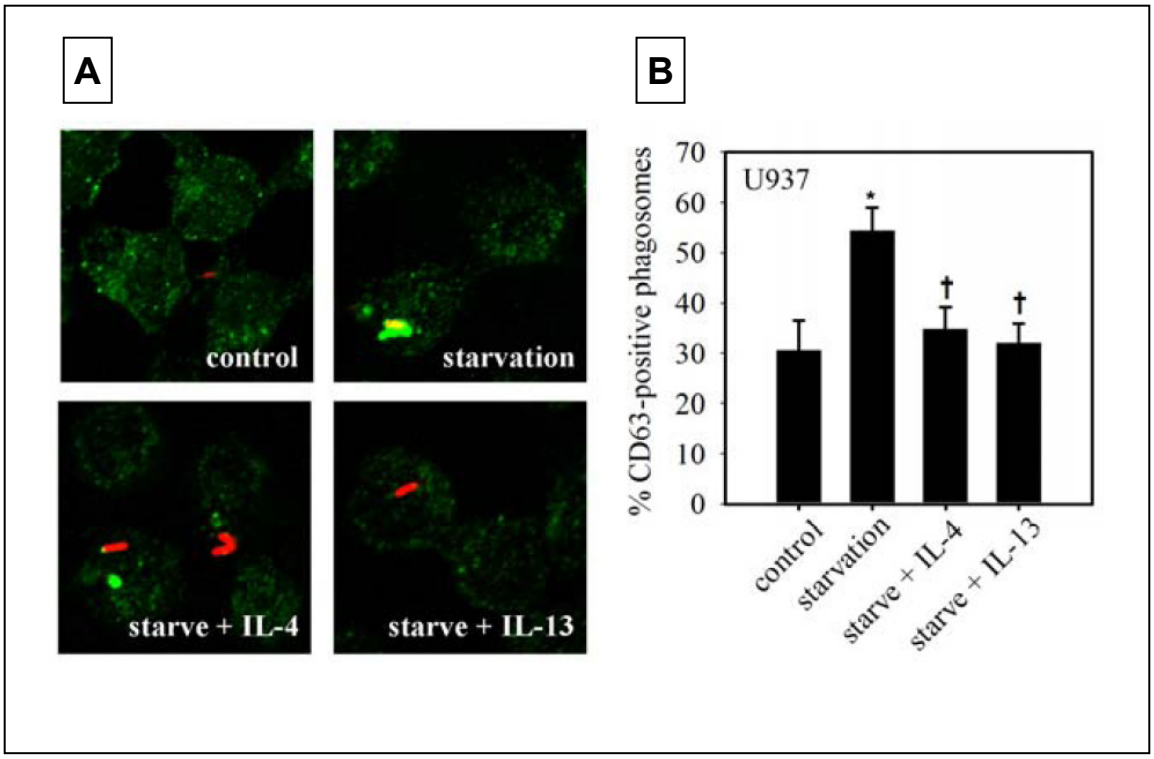

Figure 3.13. IL-4 and IL-13 Inhibit Autophagy-Induced BCG Phagosome Maturation in Human U937 cells.
(**A**) Confocal images of human U937 macrophages infected with Texas-red-BCG for 2 hr. Cells were amino acid- and serum-starved for 2 hr (during infection) with or without IL-4 or IL-13 (30 ng/ml). After infection, cells were fixed and stained with anti-CD63 (green). (**B**) The percent of $CD63^{+}$ was quantified. Data are presented as means ± SEM; * $p < 0.05$ and †$p \geq 0.05$.

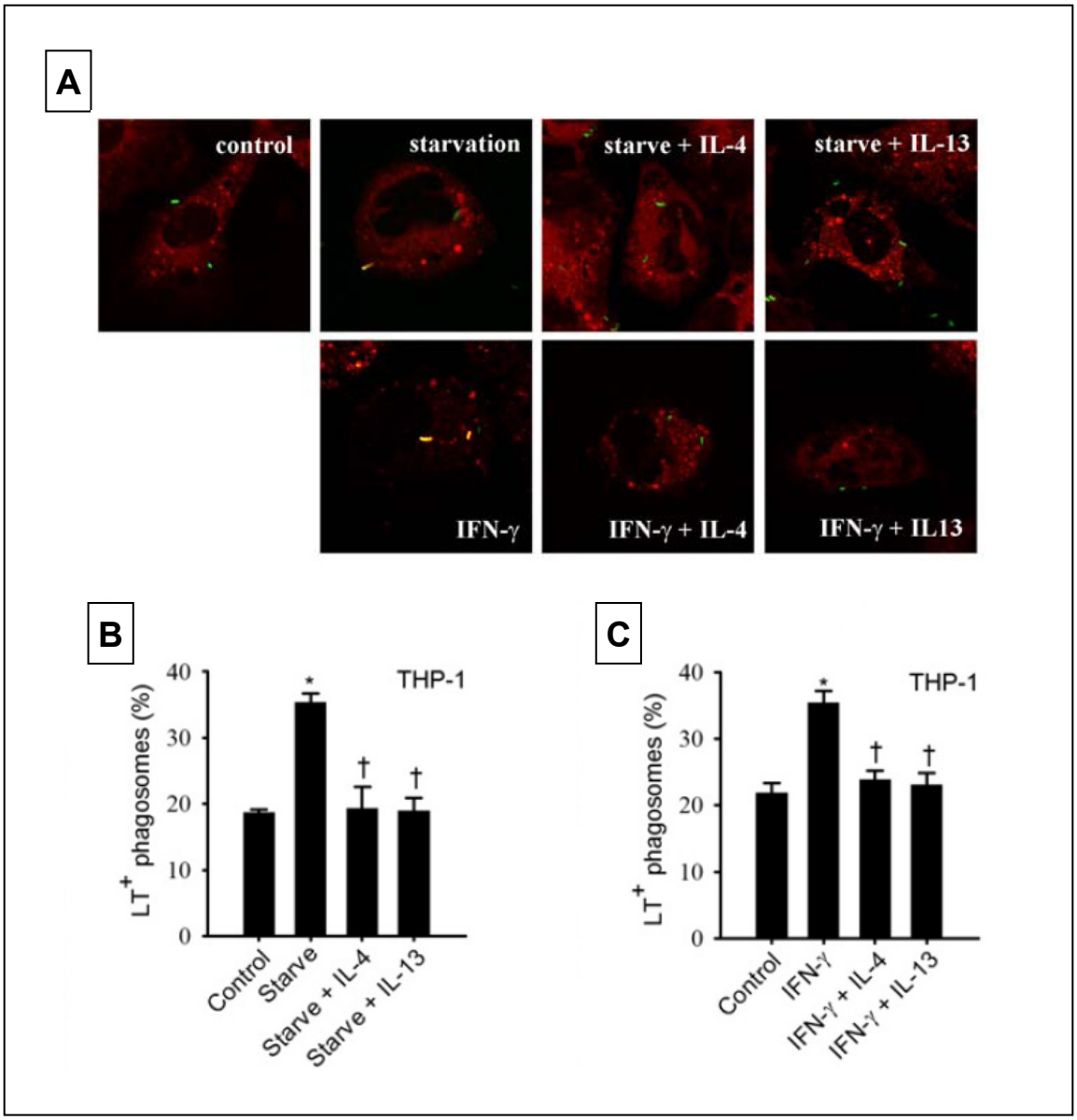

Figure 3.14. IL-4 and IL-13 Inhibit Autophagy-Induced BCG Phagosome Maturation in Human THP-1 Cells.
(**A**) Representative confocal images of PMA-differentiated human THP-1 monocytes infected with GFP-BCG for 2 hr. Cells were amino acid- and serum-starved for 2 hr (during infection), or pretreated with IFN-γ for 24 hr, with or without IL-4 or IL-13 (30 ng/ml), in the presence of LysoTracker red (red fluorescence). The percent of LysoTracker $(LT)^+$ during starvation (**B**) and IFN-γ (**C**) treatments were quantified. Data are presented as means ± SEM; * $p < 0.05$ and †$p \geq 0.05$. **(Courtesy of Dr. James Harris)**.

Phagosome maturation was autophagy dependent, as demonstrated by siRNA knockdown of Beclin 1, a critical mammalian autophagy factor [158] (Figures 3.15A-C). Although RAW cells transfected with scrambled siRNA showed an increase in BCG phagosome maturation in response to starvation (Figures 3.15A and 3.15D) or IFN-γ (Figures 3.15B and 3.15D), cells treated with Beclin 1 siRNA did not, demonstrating that these responses are dependent on functional autophagic machinery. In the presence of full-nutrient media, treatment of RAW macrophages with IL-4 or IL-13 had no effect on the colocalization of CD63 with either live or heat-killed BCG phagosomes (Figure 3.16). Thus, these Th2 cytokines do not inhibit mycobacterial phagosome maturation under normal conditions but rather specifically inhibit autophagy-dependent maturation.

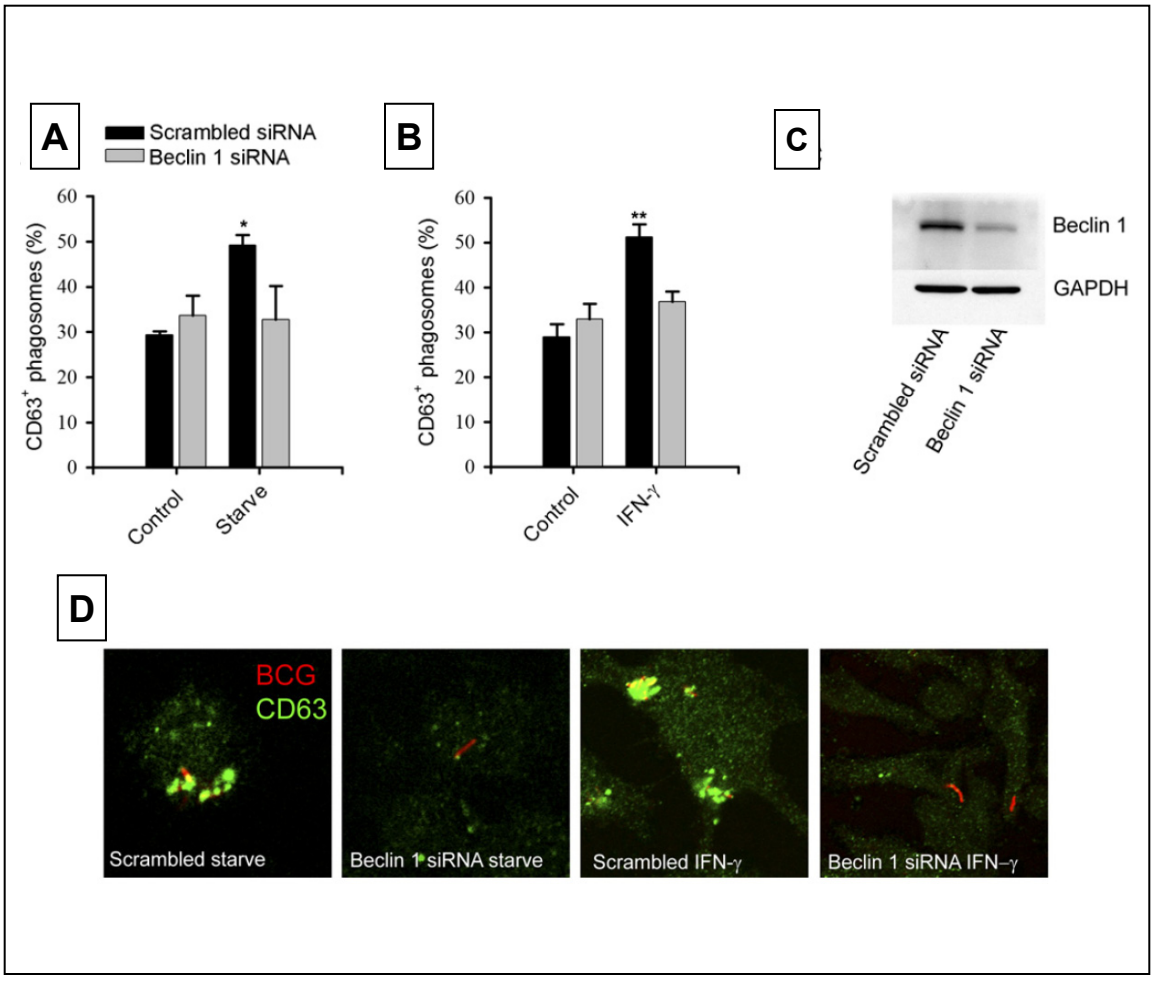

Figure 3.15. Starvation- and IFN-γ-Induced BCG Phagosome Maturation is Autophagy Dependent.

(**A**) Quantitative analysis of $CD63^+$ BCG phagosomes that were in murine RAW264.7 macrophages transiently transfected with either nontargeting (scrambled) siRNA or Beclin 1 siRNA and that were amino acid- and serum-starved for 2 hr. (**B**) $CD63^+$ BCG phagosomes in RAW264.7 cells transiently transfected with scrambled or Beclin 1 siRNA and treated with IFN-γ (200 U/ml) for 24 hr. Data are presented as means ± SEM; $*p < 0.05$, $**p < 0.01$, and $\dagger p \geq 0.05$; n = 3 for (A) and n = 6 for (B). (**C**) Immunoblot confirmation of Beclin 1 knockdown by siRNA. (**D**) Immunofluorescence panels of BCG colocalization with the late endosomal marker CD63, exemplifying the data in (A) and (B). CD63 is shown in green; BCG is shown in red.

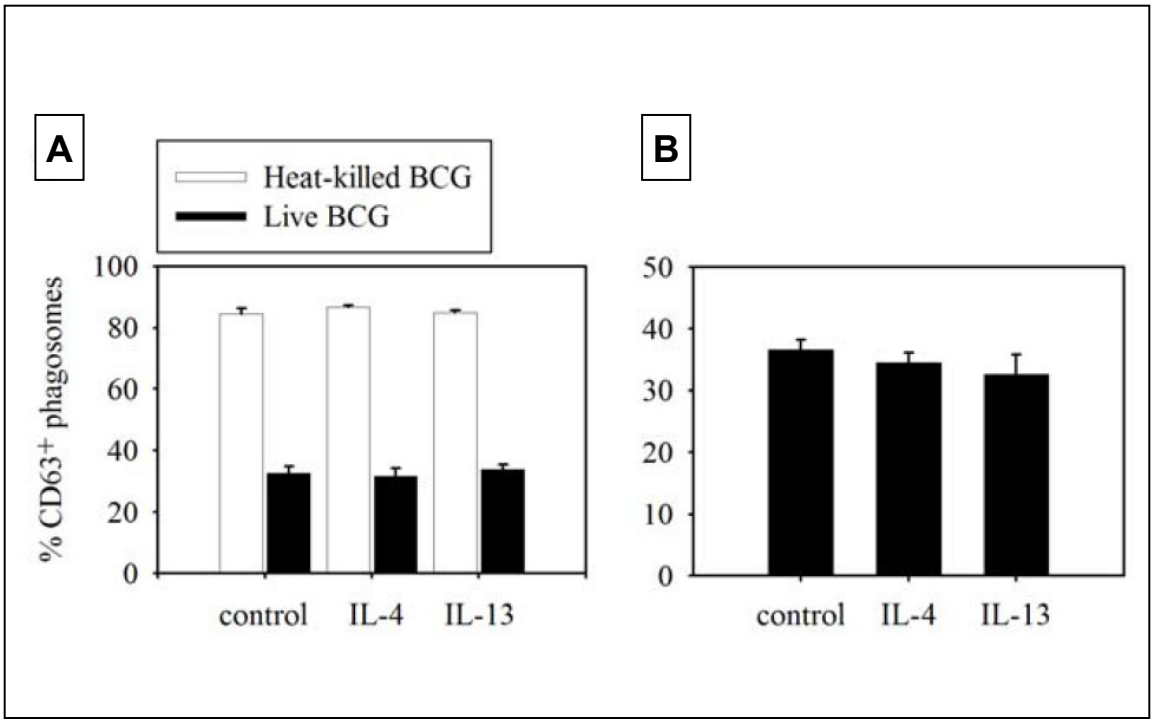

Figure 3.16. IL-4 and IL-13 Do Not Affect Phagosome Maturation in the Absence of Autophagy.
(**A**) Co-localization of CD63 with Texas-red labeled BCG was measured in RAW 264.7 cells incubated with heat-killed (dark bars) or live (light bars) BCG for 2 hr in the presence of IL-4 or IL-13 (30 ng/ml). (**B**) RAW cells incubated with IL-4 or IL-13 for 24 hr prior to infection with live BCG for 2 Hr. **(Courtesy of Dr. James Harris)**.

3.4. IL-4 and IL-13 inhibit autophagic phagosome maturation in primary human cells

To confirm that IL-4 and IL-13 inhibit autophagy-induced effects on mycobacterial phagosomes in human primary macrophages, we tested whether IL-4 and IL-13 influenced autophagy-induced mycobacterial phagosome maturation. Human peripheral blood monocyte-derived macrophages (MDMs) were infected with BCG and induced for autophagy by starvation. The effect of starvation-induced autophagy on mycobacterial phagosome maturation was decreased by the addition of either IL-4 or IL-13 (Figures 3.17A and 3.17B). Similarly, both IL-4 and IL-13 had an inhibitory effect on IFN-γ-induced mycobacterial phagosome maturation (Figures 3.17A and 3.17B). These results validate in human primary macrophages the conclusion that Th2 cytokines counteract autophagy in its ability to deliver mycobacteria into the phagolysosome.

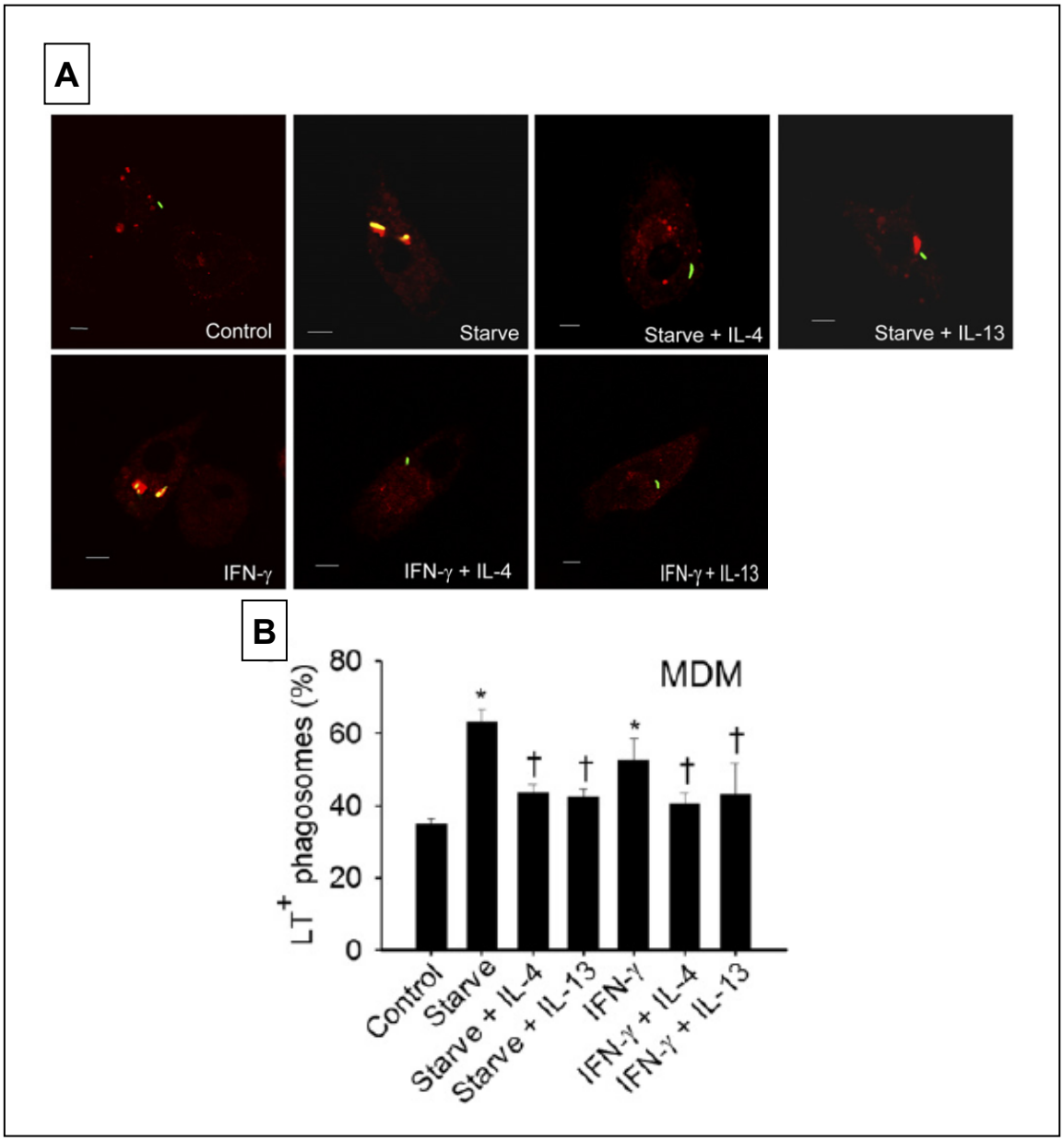

Figure 3.17. IL-4 and IL-13 Counteract Autophagy-Induced Phagosome Maturation in Primary Human Macrophages.
(**A**) Primary human macrophages derived from peripheral blood monocytes were infected with BCG. Transfer of mycobacteria to phagolysosome dependent on autophagy was scored with the acidotropic dye LysoTracker (LT). Autophagy was induced for 2 hr by starvation or by treatment with 200 U/ml human IFN-γ. Cells were incubated in the absence or presence of 30 ng/ml of human IL-4 or IL-13, as indicated. (**B**) Quantification of experiments. Data are presented as means ± SEM; *$p < 0.05$ and †$p \geq 0.05$; n = 3 (three independent donors). **(Courtesy of Dr. James Harris)**.

3.5. IL-4 and IL-13 inhibit autophagy-dependent killing of intracellular mycobacteria

To determine whether IL-4 and IL-13 affect autophagic elimination of mycobacteria [115], we infected RAW cells with BCG and induced autophagy by amino acid starvation for 2 hr. Induction of autophagy decreased BCG survival in RAW cells (Figure 3.18A). Addition of IL-4 or IL-13 to the starvation media abrogated this effect (Figure 3.18A). Similar results were obtained when survival of virulent *M. tuberculosis* H37Rv was tested with starvation or IFN-γ used as autophagy agonists (Figures 3.17B and 3.18C). Figure 3.18D shows that these relationships hold true in primary macrophages because IL-4, used as an example, abrogated killing of virulent *M. tuberculosis* H37Rv by starvation-induced autophagy in murine BMMs. These findings demonstrate that IL-4 or IL-13 inhibit autophagy-induced killing of mycobacteria by macrophages.

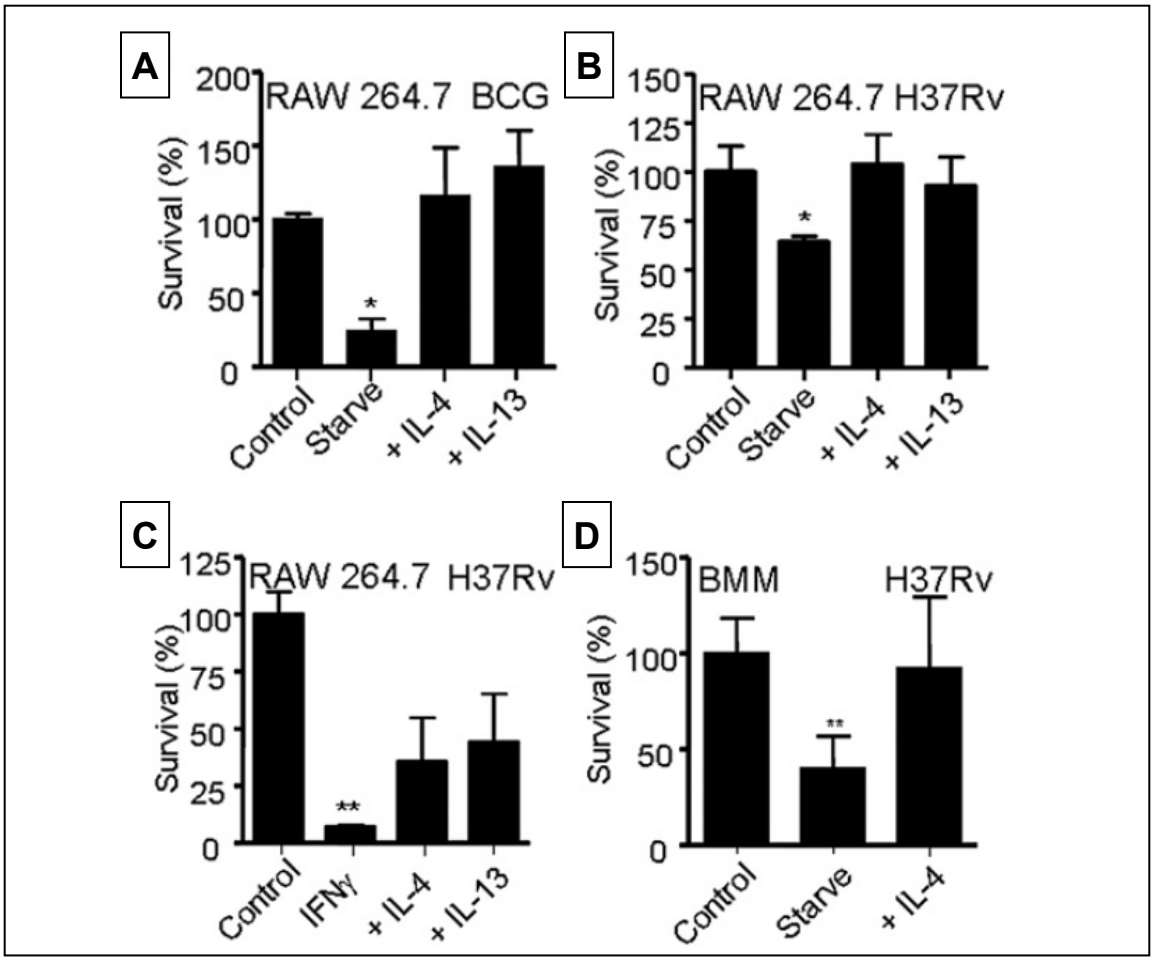

Figure 3.18. IL-4 and IL-13 Inhibit Autophagy-Dependent Killing of *M. tuberculosis* in Murine Macrophages.

(**A**) Murine RAW264.7 macrophages were infected with BCG for 1 hr and were amino acid- and serum-starved with or without IL-4 or IL-13 (30 ng/ml) for 2 h. Cells were washed and lysed for viability determination and survival expressed as a percentage of the control. (**B**) Murine RAW264.7 macrophages were infected with virulent *M. tuberculosis* H37Rv for 1 h and were amino acid- and serum-starved with or without IL-4 or IL-13 (30 ng/ml) for 2 h. Cells were washed and lysed for viability determination. (**C**) Murine RAW264.7 macrophages were either untreated or treated with 200 U/ml of m-IFN-γ for 24 h prior to infection with *M. tuberculosis* H37Rv for 1 hr with or without IL-4 or IL-13 (30 ng/ml) for 2 h. Cells were washed and lysed for viability (cfu) determination. Data are presented as means ± SEM, $*p < 0.05$, $**p < 0.01$, and $\dagger p \geq 0.05$; n = 3. (**D**) IL-4 inhibits autophagy-dependent killing of M. tuberculosis H37Rv in primary murine macrophages. Murine bone marrow macrophages were infected with virulent *M. tuberculosis* (strain H37Rv) for 1 h and were amino acid- and serum-starved with or without IL-4 (30 ng/ml) for 2 h. Mycobacterial viability (colony counts) is expressed as a percentage of the control. **(Courtesy of Dr. Sharon Master)**.

3.6. Inhibition of starvation-induced autophagy by IL-4 and IL-13 is Akt dependent

To determine the signaling involved in the actions of IL-4 and IL-13 on autophagy in macrophages, we examined whether the Akt pathway, known to activate TOR and inhibit autophagy [129], was involved. In both RAW264.7 (Figure 3.19A) and U937 (Figure 3.19B) macrophages, IL-4 and IL-13 increased phosphorylation of Akt under starvation conditions (Figure 3.19B, top two panels and graph). We next tested the activity of TOR, a key negative regulator of autophagy (when TOR is active, autophagy is inhibited) on the basis of reports that TOR is activated by Akt [129]. We investigated TOR activation by monitoring phosphorylation of p70 S6 kinase (S6k) and 4E-BP1, two TOR substrates commonly used as indicators of its activation state [129]. Treatment of U937 cells with IL-4 or IL-13 increased phosphorylation of S6k (Figure 3.19B). In RAW264.7 macrophages, IL-4 and IL-13 treatment increased phosphorylation of 4E-BP1 under starvation conditions in a dose-dependent manner (Figure 3.20B and Figure 3.21A). A finer-resolution analysis of 4E-BP1 phosphorylation was carried out next. TOR induces 4E-BP1 inactivation via multiple hierarchical phosphorylations [159]. A fine-resolution immunoblot analysis can be used for revealing 4E-BP1 hypophosphorylated α and β forms, and hyperphosphorylated γ form, resolved in the order of their decreasing electrophoretic mobility. Active Akt elicits 4E-BP1(γ) hyperphosphorylation through TOR activity [159]. Our analyses revealed that IL-4 and IL-13 treatment specifically increased phosphorylation of 4E-BP1(γ), under starvation conditions, in primary murine BMMs (Figure 3.20A and Figure 3.21B), and in human MDMs (Figure 3.22).

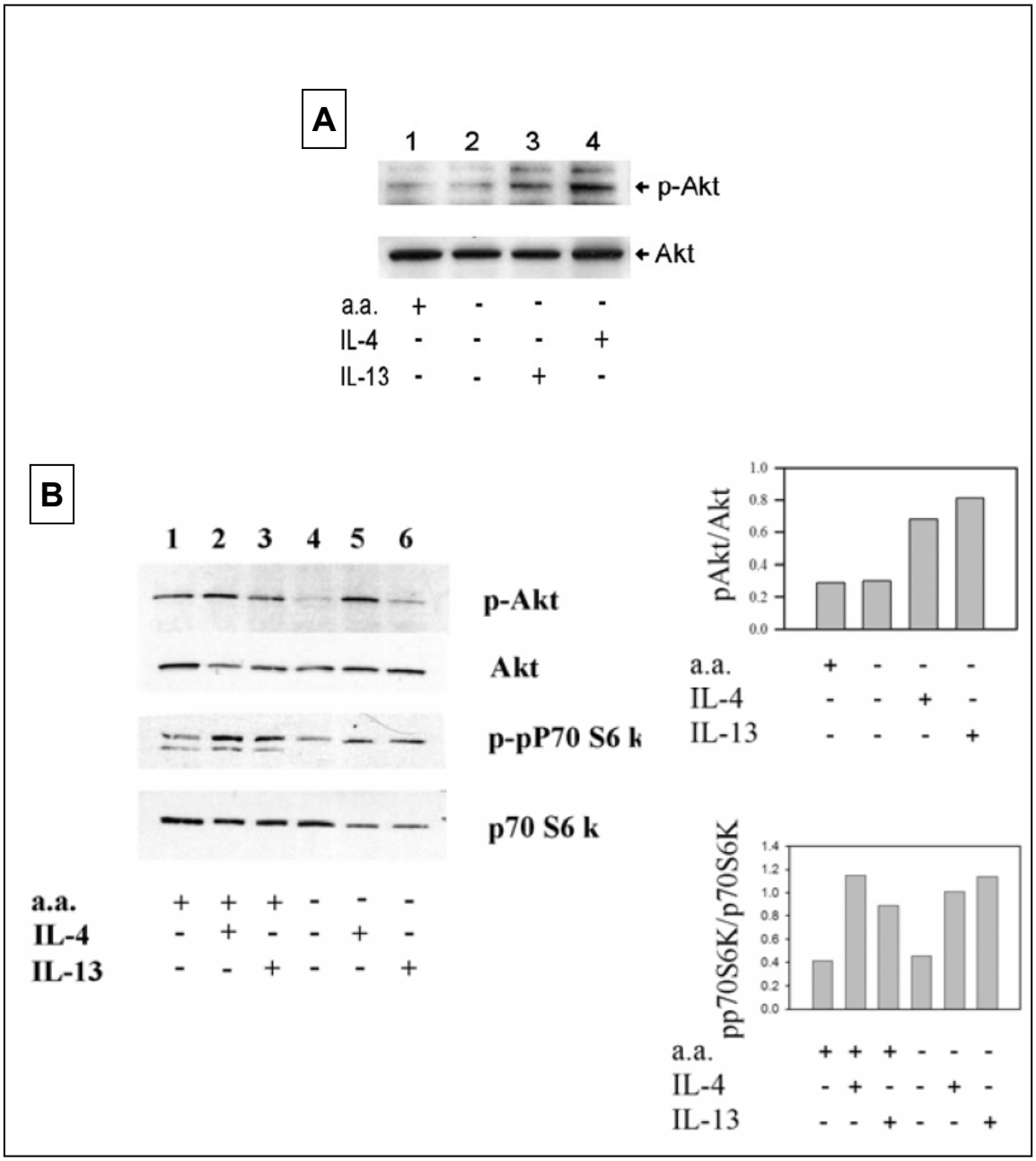

Figure 3.19. IL-4 and IL-13 Activate TOR Via the Akt Pathway.
(**A**) Immunoblot analysis of Akt phosphorylation in RAW264.7 macrophages amino acid- and serum starved for 2 hr with or without IL-4 or IL-13 (30 ng/ml). (**B**) Immunoblot analysis of Akt (top two blots) and p70S6K (bottom two blots) phosphorylation in human U937 macrophages in full media or amino acid- and serum- starved for 2 h with or without IL-4 or IL-13 (30 ng/ml). Ratios of phosphoproteins (p-Akt or p-p70 S6 k) to total corresponding protein (Akt or p70 S6 k) are shown in graphs to the right.

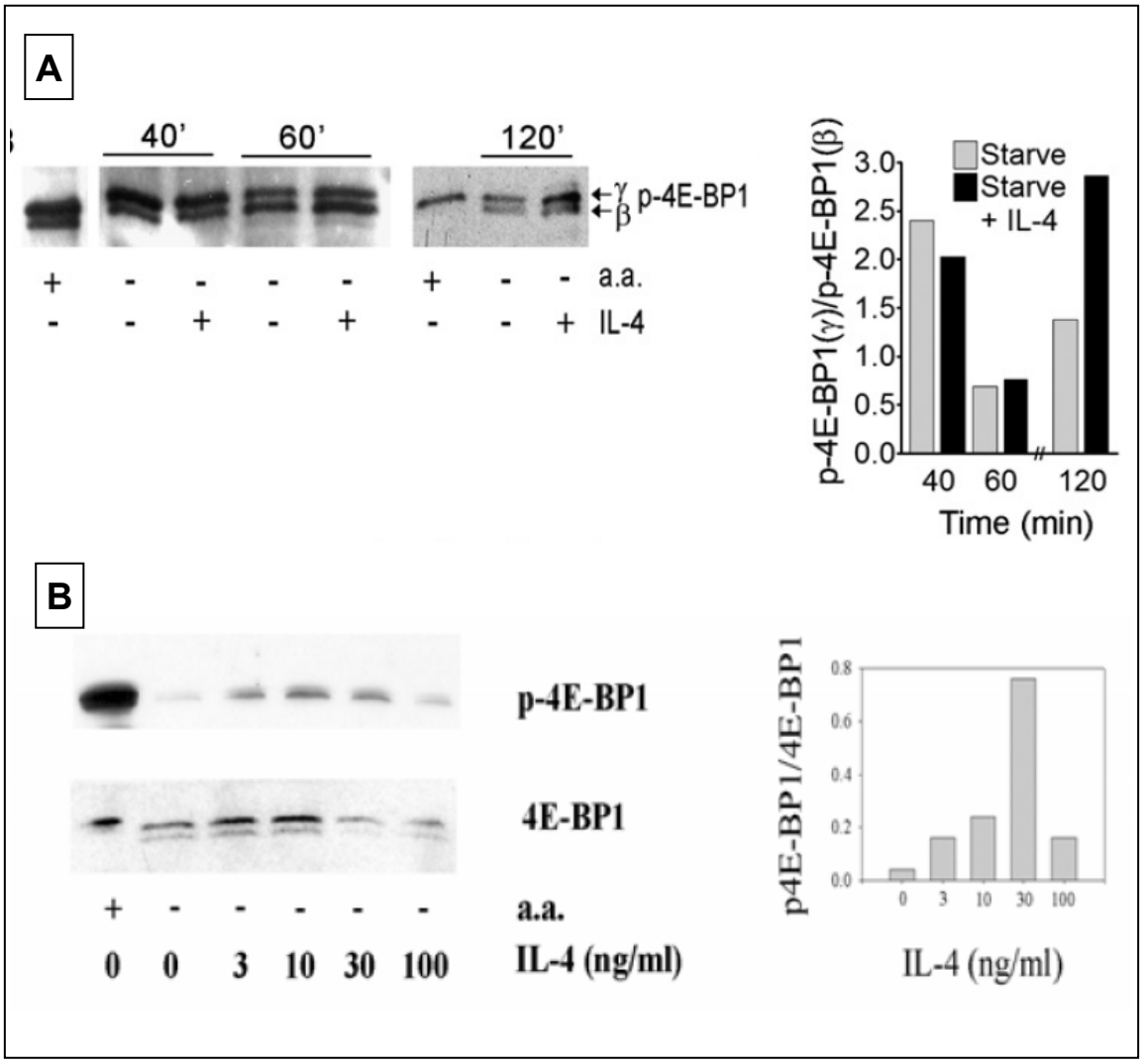

Figure 3.20. IL-4 Activates TOR Via the Akt Pathway in Murine Macrophages.

(**A**) Immunoblot analysis of 4E-BP1 hierarchical phosphorylation in murine bone marrow macrophages (BMMs) starved and treated with IL-4 for 40, 60, and 120 min. Graph shows the ratio of 4E-BP1(γ) vs. 4E-BP1(β) (n = 2) (**B**) Immunoblot analysis of 4E-BP1 phosphorylation in murine RAW 264.7 macrophages starved and treated for 2 hr with varying concentrations of IL-4. Graph shows the ratio of phosphorylated vs. total 4E-BP1 (n = 3).

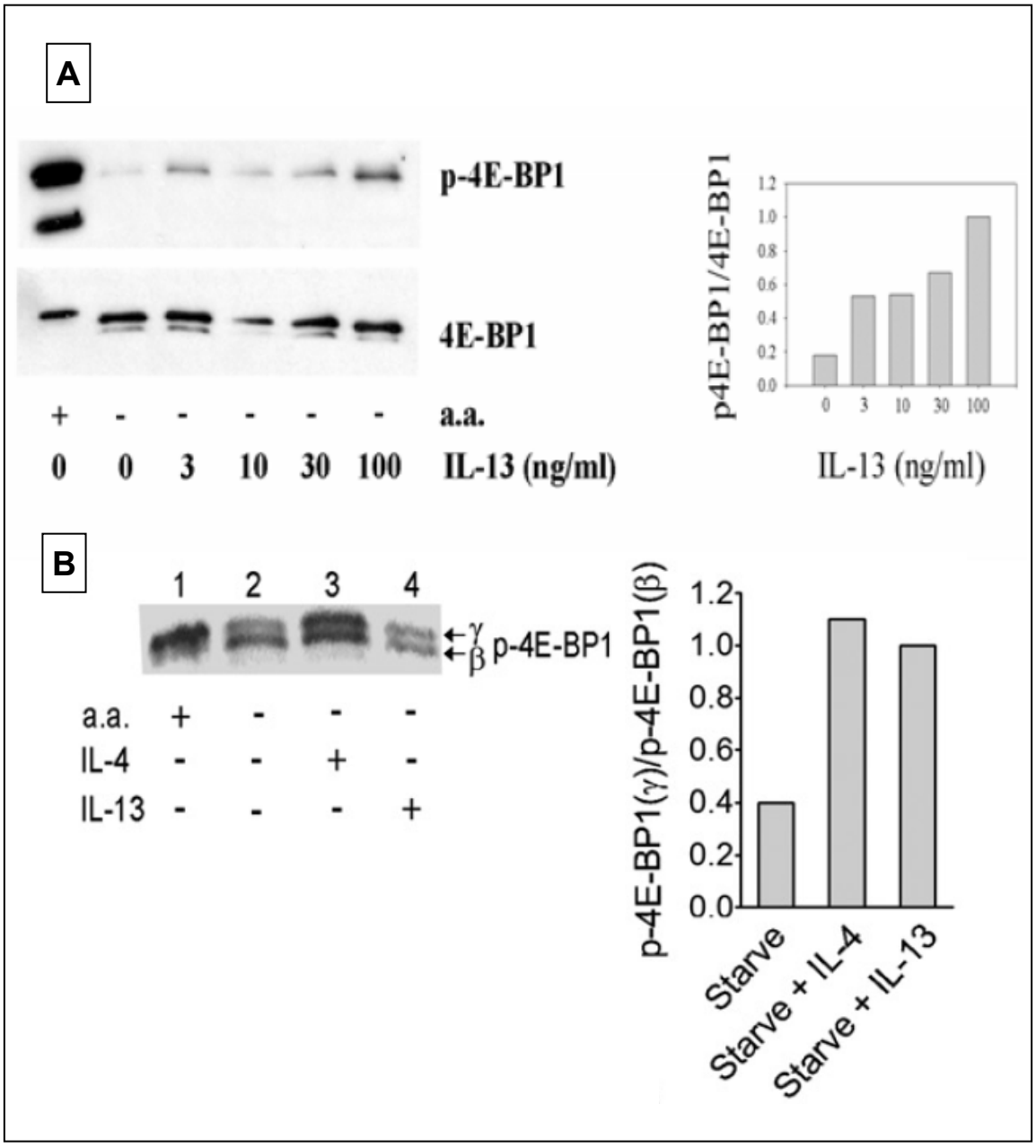

Figure 3.21. IL-4 and IL-13 Activate TOR Via the Akt Pathway in Murine Macrophages.

(**A)** Immunoblot analysis of 4E-BP1 phosphorylation in murine RAW 264.7 macrophages starved and treated for 2 hr with varying concentrations of IL-13. Graphs show the ratio of phosphorylated vs. total 4E-BP1 (n = 3). (**B**) Immunoblot analysis of 4E-BP1 in murine bone marrow macrophages (BMMs). Graph shows the ratio of 4E-BP1(γ) vs. 4E-BP1(β) (n = 2).

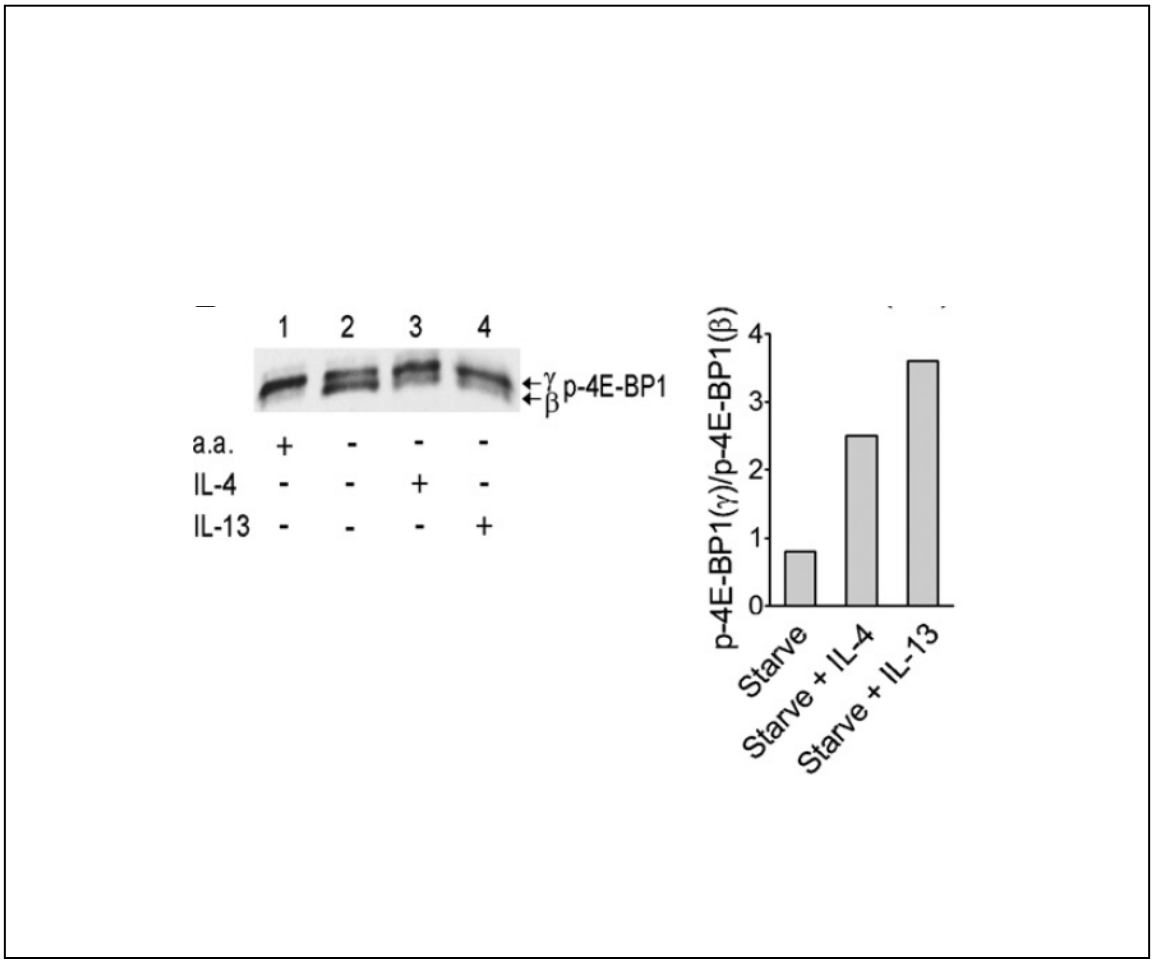

Figure 3.22. IL-4 and IL-13 Activate TOR Via the Akt Pathway in Primary Human Macrophages.
Immunnoblot analysis of human monocyte-derived macrophages (MDMs) starved for 2 hr with or without IL-4 or IL-13. Graph show (n = 2; one out of two experiments with similar results) the intensity ratios of p-4E-BP1(γ) to p-4E-BP1(β) bands.

Furthermore, a time course of 4E-BP1 showed that IL-4 exerted its effects by increasing 4E-BP1(γ) hyperphosphorylation at 2 hr but had no discernible effects at earlier time points (Figure 3.20A), consistent with other effects measured in our study. These results demonstrate that IL-4 and IL-13 activate the Akt pathway in macrophages, resulting in the activation of TOR and inhibition of starvation-induced autophagy.

To establish the role of Akt in the IL-4- and IL-13-induced inhibition of autophagy in macrophages, we knocked down Akt1 and Akt2 with siRNA in vitro (Figure 3.23A). Compared with the control samples (scrambled siRNA), knockdown of Akt1 and Akt2 in RAW macrophages increased autophagy, monitored by $LC3^{+}$ vacuole formation (Figures 3.23B and 3.23C), indicating that basal levels of autophagy are controlled, at least in part, by baseline Akt activation. Double knockdown of Akt1 and Akt2 in RAW cells abrogated the effects of IL-4 and IL-13 on starvation-induced autophagy (Figures 3.23B and 3.23C). In BCG-infected RAW cells, knockdown of Akt1 and Akt2 also inhibited the effects of IL-4 and IL-13 on starvation-induced transfer of BCG to autophagosomes (Figures 3.24A and 3.24C).

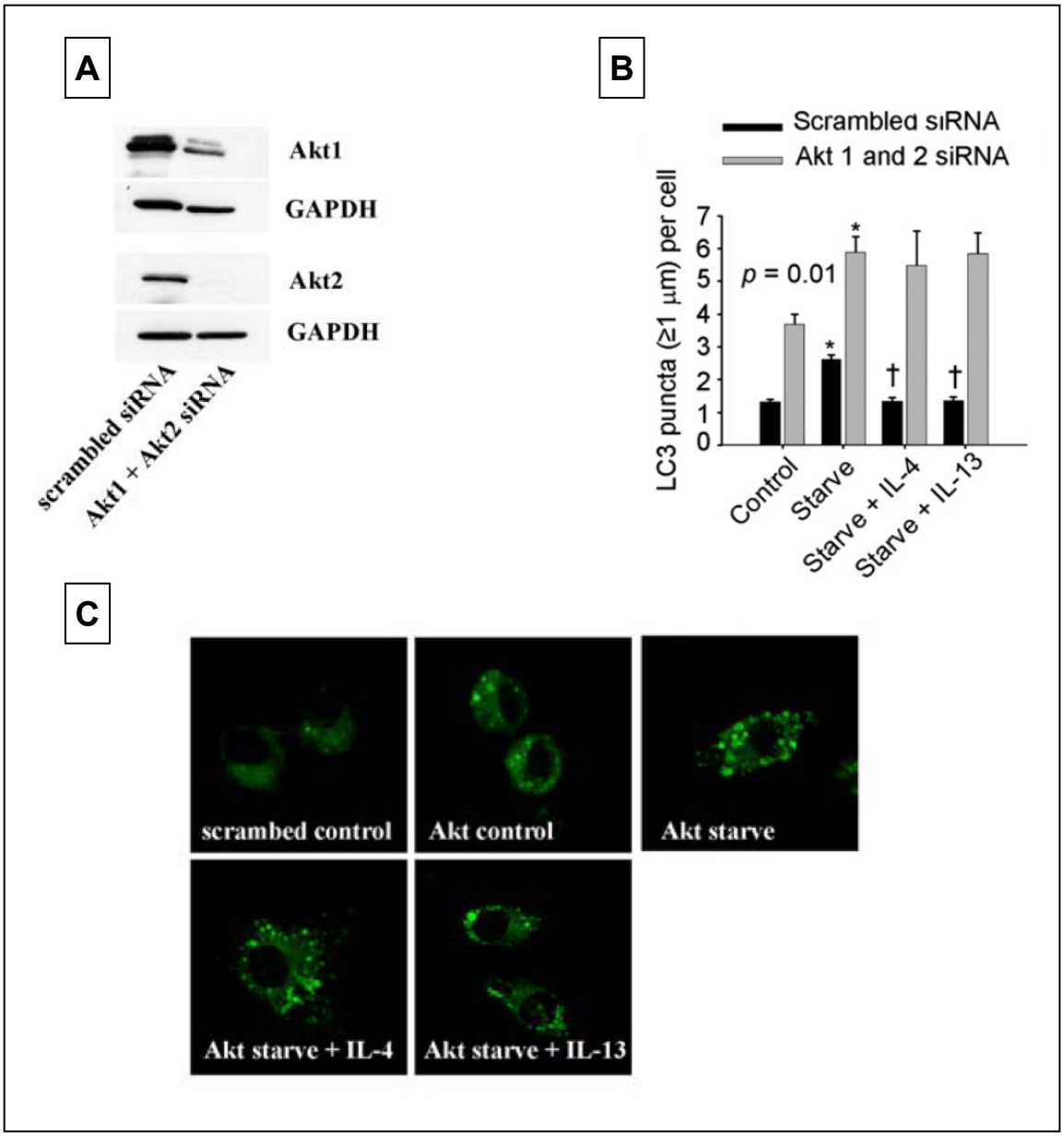

Figure 3.23. IL-4 and IL-13 Inhibit Starvation-induced Autophagy Via Akt Pathway.

(**A**) Immunoblot confirmation of Akt 1 and Akt2 knockdown in RAW264.7 cells transiently transfected with control (scrambled) or Akt 1 and Akt 2 siRNA. (**B**) Quantification of large (≥1 μm) pEGFP-LC3 puncta per cell in RAW 264.7 macrophages transiently transfected with pEGFP-LC3 and either scrambled siRNA or Akt 1 + Akt 2 siRNA and starved for 2 hr with or without IL-4 or IL-13. (**C**) Representative confocal images of RAW cells transiently transfected with pEGFPLC3 in combination with scrambled or Akt 1 and Akt 2 siRNA and amino acid- and serum-starved for 2 h with or without IL-4 or IL-13 (30 ng/ml).

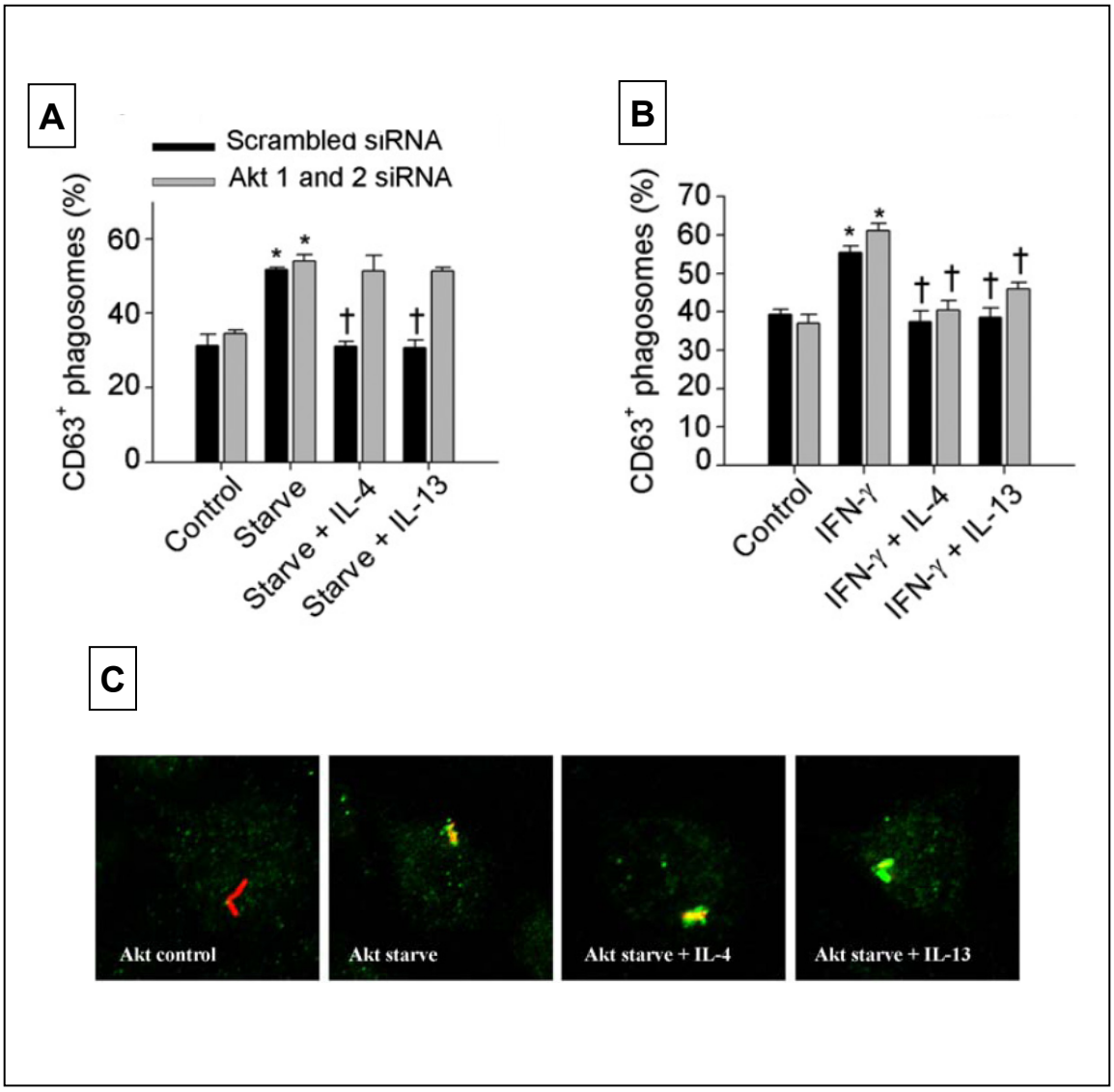

Figure 3.24. IL-4 and IL-13 Inhibit Starvation-Induced Transfer of BCG to Autophagosome Via Akt Pathway.
(**A**) Quantitative analysis of $CD63^+$ BCG phagosomes in RAW264.7 cells transiently transfected with scrambled siRNA or Akt 1 + Akt 2 siRNA and infected with BCG for 2 hr. Infected cells were amino acid- and serum-starved with or without IL-4 or IL-13. (**B**) Percentage of $CD63^+$ BCG phagosomes in RAW264.7 cells transiently transfected with scrambled siRNA or Akt1 + Akt 2 siRNA, treated with IFN-γ with or without IL-4 or IL-13 for 24 hr, and infected with BCG for 2 hr. (**C**) Representative confocal images of RAW cells transfected with Akt 1 and Akt 2 siRNA, infected with Texas red-labeled BCG and starved for 2 h with or without IL-4 or IL-13. CD63 staining was revealed with Alexa-488 (green).
(Courtesy of Dr. James Harris).

3.7. Inhibition of IFN-γ-induced autophagy is STAT6 dependent

In contrast to the effects with IL-4 and IL-13 on starvation-induced autophagy, a double knockdown of Akt1 and Akt2 with siRNA had no effect on the inhibition of IFN-γ-induced phagosome maturation by IL-4 and IL-13 (Figure 3.24B), suggesting that the suppressive effects of these Th2 cytokines on the activation by IFN-γ involved alternative or additional pathways, independent of Akt signaling. Akt3 was detectable only at very low amounts in these cells, and its expression was not affected by knockdown of Akt1 and Akt2 (data not shown). We therefore tested whether the inhibitory effect of IL-4 and IL-13 on IFN-γ-induced autophagy was regulated via a different pathway. IL-4 and IL-13 receptors, in addition to signaling via the Akt pathway, are more commonly known for their activation of STAT6 [143]. Thus, we tested whether STAT6 was required for Th2 cytokine inhibition of autophagy activation by IFN-γ. When STAT6 was knocked down (Figure 3.25A), IL-13 could no longer suppress IFN-γ-induced autophagosome formation in macrophages (Figures 3.25B-E). Collectively, our data on IL-4 and IL-13 inhibition of starvation- and IFN-γ-induced autophagy demonstrate that Th2 cytokines can employ either of the signaling pathways associated with ligation of the IL-4 and IL-13 receptors to exert this effect. Suppression of starvation-induced autophagy occurs through activation of Akt, whereas STAT6 is needed for suppression of IFN-γ-induced autophagy.

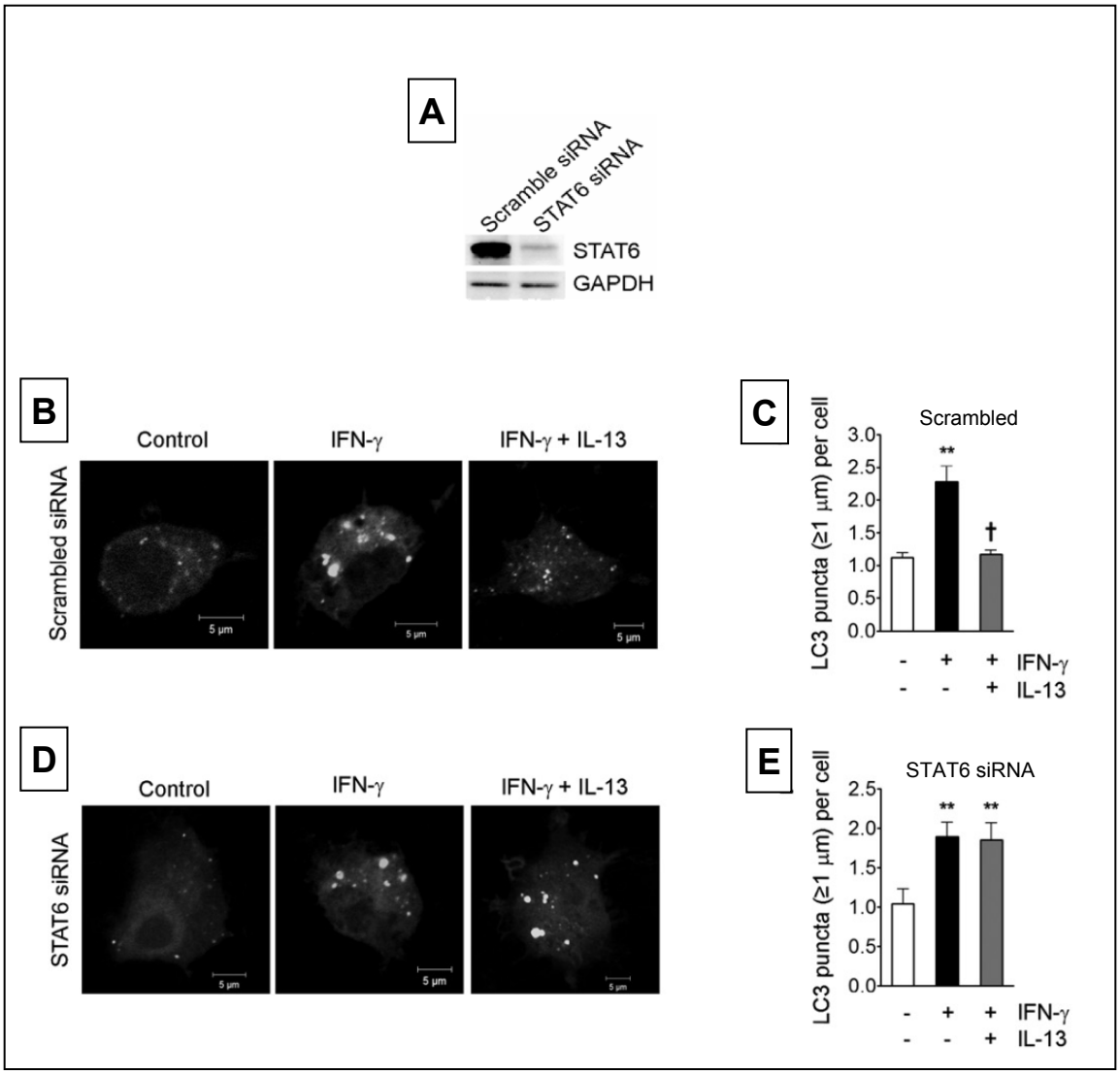

Figure 3.25. IL-13 Inhibits IFN-γ-Induced Autophagy Via STAT6 Pathway. (**A**) Immunoblot confirmation of STAT6 knockdown in RAW264.7 macrophages transiently transfected with control (scrambled) or STAT6 siRNA. Polyclonal anti-STAT6 rabbit antibodies (Bethyl Laboratories) were used for immunoblotting. (**B-E**) Th2 inhibition of IFNγ-dependent autophagy is STAT6 dependent: (**B** and **C**) display confocal-microscopy images and quantification of GFP-LC3 puncta formation in response to IFN-γ, with or without IL-13, in cells treated with control (scrambled) siRNA. (**D** and **E**) display images and quantification of GFP-LC3 puncta formation in response to IFN-γ, with or without IL-13, in cells treated with control STAT6 siRNA. Data in all graphs presented as means ± SEM; n ≥ 3; **p* < 0.05, ***p* < 0.01, and †*p* ≥ 0.05. (**Courtesy of Dr. Esteban A. Roberts**).

4. DISCUSSION

The major result of the present study is that the Th2 cytokines IL-4 and IL-13 inhibited the autophagy-induced elimination of mycobacteria by murine and human macrophages. This inhibition was accomplished by the cytokines impairing the autophagic transfer of mycobacteria from an immature phagosome to the autolysosome; this action prevented the elimination of intracellular bacilli by autophagy. This study also demonstrated that the effect of IL-4 and IL-13 on starvation-induced autophagy is dependent on signaling via the Akt pathway. The finding that the Th2 cytokines depend on Akt signaling was not surprising since published studies have reported that IL-13 stimulates the Akt pathway, which activates TOR, a repressor of autophagy [141, 142]. However, what was unexpected was the finding that the effect of IL-4 and IL-13 on IFN-γ-induced autophagy was independent of Akt; instead, it depended on signaling via the STAT6 pathway.

Autophagy plays a role in the response to a number of intracellular pathogens [85, 112-115]. *Mycobacterium tuberculosis* has developed the ability to inhibit maturation of the macrophage phagosome to promote its survival [157]. However, our lab has previously shown that the induction of autophagy in *Mycobacterium tuberculosis*-infected macrophages led to an increase in phagosome maturation and a decrease in intracellular survival of the bacilli [115]. Consequently, autophagy represents a mechanism by which macrophages are able to overcome the phagosome maturation block imposed by mycobacteria, leading ultimately to enhanced mycobacterial elimination.

4.1. Autophagy-mediated clearance of *Mycobacterium tuberculosis* and Th1-Th2 polarization

After phagocytosis by macrophages, bacterial pathogens are trapped within the phagosome and subsequently degraded by lysosomes [160]. Mycobacteria escape elimination by arresting maturation of the phagosome in which they are trapped. However, amino acid starvation and IFN-γ stimulate autophagy in macrophages to overcome this phagosome maturation block and increase elimination of mycobacteria. In this study, we analyzed the effects of the Th2 cytokines IL-4 and IL-13 in murine and human macrophage cell lines during the autophagic response. The maturation of the autophagosome when macrophages were starved or exposed to IFN-γ, as determined by the accumulation of LC3-II on the autophagosome and transfer of bacteria to the autophagosome, was hindered when the macrophage was stimulated with the Th2 cytokines. The concentrations of IL-4 and IL-13 used in our titration experiments were in the range of the *in vivo* and *ex vivo* tissue levels of these cytokines under a variety of conditions [161-163], including studies involving mycobacterial antigens [164]. Therefore, this study showed that the microbicidal effect of autophagy is abrogated by the cytokines IL-4 and IL-13 through the inhibition of mycobacterial phagosome maturation.

4.2. Effects of IL-4 and IL-13 in starvation-induced autophagy

While amino acid starvation stimulates autophagy, the Th2 cytokines IL-4 and IL-13 generated an opposing effect. In HT-29 epithelial cells, IL-13 inhibits starvation-induced autophagy through the activation of the Akt pathway [141, 142]. The two cytokines signal through IL-4Rα, which then signal via the insulin

receptor substrate (IRS)-1/2 [143]. IRS-1/2 signaling activates the type I phosphatidylinositol 3 kinase (PI3K), ultimately activating the Akt pathway and TOR [129]. The activation of TOR results in the negative regulation of autophagy. In this study, we analyzed the effects of the Th2 cytokines IL-4 and IL-13 in murine and human macrophage cell lines on the Akt pathway and TOR activity during starvation-induced autophagy. The activity of TOR decreases when cells are starved, as determined by the decrease in phosphorylation of TOR activation state indicators, resulting in the induction of autophagy in both murine and human macrophages. However, treatment with IL-4 and IL-13 increased phosphorylation of the TOR activation state indicators under starvation conditions, suggesting the Th2 cytokines stimulate TOR activity in macrophages. The *in vitro* double knockdown of Akt1 and Akt2 in macrophages negated the effects of IL-4 and IL-13 on starvation-induced autophagy, thus increasing autophagic activity, as monitored by $LC3^{+}$ vacuole formation. This data demonstrated that stimulation with IL-4 and IL-13 increased Akt and TOR activity, suggesting the effect of IL-4 and IL-13 on starvation-induced autophagy is dependent on signaling via the Akt pathway.

4.3. Effects of IL-4 and IL-13 on IFN-γ-induced autophagy

IL-4 and IL-13 inhibit IFN-γ-induced autophagy independent of the Akt and TOR pathway. Unlike the Akt-dependent inhibitory effects of IL-4 and IL-13 on starvation-induced autophagy demonstrated in this study, this investigation revealed that the Th2 cytokines inhibit autophagy by signaling through the STAT6 pathway. The effects of STAT6 on autophagy by IL-4 and IL-13 may depend on a number of pathways. One such pathway involves the increase of the

antiapoptotic and also recognized inhibitors of autophagy, Bcl-2 and Bcl-X_L protein levels by STAT6 [165] [166, 167]. The inhibition of autophagy occurs as Bcl-2 and Bcl-X_L interact with the BH3 domain of Beclin 1 under normal steady-state growth conditions, whereas its dissociation from Bcl-2 mediates autophagy [166, 167]. Beclin 1 interacts with several activators (Liang et al, 2006; Fimia et al, 2007; Takahashi et al, 2007), which positively regulate autophagy by promoting the activation of the PI(3)K protein, VPS34, and the formation of autophagosomes.

IFN-γ has also been shown to increase the expression of DAPK [168], which recent reports indicate phosphorylates Beclin 1 at its BH3 domain; subsequently, this phosphorylation promotes the dissociation of Beclin 1 from Bcl-X_L, resulting in the induction of autophagy [169]. The IL-4 and IL-13 inhibition of IFN-γ-induced autophagy via STAT6, observed in this study, is supported by the findings that Bcl-2 and Bcl-X_L association with Beclin 1 blocks stimulation of autophagy [166], and the rapid induction of STAT6 via IL-4 and IL-13 stimulation may increase Bcl-X_L availability [165]. This surprise finding suggests STAT6 as an immunologically relevant regulator of autophagy. Furthermore, it elucidates how Th2 cytokines counter autophagy-dependent protection against intracellular pathogens provided by Th1 polarization.

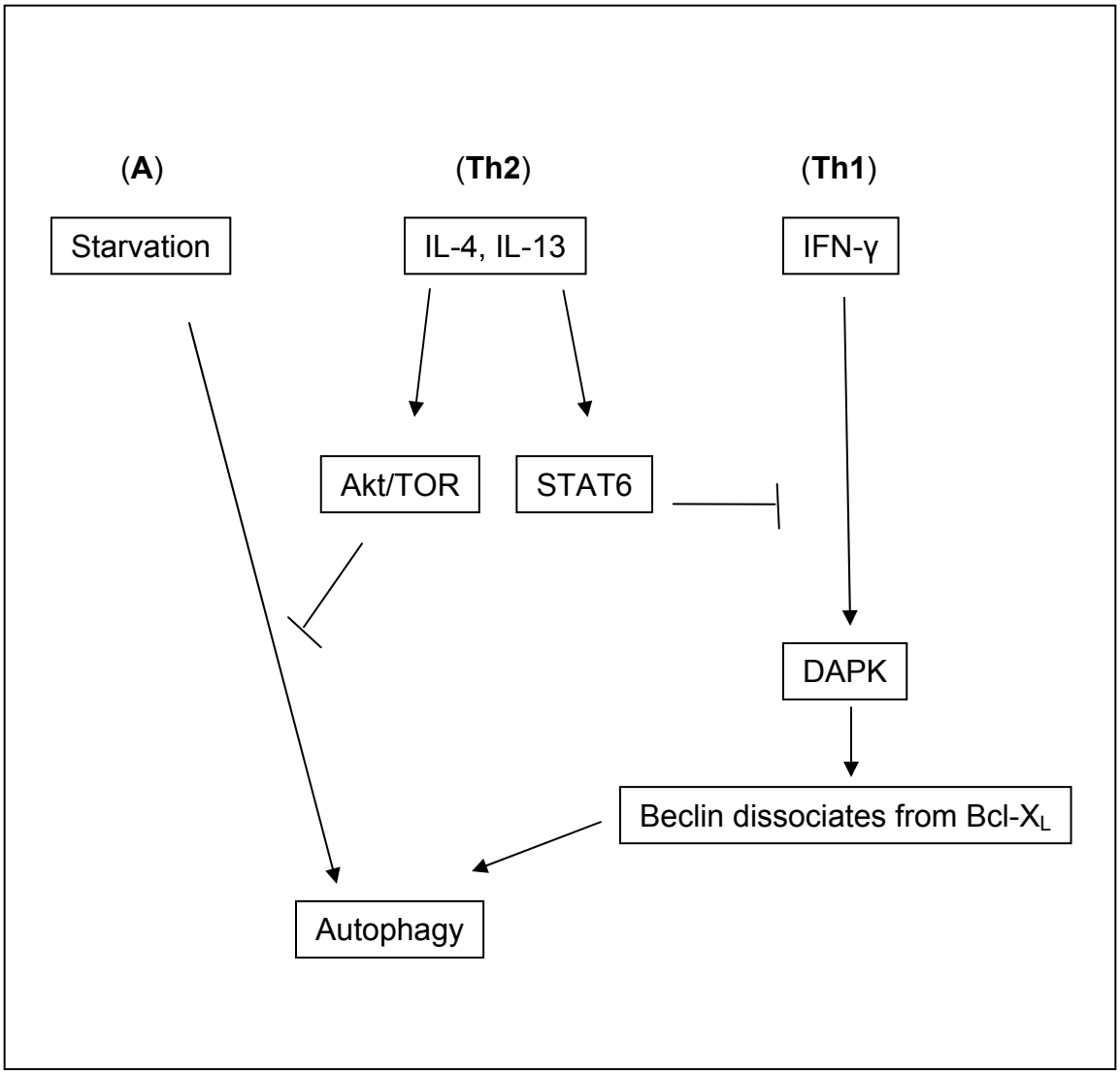

Figure 4.1. A Proposed Model for IL-4 and IL-13 Regulation of Autophagy. (**A**) IL-4 and IL-13 inhibit starvation-induced autophagy by activating Akt and TOR, two negative regulators of autophagy. (**B**) IFN-γ-induced autophagy is inhibited by the activation of STAT6. The Bcl-X_L association with Beclin blocks stimulation of autophagy. IFN-γ activates DAPK, which phosphorylates Beclin. This phosphorylation promotes the dissociation of Beclin from Bcl-X_L, resulting in the induction of autophagy. The activation of STAT6, via IL-4 and IL-13 stimulation, may increase the Bcl-X_L association with Beclin, thus inhibiting autophagy.

4.4. Cytokines in the immune response to mycobacteria

In human tuberculosis, analyses of the patterns of T cell cytokines have met with conflicting conclusion. Patients with pulmonary tuberculosis in general have lymphocytes in their lungs with a Th1 phenotype producing IFN-γ [59, 60]. Nevertheless, lung tissue from patients with cavitary tuberculosis has been observed to display a Th2 lymphocyte subset phenotype relative to the Th1 subsets demonstrated in non-cavitary disease [59]. The Th2 phenotype shows a relationship of a higher production of IL-4 in the periphery of patients with cavitary disease [64], suggesting that IL-4 levels possibly indicate severity of disease. Another study has shown that lymphocytes obtain from the cavitary wall secrete more IFN-γ when stimulated with mycobacterial antigens than cells from non-progressive tuberculoma tissues [170], indicating a higher Th1 phenotype. In some cases, granulomas can be positive for IFN-γ and IL-4 [171, 172]. Some studies have reported a Th1 response in the periphery of patients with mild pulmonary tuberculosis and Th2 responses in patients lacking immunity or those with more severe disease [146, 147]. The phenotype of infected macrophages and dendritic cells within the granuloma is less well defined, but in vitro studies in human peripheral blood monocytes have demonstrated that virulent strains of tuberculosis preferentially up-regulate the production of Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13, while non-virulent strains induce a Th1-type response [70, 148, 149]. Thus, it is possible that in the typically Th1 environment of the granuloma, local secretion of IL-4 and IL-13 could specifically impair the response of infected macrophages to IFN-γ. However, secretion of anti-inflammatory cytokines like IL-4, TGF-β, and IL-10 may be necessary to dampen

the pathological effects of a prolonged or excessive Th1 response, although in progressive tuberculosis disease IL-4 can actually increase immunopathology [68].

Mycobacterium tuberculosis HN878 and other W/Beijing isolates induce IL-13 expression, which, along with IL-4 is associated with Th2 responses, are considered to be more virulent than others [70, 173]. *M. tuberculosis* strains differ in their epidemiological features including rates of active disease among exposed/infected individuals [174]. Significantly, these effects have recently been linked to specific cytokine profiles, with excessive induction of IL-13 in macrophages infected with hypervirulent strains of *M. tuberculosis* (HN878 and W/Beijing) [70]. In contrast, CDC1551 induces more IL-12 and other molecules associated with phagocyte activation and Th1 protective immunity. These differences have been ascribed to mycobacterial lipids [173] and modulation of cytokine production [70]. This differential response mediated by mycobacterial lipids, with phenolic glycolipid (PGL) being a candidate [173]. These and additional considerations suggest the phenolic glycolips of *Mycobacterium tuberculosis* virulent strains increase levels of Th2 cytokines to inhibit autophagic activity and enhance their survival.

4.5. Limitations

The present study contains certain inherent limitations that need to be addressed. First, the study was accomplished on cultured cells. Consequently, the monocytes/macrophage cells were analyzed secluded from the dynamics existing in their microenvironment and may not reflect accurate *in vivo* cell behavior. Second, in this study, we did not specifically examine alternative activation of macrophages by IL-4 and IL-13 [69]; instead, the focus was on the effects of short-term treatment of 2 hours in combination with IFN-γ. Treatment of macrophages with IL-4 or IL-13 for 6 days has been shown to enhance fluid-phase pinocytosis and mannose receptor-mediated endocytosis through activation of phosphatidylinositol 3 kinase; both cytokines increase tubular-vesicle formation at pericentriolar sites under the plasma membrane, concurrent with decreased particle sorting to the lysosomes [175]. Although these previous observations could explain the inhibitory effects of IL-4 and IL-13 on autophagolysosome formation, IL-4 and IL-13 had no effect on BCG phagosome maturation in the absence of starvation- or IFN-γ-induced autophagy, demonstrating that the effects observed in our experiments are autophagy specific. A study on the effects of alternative activation of macrophages with Th2 cytokines, in the context of autophagy, may be difficult to address because prolonged autophagy can lead to type-II programmed cell death, and most cells will downregulate this process to ensure their survival [167].

4.6. Conclusion and future directions

This study shows that Th2 cytokines IL-4 and IL-13 inhibited autophagy and undermined the ability of macrophages to eliminate intracellular mycobacteria. Th2 cytokines inhibited both physiologically and immunologically induced autophagy in murine and human monocytes and macrophage. Furthermore, these cytokines inhibited autophagy-dependent maturation of the mycobacterial phagosome averting elimination of mycobacteria. These findings have ramifications in Th1-Th2 polarization of the immune response to *Mycobacterium tuberculosis*. By antagonizing autophagy, IL-4 and IL-13 inhibit Th1-dependent protection against mycobacteria, possibly explaining why Th2 cytokines antagonize protection against other intracellular microbes, such as *Listeria moncytogenes* and *Legionella pneumophila*. The regulation of autophagy may represent a critical aspect of Th1-Th2 polarization in the host response to intracellular pathogens. Thus, the balance of Th1 and Th2 cytokines is clearly critical in the host response to *Mycobacterium tuberculosis*. Based on Th1 inducing autophagy and Th2 dampening that response, this study sheds more light on this dichotomy.

Higher level of IL-4 may indicate severity of disease in patients with cavitary tuberculosis [64]. Therefore, in individuals with a mixed Th1-Th2 response to mycobacteria, it may be more beneficial to block the Th2 component than to boost the Th1 response. This would be especially important if there is an already adequate Th1 component. The possibility of restraining the Th2 response by stimulating suitable types of regulatory T cells has been pursued in studies of allergic disorders, where much larger Th2 responses are present [176].

Although the importance of autophagy in the elimination of *Mycobacterium tuberculosis* has been established *in vitro*, existing studies have not determined to what extent autophagy contributes to the immune response against mycobacteria *in vivo*. Therefore, it would be of great relevance to study the autophagic status of alveolar macrophages and dendritic cells, inside the granuloma and its periphery, during pulmonary tuberculosis. The role of various different cytokines on this process may prove insightful, particularly those of potential importance in the innate immune response and granuloma formation, such as IL-10 and TGF-β. The answers to these questions could advance the evolutionary arms race of the host to prevail in the fight against *Mycobacterium tuberculosis.*

5. APPENDICES

Appendix A. T Helper 2 Cytokines Inhibit Autophagic Control of Intracellular Mycobacterium tuberculosis.

Immunity
Article

T Helper 2 Cytokines Inhibit Autophagic Control of Intracellular *Mycobacterium tuberculosis*

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SUMMARY

Autophagy is a recently recognized immune effector mechanism against intracellular pathogens. The role of autophagy in innate immunity has been well established, but the extent of its regulation by the adaptive immune response is less well understood. The T helper 1 (Th1) cell cytokine IFN-γ induces autophagy in macrophages to eliminate *Mycobacterium tuberculosis*. Here, we report that Th2 cytokines affect autophagy in macrophages and their ability to control intracellular *M. tuberculosis*. IL-4 and IL-13 abrogated autophagy and autophagy-mediated killing of intracellular mycobacteria in murine and human macrophages. Inhibition of starvation-induced autophagy by IL-4 and IL-13 was dependent on Akt signaling, whereas the inhibition of IFN-γ-induced autophagy was Akt independent and signal transducer and activator of transcription 6 (STAT6) dependent. These findings establish a mechanism through which Th1-Th2 polarization differentially affects the immune control of intracellular pathogens.

INTRODUCTION

Autophagy is a fundamental homeostatic mechanism in which cells sequester discrete portions of the cytoplasm into an autophagosome, a specialized vacuole with a double membrane, which in turn delivers them to lysosomes for degradation (Levine, 2005; Shintani and Klionsky, 2004). This process removes damaged or surplus organelles such as leaky mitochondria and excess peroxisomes and, by degrading long-lived cytoplasmic macromolecules during periods of starvation, promotes cell survival (Kuma et al., 2004). Autophagy has recently been shown to play a role in innate immunity against intracellular pathogens, including Epstein-Barr Virus (Paludan et al., 2005), *Shigella flexneri* (Ogawa et al., 2005), *Salmonella typhimurium* (Birmingham et al., 2006), *Toxoplasma gondii* (Ling et al., 2006), and *Mycobacterium tuberculosis* (Gutierrez et al., 2004). In addition, autophagy has been implicated in adaptive immunity, playing a role in endogenous antigen presentation (Dengjel et al., 2005; Deretic, 2005; Paludan et al., 2005; Schmid et al., 2006b).

Autophagy can be induced pharmacologically with rapamycin, which inhibits TOR, a conserved Ser and Thr kinase that regulates cell growth and metabolism in response to growth factors, energy inputs, and nutritional demands (Wullschleger et al., 2006). TOR integrates various inputs; its activation stimulates anabolic processes and biomass production, whereas its inhibition enhances catabolic processes, including autophagy. A classical example of this system in action is demonstrated by amino acid starvation, which leads to inhibition of TOR and induction of autophagy. Conversely, TOR can be activated by growth factors via the Akt (also known as PKB) pathway, resulting in the inhibition of autophagy (Wullschleger et al., 2006). In some cases, withdrawal of growth factors is sufficient for induction of autophagy, even in the presence of adequate nutrients. For example, removal of IL-3 from cultures of an IL-3-dependent hemopoietic cell line that also lacks the apoptotic regulators Bax and Bak has been shown to induce autophagy, and if left unchecked, the cells eventually die (Lum et al., 2005). However, the cells die more rapidly when autophagy is blocked, suggesting that autophagy is a survival mechanism in these cells (Lum et al., 2005). Autophagy can be modulated by cytokines and other immunological signals (Andrade et al., 2006; Djavaheri-Mergny et al., 2006; Singh et al., 2006). For example, TNF-α can induce autophagy in Ewing sarcoma cells in the absence of NF-κB activation (Djavaheri-Mergny et al., 2006), whereas in macrophages and other cells, IFN-γ, a classical T helper 1 (Th1) cell cytokine and a critical antituberculosis immune mediator, induces or augments autophagy (Gutierrez et al., 2004; Inbal et al., 2002; Pyo et al., 2005). Moreover, the protective role of IFN-γ against mycobacteria has been associated with autophagy (Gutierrez et al., 2004; Singh et al.,

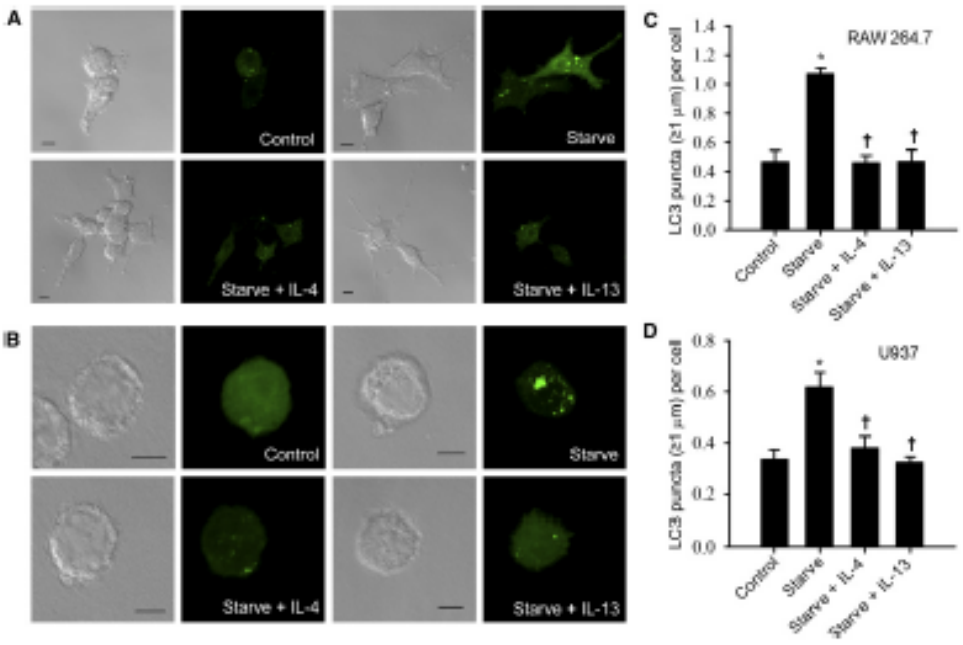

Figure 1. Inhibition of Starvation-Induced Autophagy by IL-4 and IL-13

(A) Murine RAW264.7 or (B) human U937 cells were transiently transfected with pEGFP-LC3, amino acid- and serum-starved for 2 hr with or without IL-4 or IL-13 (30 ng/ml), and analyzed by confocal microscopy. The number of large (≥1 μm) LC3 puncta per cell were quantified (C and D). Data are presented as means ± SEM; *$p < 0.05$ and †$p \geq 0.05$; $n = 3$. Scale bars represent 5 μm.

2006), indicating that this process is an important effector mechanism of the Th1 response.

The two classical Th2 cytokines, IL-4 and IL-13, signal through the receptor IL-4Rα, which forms a heterodimer either with the gamma common (γc) chain (for IL-4) or the IL-13Rα1 (for IL-13) (Nelms et al., 1999). In addition, IL-13 can signal through the high-affinity IL-13Rα2, initially thought to be a decoy, nonsignaling receptor (Donaldson et al., 1998; Fichtner-Feigl et al., 2006; Kawakami et al., 2001; Zhang et al., 1997). Ligation of the IL-4 and IL-13 receptor complexes results in signaling via the insulin receptor substrate (IRS)-1 and 2 and STAT-6 pathways (Nelms et al., 1999). Although STAT-6 is involved in IL-4- and IL-13-induced gene expression, IRS-1 and 2 signaling activates the type I phosphatidylinositol 3-kinase (PI3K) pathway and subsequently the Akt pathway. On the basis of the latter signaling pathway, we hypothesized that IL-4 and IL-13 would inhibit the autophagy-induced killing of mycobacteria by macrophages. Here, we showed that IL-4 and IL-13 inhibited autophagy induced by either amino acid starvation or by IFN-γ in murine and human macrophages but that different signaling pathways were used to suppress starvation-induced versus IFN-γ-induced autophagy. This IL-4 and IL-13 action specifically inhibited the transfer of mycobacteria into lysosomal compartments and enhanced mycobacterial survival within infected macrophages by inhibiting autophagy.

RESULTS

IL-4 and IL-13 Inhibit Starvation-Induced Autophagy

Induction of autophagy in the murine RAW264.7 and human U937 monocyte or macrophage cell lines was monitored with morphometric analysis after the formation of pEGFP-LC3-labeled (Kabeya et al., 2000) autophagosomes (≥1 μm). LC3 is the mammalian equivalent of yeast Atg8, a specific marker that translocates from the cytosol to autophagosomal membranes (Kabeya et al., 2000). Amino acid starvation resulted in a significant increase in the number of pEGFP-LC3$^+$ puncta per cell (Figure 1). Translocation of cytosolic LC3 to autophagosomal organelles was also detected by 4D live confocal microscopy (Chua and Deretic, 2004; Kyei et al., 2006; Roberts et al., 2006) in macrophages transfected with tdTomato-LC3 (Bjorkoy et al., 2005) (Movie SM1 in the Supplemental Data available online). Amino acid-starvation-induced formation of LC3 puncta was abrogated with the addition of 3-MA (Figure S1), a classical inhibitor of autophagy (Blommaart et al., 1997). After ascertaining the expression of IL-4Rα by flow cytometry (Figure S2), IL-4 and IL-13 were added to macrophages induced for autophagy by amino acid starvation. Either one of the Th2 cytokines tested abrogated autophagy, as shown by inhibition of pEGFP-LC3$^+$ puncta formation in both RAW and U937 cells

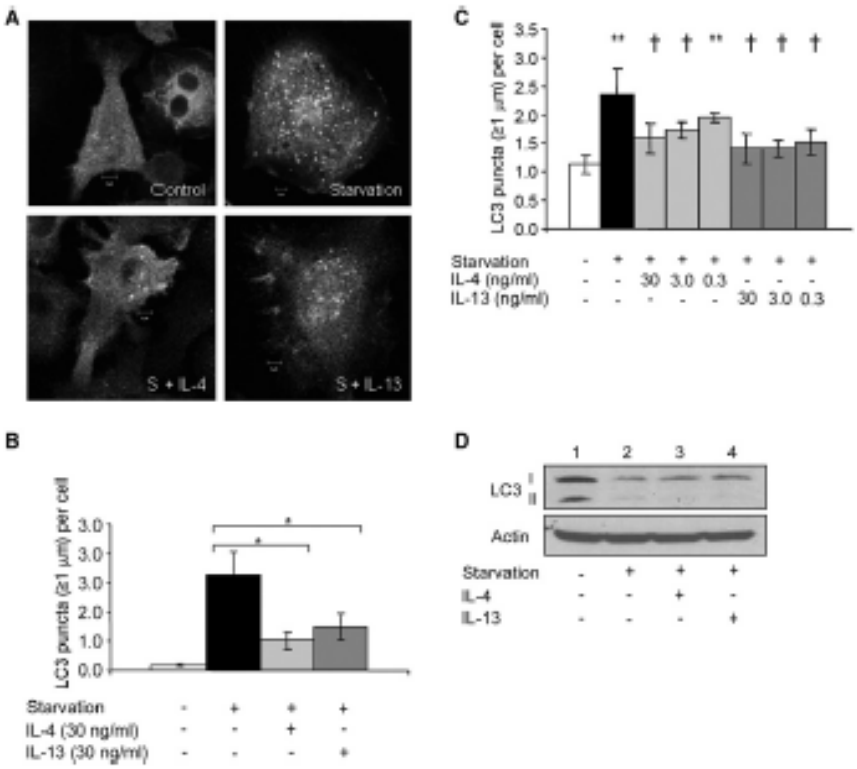

Figure 2. Inhibition of Starvation-Induced Autophagy by IL-4 and IL-13 in Murine Macrophages

(A) Bone marrow macrophages (BMMs) from GFP-LC3 transgenic mice were amino acid- and serum-starved (S) for 2 hr with or without IL-4 or IL-13 (30 ng/ml) and analyzed by confocal microscopy.

(B) Quantification of large (>1 μm) GFP-LC3 puncta per cell (within the denominator; calculations included cells without any large puncta); n = 3 (separate slides).

(C) Quantification of large (≥ 1 μm) LC3 puncta in RAW264.7 cells transfected with GFP-LC3 and induced for autophagy by starvation in the presence of decreasing concentrations of IL-4 or IL-13; n = 3 (separate slides; shown is one out of two independent experiments).

(D) Immunoblot analysis of LC3 lipidation state in RAW264.7 cells starved and incubated without (none) or with IL-4 or IL-13. Full; cells grown in full medium. Cells were amino acid- and serum-starved for 2 hr (lanes 2–4). Actin was used as the loading control. Data are presented as means ± SEM; *$p < 0.05$, **$p < 0.01$, and †$p \geq 0.05$.

(Figure 1). Similar results were obtained with primary bone marrow-derived macrophages (BMMs) from GFP-LC3 transgenic mice (Mizushima et al., 2004) (Figures 2A and 2B). A titration of IL-4 and IL-13 in RAW264.7 cells showed that at 0.3 ng/ml, both cytokines still exerted their effects, albeit IL-4 was losing some of its potency at the lowest concentration tested (Figure 2C).

The inhibitory effects of IL-4 and IL-13 on starvation-induced autophagy were independently confirmed by an assay that measures conversion of LC3-I (nonlipidated form with lower electrophoretic mobility) to LC3-II (LC3 form C-terminally lipidated by phosphatidylethanolamine, displaying higher electrophoretic mobility) with immunoblots (Kabeya et al., 2000). As expected, induction of autophagy by starvation reduced both LC3-I and LC3-II band intensity in RAW264.7 cell (Figure 2D and Figure S2B, lanes 1 and 2) because of the autophagic consumption of LC3 (Kabeya et al., 2000). At the same time, the intensity of the LC3-II band changed (increased) relative to the intensity of LC3-I band (Figure 2D and Figure S2B, lanes 1 and 2). When IL-4 and IL-13 were added to starved macrophages, two changes occurred: (1) The intensity of the LC3-I band increased (Figure 2D, lanes 3 and 4, relative to lane 2), indicative of a reduced LC3-I-to-LC3-II conversion, consistent with the inhibition of autophagy induction by IL-4 and IL-13. An increase in LC3-I band was also observed in primary murine BMM (Figure S2C). (2) The LC3-II band intensity decreased in starved RAW264.7 macrophages treated with IL-4 and IL-13 (Figure 2D, lanes 3 and 4, compared to lane 2). This could be the result of reduced LC3-I-to-LC3-II conversion, in keeping with an inhibition of autophagy induction. However in BMM, only an LC3-I increase but no LC3-II decrease was observed (Figure S2C). The differences in

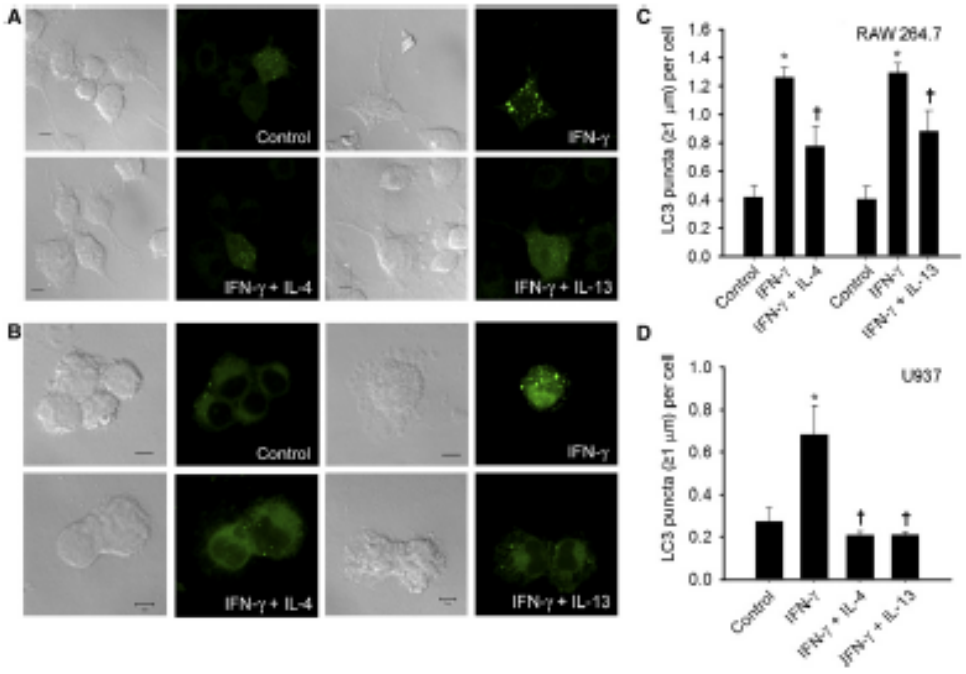

Figure 3. Inhibition of IFN-γ-Induced Autophagy by IL-4 and IL-13

(A) Murine RAW264.7 or (B) human U937 cells were transiently transfected with pEGFP-LC3 and treated with 200 U/ml IFN-γ or IFN-γ in combination with IL-4 or IL-13 (30 ng/ml) for 24 hr and were analyzed by confocal microscopy. Large (≥ 1 μm) LC3 puncta per cell were quantified (C and D). Data are presented as means ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, †$p \geq 0.05$; n = 3. Scale bars represent 5 μm.

LC3-II band intensity between RAW264.7 and BMM may be the net result of differences in the rates of LC3-I-to-LC3-II conversion versus LC3-II depletion through degradation in autolysosomes. This was substantiated with lysosomal and autolysosomal protease inhibitors (Figure S2, lanes 3 and 4, compared to lane 2), which, as expected, increased LC3-II amounts by blocking its degradation both in the presence or absence of IL-4. Consistent with the conclusion that Th2 cytokines inhibit LC3-I-to-LC3-II conversion, IL-4 presence increased LC3-I band intensity (Figure S2B, lane 4 compared to lane 3). Because the presence of IL-4 did not diminish LC3-II band levels relative to protease inhibitors alone (Figure S2, lane 4 versus lane 3), as one might expect from lower LC3-I-to-LC3-II conversion, Th2 cytokines may have an additional effect on the fate of LC3-II by partially inhibiting its delivery to or its degradation in autolysosomes. This conclusion is consistent with the apparent preservation of the LC3-II band in BMM (Figure S2C). Of note is that IL-4 and IL-13 did not change expression of the murine LC3 gene MAP1LC3b under starvation conditions (Figure S2D). In conclusion, IL-4 and IL-13 inhibit LC3-I-to-LC3-II conversion and initiation of autophagy but may have additional effects on the autophagic pathway; these effects become apparent when different cells are examined.

IL-4 and IL-13 Inhibit IFN-γ-Induced Formation of Autophagosomes

Treatment of macrophages with IFN-γ promotes the formation of autophagosomes (Gutierrez et al., 2004; Singh et al., 2006). We tested whether this effect could be inhibited by treating RAW and U937 cells with IFN-γ in combination with either IL-4 or IL-13. In both cell types, IL-4 and IL-13 significantly reduced the number of IFN-γ-induced pEGFP-LC3^{+} puncta per cell (Figure 3). In addition, we found that IFN-γ treatment increased the percentage of RAW cells with large vacuoles that stained positively for monodansylcadaverine (MDC), another marker for autophagic vacuoles (Biederbick et al., 1995), and this effect was inhibited by IL-4 or IL-13 (Figures S3A and S3B). By using the MDC assay, we confirmed the effects of IL-4 and IL-13 in primary human, peripheral blood monocyte-derived macrophages (MDMs) and found, in titration experiments, a similar concentration-dependence pattern to that seen in murine macrophages (Figure S3C).

IL-4 and IL-13 Inhibit Autophagy-Dependent BCG Phagolysosome Maturation

Mycobacterium tuberculosis normally resides in phagosomes that do not acquire phagolysosomal properties, such as lumenal acidification and the presence of

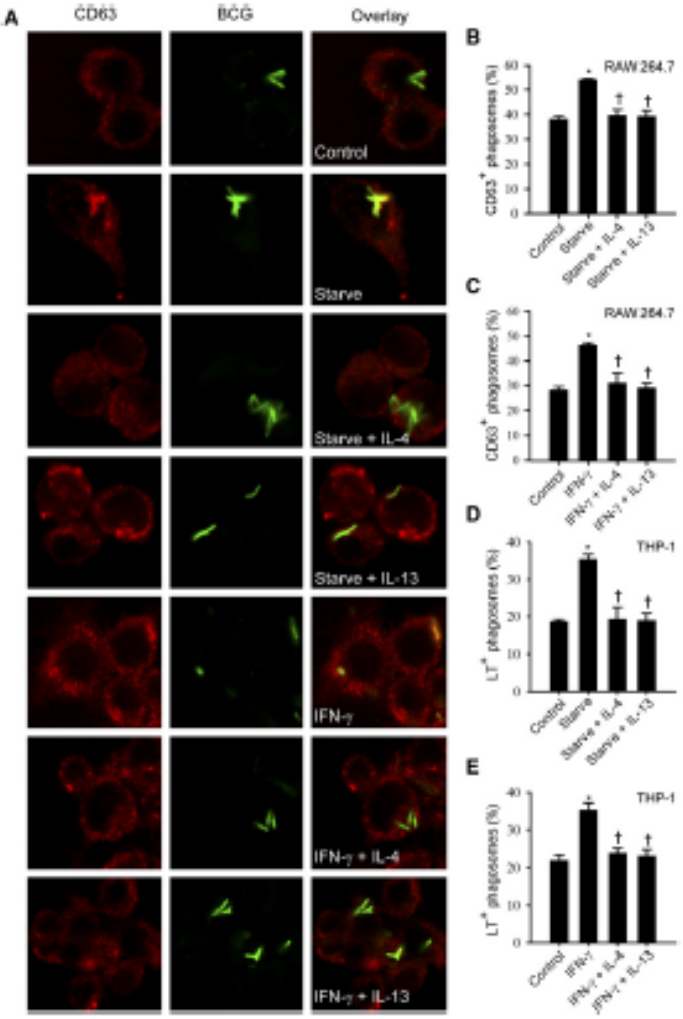

Figure 4. Inhibition of Starvation- and IFN-γ-Induced BCG Phagosome Maturation by IL-4 and IL-13

(A) Confocal images of murine RAW264.7 macrophages infected with GFP-BCG for 2 hr. Cells were amino acid- and serum-starved for 2 hr (during infection) or treated with IFN-γ (200 U/ml) for 24 hr prior to infection, with or without IL-4 or IL-13 (30 ng/ml). After infection, cells were fixed and stained for CD63. The percentage of $CD63^+$ BCG phagosomes was quantified (B and C). The percentage of Lysotracker red $(LT)^+$ BCG phagosomes was recorded in PMA-differentiated human THP-1 monocytes after (D) starvation or (E) IFN-γ (200 U/ml) treatment with or without IL-4 or IL-13 (30 ng/ml). Representative images are shown in Figure S6. Data are presented as means ± SEM; *$p < 0.05$ and †$p \geq 0.05$; $n = 3$.

lysosomal hydrolases (Vergne et al., 2004). Induction of autophagy has been shown to promote the transfer of mycobacteria into degradative autolysosomal organelles (Gutierrez et al., 2004). To investigate whether IL-4 and IL-13 counteract this effect, we examined the maturation of *M. tuberculosis* variant *bovis* BCG (BCG) phagosomes by monitoring the late endosomal or lysosomal marker CD63. Induction of autophagy by starvation in BCG-infected RAW cells significantly increased colocalization of GFP-BCG with CD63 (Figures 4A and 4B). The effect of starvation-induced autophagy on BCG phagosome maturation was inhibited by the addition of either IL-4 or IL-13 (Figures 4A and 4B). Both IL-4 and IL-13 also inhibited IFN-γ-induced phagosome maturation in BCG-infected cells (Figures 4A and 4C). Starvation increased phagosome maturation in U937 cells, an effect that was inhibited with either IL-4 or IL-13 (Figure S4). For unknown reasons, IFN-γ did not significantly increase BCG phagosome maturation in U937 cells (data not shown). Instead, we tested PMA-differentiated human THP-1 cells, by using the acidotropic dye LysoTracker Red (LT) to visualize lysosomal compartments. As with RAW cells, phagosome maturation was increased by either starvation or IFN-γ; this was inhibited by treatment with either IL-4 or IL-13 (Figures 4D and 4E and Figure S5).

Phagosome maturation was autophagy dependent, as demonstrated by siRNA knockdown of Beclin 1, a critical mammalian autophagy factor (Liang et al., 1999) (Figures 5A–5C). Although RAW cells transfected with scrambled siRNA showed an increase in BCG phagosome maturation in response to starvation (Figures 5A and 5D) or IFN-γ (Figures 5B and 5D), cells treated with Beclin 1 siRNA did not, demonstrating that these responses are dependent on functional autophagic machinery. In the

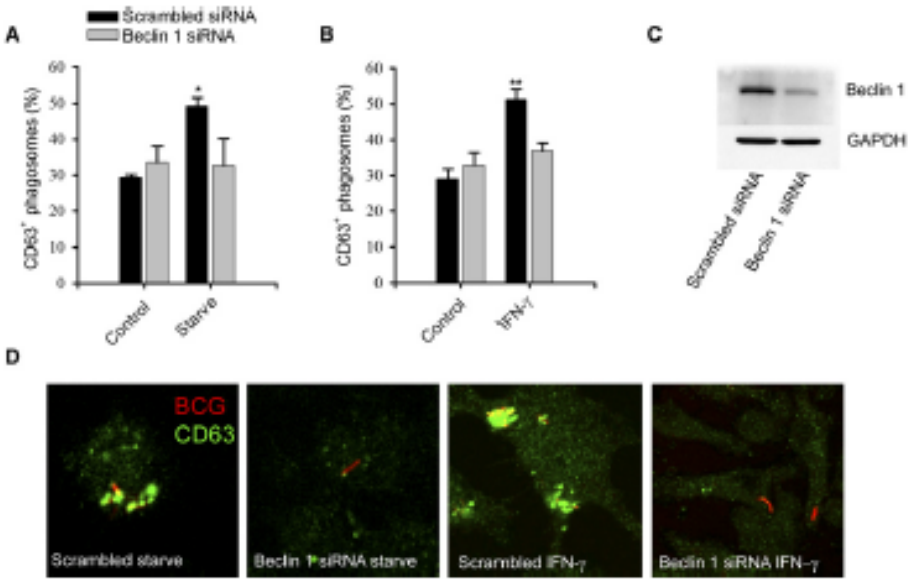

Figure 5. Starvation- and IFN-γ-Induced BCG Phagosome Maturation Is Autophagy Dependent

(A) Quantitative analysis of CD63$^+$ BCG phagosomes that were in murine RAW264.7 macrophages transiently transfected with either nontargeting (scrambled) siRNA or Beclin 1 siRNA and that were amino acid- and serum-starved for 2 hr.

(B) CD63$^+$ BCG phagosomes in RAW264.7 cells transiently transfected with scrambled or Beclin 1 siRNA and treated with IFN-γ (200 U/ml) for 24 hr. Data are presented as means ± SEM; $^*p < 0.05$, $^{**}p < 0.01$, and $\dagger p \geq 0.05$; n = 3 for (A) and n = 6 for (B).

(C) Immunoblot confirmation of Beclin 1 knockdown by siRNA.

(D) Immunofluorescence panels of BCG colocalization with the late endosomal marker CD63, exemplifying the data in (A) and (B). CD63 is shown in green; BCG is shown in red.

presence of full-nutrient media, treatment of RAW macrophages with IL-4 or IL-13 had no effect on the colocalization of CD63 with either live or heat-killed BCG phagosomes (Figure S6). Thus, these Th2 cytokines do not inhibit mycobacterial phagosome maturation under normal conditions but rather specifically inhibit autophagy-dependent maturation.

IL-4 and IL-13 Inhibit Autophagy-Dependent Killing of Intracellular Mycobacteria

To determine whether IL-4 and IL-13 affect autophagic elimination of mycobacteria (Gutierrez et al., 2004), we infected RAW cells with BCG and induced autophagy by amino acid starvation for 2 hr. Induction of autophagy decreased BCG survival in RAW cells (Figure 6A). Addition of IL-4 or IL-13 to the starvation media abrogated this effect (Figure 6A). Similar results were obtained when survival of virulent *M. tuberculosis* H37Rv was tested with starvation or IFN-γ used as autophagy agonists (Figures 6B and 6C). Figure 6D shows that these relationships hold true in primary macrophages because IL-4, used as an example, abrogated killing of virulent *M. tuberculosis* H37Rv by starvation-induced autophagy in murine BMMs. These findings demonstrate that IL-4 or IL-13 inhibit autophagy-induced killing of mycobacteria by macrophages.

IL-4 and IL-13 Inhibit Autophagic Phagosome Maturation in Primary Human Cells

To confirm that IL-4 and IL-13 inhibit autophagy-induced effects on mycobacterial phagosomes in human primary macrophages, we tested whether IL-4 and IL-13 influenced autophagy-induced mycobacterial phagosome maturation. Human peripheral blood monocyte-derived macrophages (MDMs) were infected with BCG and induced for autophagy by starvation. The effect of starvation-induced autophagy on mycobacterial phagosome maturation was decreased by the addition of either IL-4 or IL-13 (Figures 6E and 6F). Similarly, both IL-4 and IL-13 had an inhibitory effect on IFN-γ-induced mycobacterial phagosome maturation (Figures 6E and 6F). These results validate in human primary macrophages the conclusion that Th2 cytokines counteract autophagy in its ability to deliver mycobacteria into the phagolysosome.

Inhibition of Starvation-Induced Autophagy by IL-4 and IL-13 Is Akt Dependent

To determine the signaling involved in the actions of IL-4 and IL-13 on autophagy in macrophages, we examined whether the Akt pathway, known to activate TOR and inhibit autophagy (Wullschleger et al., 2006), was involved. In both RAW (Figure 7A) and U937 (Figure S7A) macrophages, IL-4 and IL-13 increased phosphorylation of Akt

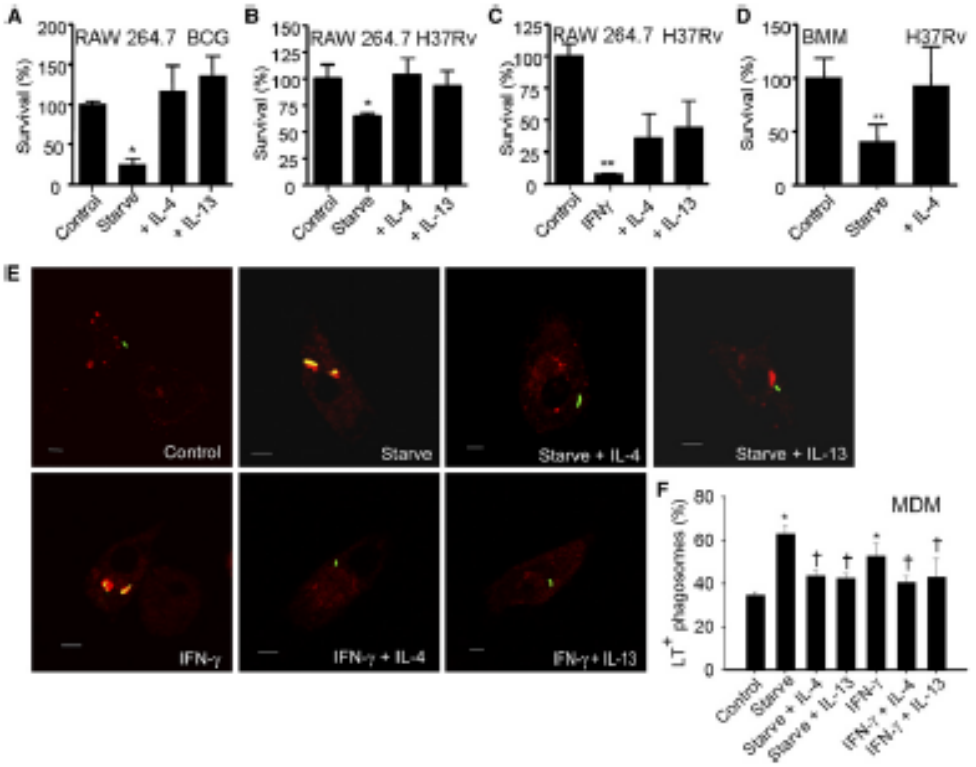

Figure 6. IL-4 and IL-13 Inhibit Autophagy-Dependent Killing of *M. tuberculosis* in Murine Macrophages and Counteract Autophagy-Induced Phagosome Maturation in Primary Human Macrophages

(A) Murine RAW264.7 macrophages were infected with BCG for 1 hr and were amino acid- and serum-starved with or without IL-4 or IL-13 (30 ng/ml) for 2 hr. Cells were washed and lysed for viability determination and survival expressed as a percentage of the control.

(B) Murine RAW264.7 macrophages were infected with virulent *M. tuberculosis* H37Rv for 1 hr and were amino acid- and serum-starved with or without IL-4 or IL-13 (30 ng/ml) for 2 hr. Cells were washed and lysed for viability determination.

(C) Murine RAW264.7 macrophages were either untreated or treated with 200 u/ml of m-IFN-γ for 24 hr prior to infection with *M. tuberculosis* H37Rv for 1 hr with or without IL-4 or IL-13 (30 ng/ml) for 2 hr. Cells were washed and lysed for viability (cfu) determination. Data are presented as means ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, and $\dagger p \geq 0.05$; n = 3.

(D) IL-4 inhibits autophagy-dependent killing of *M. tuberculosis* H37Rv in primary murine macrophages. Murine bone marrow macrophages were infected with virulent *M. tuberculosis* (strain H37Rv) for 1 hr and were amino acid- and serum-starved with or without IL-4 (30 ng/ml) for 2 hr. Mycobacterial viability (colony counts) is expressed as a percentage of the control.

(E and F) Primary human macrophages derived from peripheral blood monocytes were infected with BCG. Transfer of mycobacteria to phagolysosome dependent on autophagy was scored with the acidotropic dye LysoTracker. Autophagy was induced for 2 hr by starvation or by treatment with 200 u/ml human IFN-γ. Cells were incubated in the absence or presence of 30 ng/ml of human IL-4 or IL-13, as indicated. (F) shows quantification of experiments shown in (E). Data are presented as means ± SEM; $^{*}p < 0.05$ and $\dagger p \geq 0.05$; n = 3 (three independent donors).

under starvation conditions (Figure S7A, top two panels and graph). We next tested the activity of TOR, a key negative regulator of autophagy (when TOR is active, autophagy is inhibited) on the basis of reports that TOR is activated by Akt (Wullschleger et al., 2006). We investigated TOR activation by monitoring phosphorylation of p70 S6 kinase (S6k) (Figure S7A, bottom two panels and graph) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (Figures 7B–7E and Figure S7), both commonly used as indicators of TOR activation state (Wullschleger et al., 2006). Treatment of U937 cells with IL-4 or IL-13 increased phosphorylation of S6k (Figure S7A). In RAW264.7 macrophages, IL-4 and IL-13 treatment increased phosphorylation of 4E-BP1 under starvation conditions in a dose-dependent manner (Figures S7B and S7C). A finer-resolution analysis of 4E-BP1 phosphorylation was carried out next. TOR induces 4E-BP1 inactivation via multiple hierarchical phosphorylations (Gingras et al., 2001). A fine-resolution immunoblot analysis can be used for revealing 4E-BP1 hypophosphorylated α and β forms, and hyperphosphorylated γ form, resolved in the order of their decreasing electrophoretic mobility. Active Akt elicits 4E-BP1(γ) hyperphosphorylation through TOR activity (Gingras et al., 2001). Our analyses in primary

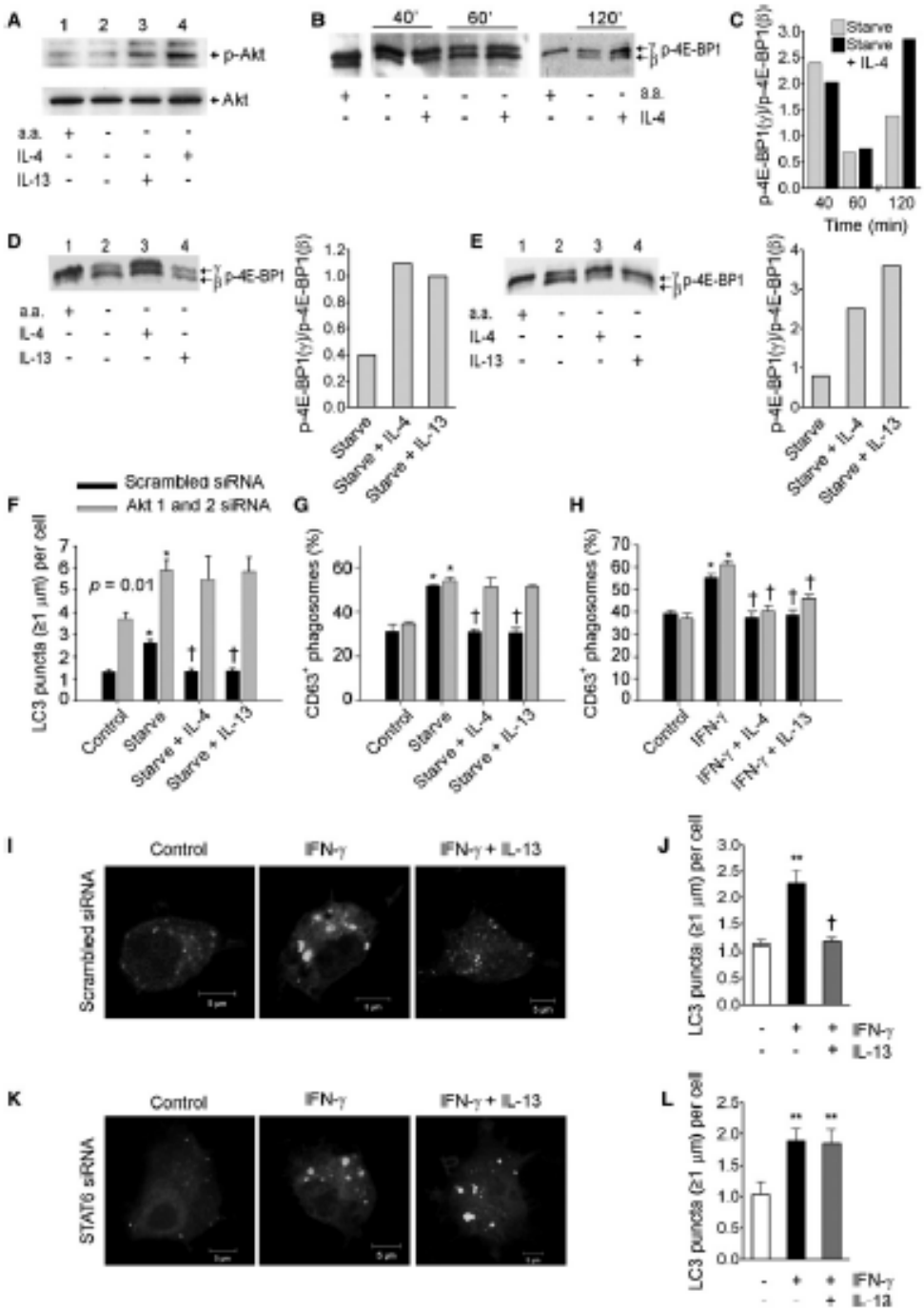

Figure 7. Role of Akt and STAT6 in Suppression by IL-4 and IL-13 of Starvation- and IFN-γ-Induced Autophagy

(A) Immunoblot analysis of Akt phosphorylation in RAW264.7 macrophages amino acid- and serum starved for 2 hr with or without IL-4 or IL-13.

(B) Immunoblot analysis of 4E-BP1 hierarchical phosphorylation in murine bone marrow macrophages (BMMs) starved and treated with IL-4 for 40, 60, and 120 min.

(C) Graph shows the ratio of p-4E-BP1(γ)/p-4E-BP1(β).

(D) Immunoblot analysis of 4E-BP1 in murine bone marrow macrophages (BMMs).

murine BMMs and in human MDM revealed that IL-4 and IL-13 treatment specifically increased phosphorylation of 4E-BP1(γ) under starvation conditions (Figures 7B–7E). Furthermore, a time course of 4E-BP1 showed that IL-4 exerted its effects by increasing 4E-BP1(γ) hyperphosphorylation at 2 hr but had no discernible effects at earlier time points (Figures 7B and 7C), consistent with other effects measured in our study. These results demonstrate that IL-4 and IL-13 activate the Akt pathway in macrophages, resulting in the activation of TOR and inhibition of starvation-induced autophagy.

To establish the role of Akt in the IL-4- and IL-13-induced inhibition of autophagy in macrophages, we knocked down Akt1 and Akt2 with siRNA in vitro (Figure S8A). Compared with the control samples (scrambled siRNA), knockdown of Akt1 and Akt2 in RAW macrophages increased autophagy, monitored by $LC3^+$ vacuole formation (Figure 7F and Figure S8B), indicating that basal levels of autophagy are controlled, at least in part, by baseline Akt activation. Double knockdown of Akt1 and Akt2 in RAW cells abrogated the effects of IL-4 and IL-13 on starvation-induced autophagy (Figure 7F and Figure S8B). In BCG-infected RAW cells, knockdown of Akt1 and Akt2 also inhibited the effects of IL-4 and IL-13 on starvation-induced transfer of BCG to autophagosomes (Figure 7G and Figure S8C).

Inhibition of IFN-γ-Induced Autophagy Is STAT6 Dependent

In contrast to the effects with IL-4 and IL-13 on starvation-induced autophagy, a double knockdown of Akt1 and Akt2 with siRNA had no effect on the inhibition of IFN-γ-induced phagosome maturation by IL-4 and IL-13 (Figure 7H), suggesting that the suppressive effects of these Th2 cytokines on the activation by IFN-γ involved alternative or additional pathways, independent of Akt signaling. Akt3 was detectable only at very low amounts in these cells, and its expression was not affected by knockdown of Akt1 and Akt2 (data not shown). We therefore tested whether the inhibitory effect of IL-4 and IL-13 on IFN-γ-induced autophagy was regulated via a different pathway. IL-4 and IL-13 receptors, in addition to signaling via the Akt pathway, are more commonly known for their activation of STAT6 (Nelms et al., 1999). Thus, we tested whether STAT6 was required for Th2 cytokine inhibition of autophagy activation by IFN-γ. When STAT6 was knocked down (Figure S9), IL-13 could no longer suppress IFN-γ-induced autophagosome formation in macrophages (Figures 7I–7L). Collectively, our data on IL-4 and IL-13 inhibition of starvation- and IFN-γ-induced autophagy demonstrate that Th2 cytokines can employ either of the signaling pathways associated with ligation of the IL-4 and IL-13 receptors to exert this effect. Suppression of starvation-induced autophagy occurs through Akt signaling, whereas STAT6 is needed for suppression of IFN-γ-induced autophagy.

DISCUSSION

Recent studies have demonstrated that autophagy represents a mechanism for eliminating intracellular pathogens (Deretic, 2005; Schmid et al., 2006a; Levine and Deretic, 2007), as shown in several bacterial, viral, and protozoan systems (Birmingham et al., 2006; Gutierrez et al., 2004; Ling et al., 2006; Ogawa et al., 2005; Orvedahl et al., 2007; Paludan et al., 2005; Singh et al., 2006). Induction of autophagy by amino acid starvation, rapamycin treatment, or macrophage activation with IFN-γ leads to an increase in mycobacterial phagolysosome maturation. This increase is concomitant with a decrease in the intracellular survival of the bacilli (Singh et al., 2006), and such a survival decrease is independent of apoptosis (Gutierrez et al., 2004), another process previously implicated in the elimination of *M. tuberculosis* (Fratazzi et al., 1999). Here, we have shown that autophagy-induced killing of mycobacteria by murine and human macrophages is inhibited by the Th2 cytokines IL-4 and IL-13. Both cytokines counteract the autophagic transfer of mycobacteria from their normally immature phagosomes with early endosomal characteristics (Vergne et al., 2004) to autolysosomes, thus preventing the elimination of intracellular bacilli by autophagy.

We have shown that the effect of IL-4 and IL-13 on starvation-induced autophagy is dependent on signaling via the Akt pathway, which activates TOR. Activation of TOR, downstream of Akt, inhibits autophagy. As expected, IL-4 and IL-13 could not influence rapamycin-induced autophagy (data not shown) because rapamycin acts directly on TOR, bypassing Akt signaling. Notably, the mechanism of IL-4 and IL-13 inhibition of IFN-γ-induced autophagy is

(E) Immunoblot analysis of human monocyte-derived macrophages (MDMs) starved for 2 hr with or without IL-4 or IL-13. Graphs in (D) and (E) show (n = 2; one out of two experiments with similar results) the intensity ratios of p-4E-BP1(γ) to p-4E-BP1(β) bands.
(F) Quantification of large (≥ 1 μm) pEGFP-LC3 puncta per cell in RAW264.7 macrophages transiently transfected with pEGFP-LC3 and either scrambled siRNA or Akt 1 + Akt 2 siRNA and starved for 2 hr with or without IL-4 or IL-13.
(G) Quantitative analysis of $CD63^+$ BCG phagosomes in RAW264.7 cells transiently transfected with scrambled siRNA or Akt 1 + Akt 2 siRNA and infected with BCG for 2 hr. Infected cells were amino acid- and serum-starved with or without IL-4 or IL-13.
(H) Percentage of $CD63^+$ BCG phagosomes in RAW264.7 cells transiently transfected with scrambled siRNA or Akt1 + Akt2 siRNA, treated with hIFN-γ with or without IL-4 or IL-13 for 24 hr, and infected with BCG for 2 hr. Representative images from these experiments and immunoblot-blot confirmation of Akt 1 and Akt 2 knockdown are shown in Figure S8.
(I–L) Th2 inhibition of IFNγ-dependent autophagy is STAT6 dependent: (I and J) display confocal-microscopy images and quantification of GFP-LC3 puncta formation in response to IFN-γ, with or without IL-13, in cells treated with control (scramble) siRNA. (K–L) display images and quantification of GFP-LC3 puncta formation in response to IFN-γ, with or without IL-13, in cells treated with control STAT6 siRNA. Immunoblot analysis of STAT6 knockdown is given in Figure S9. Data in all graphs (when error bars are shown) are presented as means ± SEM; $n \geq 3$; $^*p < 0.05$, $^{**}p < 0.01$, and $\dagger p \geq 0.05$.

Akt independent. This finding was surprising given the established role of Akt in inhibiting autophagy in general (Petiot et al., 2000) and given the inhibitory effects of Akt in starvation-induced autophagy in macrophages demonstrated here for IL-4 and IL-13 and in nonphagocytic cells elsewhere for IL-13 (Arico et al., 2001). To explain these observations, we have now uncovered a previously unknown signaling relationship; STAT6 modulates the Th2 antiautophagic function when macrophages are stimulated for autophagy by IFN-γ. This places STAT6 within the ranks of the immunologically relevant regulators of autophagy. Most importantly, it provides an explanation for how Th2 cytokines counter autophagy-dependent protection against intracellular pathogens afforded by Th1 responses. The effects of STAT6 on autophagy downstream of IL-4 and IL-13 action may involve a number of pathways, one of which could be linked to the intriguing ability of STAT6 to increase the Bcl-2 and Bcl-X_L antiapoptotic proteins (Wurster et al., 2002). Bcl-2 and Bcl-X_L are now also recognized as being antiautophagic effector proteins (Maiuri et al., 2007; Pattingre et al., 2005). Bcl-2 and Bcl-X_L interact directly (Pattingre et al., 2005) through the recently recognized BH3 domain (Maiuri et al., 2007) within Beclin 1, the key activator of autophagy. Because Bcl-2 and Bcl-X_L association with Beclin 1 blocks autophagy induction (Maiuri et al., 2007), and the rapid induction of STAT6 downstream of IL-4 and IL-13 stimulation may increase Bcl-X_L availability (Wurster et al., 2002), the IL-4-IL-13-STAT6-dependent inhibition of autophagy observed in our work could reflect these recently uncovered relationships.

In this work, we did not specifically study alternative activation of macrophages by IL-4 and IL-13 (Gordon, 2003) but instead focused on the effects of short-term exposure (2 hr) or treatment in combination with IFN-γ. Treatment of macrophages with IL-4 or IL-13 for 6 days has been shown to enhance fluid-phase pinocytosis and mannose receptor-mediated endocytosis through activation of phosphatidylinositol 3 kinase; both cytokines increase tubular-vesicle formation at pericentriolar sites under the plasma membrane, concurrent with decreased particle sorting to the lysosomes (Montaner et al., 1999). Although these previous observations could explain the inhibitory effects on autophagolysosome formation, IL-4 and IL-13 had no effect on BCG phagosome maturation in the absence of starvation- or IFN-γ-induced autophagy, demonstrating that the effects observed in our experiments are autophagy specific. A study on the effects of alternative activation of macrophages with Th2 cytokines, in the context of autophagy, may be difficult to address because prolonged autophagy can lead to type-II-programmed cell death, and most cells will downregulate this process to ensure their survival (Pattingre et al., 2005). Nevertheless, alternative activation of macrophages with IL-4 in vitro delays and inhibits antibacterial responses to intracellular *M. tuberculosis* (Kahnert et al., 2006) by an unknown mechanism that may be related to the processes reported here.

The concentrations of IL-4 and IL-13 used in our titration experiments fall well within the in vivo and ex vivo tissue levels of these cytokines under various conditions (Aspord et al., 2007; Huang et al., 1995; Zhu et al., 1999), including studies with mycobacterial antigens (Chensue et al., 1997). Because local concentrations in the vicinity of T cells secreting IL-4 or IL-13 in vivo can be much higher, our range included 10-fold increments. Although lymphocytes in the lungs of patients with pulmonary tuberculosis typically have a Th1 phenotype, secreting IFN-γ and IL-12 (Mazzarella et al., 2003; Taha et al., 1997), little is known of the phenotype or cytokine-secretion profiles of infected macrophages and dendritic cells within the granuloma. However in vitro studies, in human peripheral blood monocyte-derived macrophages have demonstrated that virulent strains of tuberculosis preferentially upregulate Th2 cytokines (IL-4, IL-5, IL-10, and IL-13), whereas nonvirulent strains induce Th1 cytokines and chemokines (Freeman et al., 2006; Manca et al., 2004; Sun et al., 2006). Moreover, Th2 lymphocyte subsets have been observed in lung tissue from patients with cavitary tuberculosis, compared with Th1 subsets in noncavitary disease (Mazzarella et al., 2003). This correlates with higher production of IL-4 in the periphery of patients with cavitary disease (van Crevel et al., 2000). Thus, it is possible that in an environment predominantly influenced by Th1 cytokines, paracrine, and autocrine secretion of IL-4 and IL-13 could specifically impair the response of infected macrophages to IFN-γ. Moreover, the peripheral response of patients with active tuberculosis disease may not necessarily mirror the response in the lungs. Up to 15% of patients with active pulmonary tuberculosis display specific anergy to tuberculin (Bloom and Small, 1998), and this has been linked with increased ratios of IL-4- and IL-10-positive lymphocytes and decreased ratios of IL-12 (Bailko et al., 1998). Some authors have reported a Th1 response in the periphery of patients with mild pulmonary tuberculosis and Th2 responses in anergic patients and those with more severe disease (Boussiotis et al., 2000; Dlugovitzky et al., 1997). Clinical observations in tuberculin-reactive patients have also demonstrated that Th1 responses, although high in the granuloma, are often paradoxically depressed in peripheral blood lymphocytes in response to mycobacterial antigens (Jo et al., 2003). In this context, it would be interesting to determine whether the cytokine profile in the periphery could influence the autophagic status of newly recruited monocytes in the granuloma. Thus, the balance of Th1 and Th2 cytokines is clearly critical in the host response to *M. tuberculosis*. On the basis of the Th1 autophagy-promoting and Th2 autophagy-dampening roles, we can now shed more light on this dichotomy.

In summary, we have shown that inhibition of autophagy by IL-4 and IL-13 impairs the ability of macrophages to kill intracellular mycobacteria. The Th2 cytokines inhibit physiologically and immunologically induced autophagy in murine and human monocyte and macrophage cells. Moreover, these cytokines inhibit autophagy-dependent maturation of the mycobacterial phagosome and subsequent killing of mycobacteria. In addition to furthering our understanding of the modulation of autophagy in macrophages, these data present evidence for a novel role of

Th1-Th2 polarization, modulating autophagy as an immune effector mechanism in opposing ways. Our studies offer an explanation as to why Th2 cytokines are incompatible with protection against certain intracellular pathogens, including *M. tuberculosis*. By antagonizing autophagy, IL-4 and IL-13 inhibit Th1-dependent protection against mycobacteria. Because Th1 and Th2 cytokines show antagonistic effects on autophagy as an immune effector, the regulation of autophagy may represent a critical aspect of Th1-Th2 polarization in the host response to intracellular pathogens.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

Unless otherwise stated, reagents were from Sigma. Recombinant murine and human IL-4 and IL-13 were purchased from R & D Systems. Rabbit polyclonal antibody against CD63 was from Santa Cruz Biotechnology, rabbit polyclonal antibody against LC3 was from T. Ueno and E. Kominami. pEGFP-LC3 was from T. Yoshimori and GFP-LC3 transgenic mice were from N. Mizushima. Tdtomato-LC3 was from G. Bjorkoy.

Cells and Bacterial Cultures

Murine RAW264.7 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and L-glutamine (full-nutrient medium). The human monoblastic cell lines, U937 and THP-1, were maintained in RPMI-1640 (Invitrogen) with 10% FBS, L-glutamine, and HEPES. Before use, U937 and THP-1 cells were differentiated with PMA (100 nM) for 24–72 hr. *Mycobacterium bovis* BCG was grown in Middlebrook 7H9 broth with 0.5% Tween, 0.2% glycerol, and albumin-dextrose-catalase supplement (BD Diagnostics, Franklin Lakes, NJ, USA). Primary human monocyte-derived macrophages (MDMs) were isolated and cultured from buffy coats by density gradient centrifugation on Ficoll-Paque Plus (GE Healthcare). PBMCs were allowed to adhere to gelatin-coated plates for at least 1 hr, washed so that nonadherent cells could be removed, and cultured in RPMI-1640 with 10% human human AB serum for 24 hr. After this time, cells were lifted, counted, and plated on tissue-culture plastic or glass coverslips for 5–7 days. Bone marrow-derived macrophages (BMMs) were isolated and cultured as described.

Flow Cytometry

After blocking with seroblock (AbD Serotec), RAW264.7 cells were stained with rat anti-mouse CD124 (IL-4Rα)-PE (BD) for 30 min on ice. U937 and THP-1 cells were blocked with human serum (10%) and stained first with anti-human CD124 for 30 min on ice, then with anti-mouse IgG1-PE (BD) for 30 min on ice. The cells were analyzed on a BD FACScaliber, and data were processed with CellQuest software.

Induction of Autophagy

Autophagy was induced either by amino acid starvation, in which cells were incubated for 2 hr in Earle's balanced salt solution (EBSS) or by treatment with IFN-γ (200 U/ml) for 24 hr. (Gutierrez et al., 2004).

Macrophage Transfection

RAW264.7 and U937 cells were transfected by nucleoporation as previously described (Chua and Deretic, 2004). In brief, cells were harvested after 2–3 days in culture and resuspended in 100 μl of the appropriate electroporation buffer (Amaxa Biosystems) with 5–10 μg plasmid DNA or 1.5 μg siGENOME SMARTpool siRNA or siCONTROL nontargeting siRNA (Dharmacon) and nucleofected with Amaxa Nucleofector apparatus. After electroporation, cells were cultured in full-nutrient medium for 24 hr before use.

Fluorescence Confocal Microscopy

Cells were cultured on glass coverslips, fixed in 2% paraformaldehyde for 20 min at room temperature, permeabilized, and blocked in PBS with 0.5% Tween 20, 1% BSA, and 2% goat serum for 30 min at room temperature. Cells were incubated with primary antibody for 1 hr and then with secondary antibody for an additional 1 hr. Alternatively, cells were incubated with Lysotracker Red DND-99 (LT, Invitrogen) for 2 hr prior to and during incubation with mycobacteria. For labeling of acidic, lipid-rich vacuoles, cells were incubated with monodansylcadaverine (MDC, 50 μM) for 15 min prior to fixing. Bone marrow macrophages expressing GFP-LC3 were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.2% saponin for 5 min, blocked for 30 min, and incubated with rabbit polyclonal antibodies against GFP (Abcam) overnight for visualization of the GFP-LC3 fusion protein (GFP fluorescence in BMMs isolated from these transgenic mice is sporadic, and GFP antibody is routinely used for visualization of GFP-LC3.). Slides were incubated with a secondary anti-mouse fluorescein isothiocyanate-conjugated antibody. Coverslips were mounted onto glass slides with Permafluor Aqueous mounting medium (Thermo Scientific, Waltham, MA, USA) and analyzed on a Zeiss LSM510 META laser-scanning confocal microscope.

Immunoblotting

Cells were washed in PBS and lysed with buffer containing 10 mM Tris HCl, 150 mM NaCl, 0.5% deoxycholate, 2 mM EDTA, 2% NP-40, 1 mM PMSF, and protease inhibitor cocktail (Roche Applied Science). A total of 50 μg of protein was loaded and separated on a 12% or 15% SDS-polyacrylamide gel (BioRad) and transferred to nitrocellulose. The membrane was blocked in 5% milk or 3% BSA in PBS/Tween 20 (0.1%) and probed with antibodies overnight at 4°C. After washing with PBS/Tween, the blot was probed with HRP-conjugated secondary antibody for 1 hr at room temperature. Staining was revealed with SuperSignal West Dura Extended Duration Substrate (Pierce).

Phagolysosome Maturation and Mycobacterial-Survival assays

For phagocytosis of mycobacteria, macrophages were incubated with Texas red-labeled BCG, GFP-BCG, or *Mycobacterium tuberculosis* strain H37Rv for 15–30 min, washed, and incubated for an additional 2 hr, in the presence of starvation media in the presence or absence of cytokines as indicated. After staining the fixed cells for the lysosomal marker CD63 (LAMP3), confocal microscopy was used so that the percentage of CD63-positive mycobacterial phagosomes from at least 100 cells could be recorded. For mycobacterial-survival assays, RAW cells or MDM were infected with BCG or H37Rv for 1 hr, washed, and incubated for an additional 2 hr with starvation media and cytokines as indicated. Cells were washed with PBS and lysed with distilled water. Serial dilutions of lysates were made, and 5 ml aliquots were inoculated on Middlebrook 7H10 agar plates supplemented with oleic acid-albumin-dextrose-catalase (BD). Plates were sealed and incubated for 2 weeks at 37°C, and colonies were counted from dilutions yielding 10–60 visible colonies. Data are expressed as colony-forming units per ml (cfu/ml).

Statistical Analysis

Data are presented as means ± SEM (approximately three independent experiments); p values (Student's t test; two-tailed) are relative to the control, unless otherwise specified.

Supplemental Data

Nine figures and one movie are available at http://www.immunity.com/cgi/content/full/27/3/505/DC1/.

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Note Added in Proof

The following errors as they appeared in the original publication of this paper (on September 20, 2007) have now been corrected online: (1) Figure S2 did not contain panel D; (2) Figures S5, S6, and S7 were not in order and were incorrectly cited in the main text (detailed below in points 3–6); (3) Figure S5 as it was published originally online should have been Figure S7; (4) Figure S6 should have been Figure S5; (5) Figure S7 should have been Figure S6; (6) on page 511 of the original main text, all citations for Figure S7 were incorrectly cited as Figure S5; (7) and in Figure 3 of the main text, panels A and B were incorrectly labeled-all instances of the label "Starve" should have read "IFN-γ." See the Correction in the October 26th issue for further details.

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