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Selective modulation of autophagy, innate immunity and adaptive immunity by small molecules

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Abstract

Autophagy is an evolutionarily conserved catabolic process that directs cytoplasmic proteins, organelles and microbes to lysosomes for degradation. Autophagy acts at the intersection of pathways involved in cellular stress, host defense, and modulation of inflammatory and immune responses; however, the details of how the autophagy network intersects with these processes remain largely undefined. Given the role of autophagy in several human diseases, it is important to determine the extent to which modulators of autophagy also modify inflammatory or immune pathways, and whether it is possible to modulate a subset of these pathways selectively. Here, we identify small-molecule inducers of basal autophagy (including several FDA-approved drugs) and characterize their effects on IL-1 β production, autophagic engulfment and killing of intracellular bacteria, and development of T_{reg}, T_H17, and T_H1 subsets from naïve T cells. Autophagy inducers with distinct, selective activity profiles were identified that reveal the functional architecture of connections between autophagy, and innate and adaptive immunity. In macrophages from mice bearing a conditional deletion of the essential autophagy gene *Atg16L1*, the small molecules inhibit IL-1 β production to varying degrees suggesting that individual compounds may possess both autophagy-dependent and autophagy-independent activity on immune pathways. The small molecule autophagy inducers constitute useful probes to test the contributions of autophagy-related pathways in diseases marked by impaired autophagy or elevated IL-1 β , and to test novel therapeutic hypotheses.

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Supporting Information Available

Additional methods, tables and figures may be found in Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved catabolic process that directs cytoplasmic proteins and organelles to the lysosome, where they are degraded for subsequent recycling. Autophagy plays important roles in normal development and differentiation, and helps maintain homeostasis in the face of nutrient and other cellular stresses. Autophagy has also been implicated in a wide variety of diseases, including cancer, neurodegenerative disease, susceptibility to pathogens, and inflammatory bowel disease.(1) Consistent with this broad disease involvement, autophagy (or individual autophagy proteins) exerts functional connections to many other cell processes, including host responses to intracellular bacteria, inflammatory signaling, and innate and adaptive immunity.(2, 3) For instance, autophagy leads to the killing of intracytoplasmic *group A Streptococcus* in an Atg5-dependent fashion,(4) and induction of autophagy decreases intracellular survival of *M. tuberculosis*. (5) Individual autophagy proteins (such as Atg5) can also mediate host defense against intracellular pathogens (such as *L. Monocytogenes* and the protozoan *T. Gondii*) in an autophagy-independent manner, highlighting that autophagy proteins may contribute to host defense through multiple mechanisms.(6)

Autophagy also modulates inflammation and adaptive immunity. In myeloid cells, activation of the NLRP3 (NOD-like receptor family pyrin containing 3) inflammasome complex leads to caspase-1-mediated release of cytokines such as IL-1 β , IL-18, and IL-33. Murine macrophages lacking the essential autophagy gene *Atg16L1* produce increased amounts of IL-1 β upon stimulation with the TLR4 ligand lipopolysaccharide (LPS).(7) Autophagy proteins also contribute to antigen presentation to CD4 and CD8 T cells. For instance, in dendritic cells, basal autophagy, as well as autophagy induced by the intracellular pattern recognition receptor NOD2, stimulates MHC class II presentation to CD4+ T cells, and antigen-specific CD4+ T cell proliferation.(8, 9)

The pathways that mediate autophagy (and connect autophagy with inflammation and innate and adaptive immunity) likely involve a network of hundreds of proteins. Multiple steps of autophagy (including nucleation and elongation of an isolation membrane, fusion of the edges of the isolation membrane to form an autophagosome containing the cytoplasmic cargo, and fusion of the autophagosome with a lysosome to form an autolysosome) are mediated by multi-protein complexes and subject to regulatory inputs. A proteomic study of basal autophagy identified a network of 751 protein/protein interactions among over 400 proteins.(10) Genome-wide siRNA screens for modulators of basal or viral autophagy also support the notion of an autophagy network involving hundreds of proteins.(11, 12)

To complement these genetic screens, and to better define the functional connections associated with autophagy, host response to pathogens, inflammation, and immunity, we undertook a chemical biology approach using FDA-approved drugs and bioactive compounds with predominantly known targets and mechanisms. We identified small molecules that induce basal autophagic flux, using image-based quantitation of autophagosomes and autolysosomes. Autophagic inducers were further characterized for their ability to reduce production of IL-1 β by bone marrow-derived macrophages; enhance localization of the intracellular bacterium *S. Typhimurium* to autophagosomes in a manner that correlates with subsequent killing; and modulate differentiation of naive T-cells into T_{reg}, T_H17 and T_H1 subsets. We find that compounds partition themselves into a variety of classes with distinct, selective activity profiles across these assays. Small molecules were further examined in macrophages from mice bearing a conditional knockout of the essential autophagy gene *Atg16L1*, where their ability to inhibit IL-1 β suggests their potential utility in a variety of disease models.

RESULTS AND DISCUSSION

Small-molecule screen to identify inducers of basal autophagy

Our goal was to identify small molecules that induced basal autophagy, and to partition them into different subcategories of activity with respect to inflammation, and innate and adaptive immunity (Figure 1a). We undertook an initial screen of ~3,700 known bioactive compounds, including FDA-approved drugs and tool compounds with known mechanisms.

We used a dual fluorescent reporter for the essential autophagosome membrane protein LC3 (mCherry-GFP-LC3) in our screen.(13, 14) This assay can better distinguish between small molecules that increase GFP+ punctae via increased autophagic flux (our desired hits) from those that impair the ability of autophagy to proceed to completion (for example, by impairing autophagosome maturation into autolysosomes); because the acidic lysosomal pH quenches the GFP signal, autolysosomes may appear as mCherry+ GFP- punctae.

A HeLa cell line stably expressing mCherry-GFP-LC3 was treated with 3,713 small molecules in duplicate for eight hours in the presence of nutrient-rich media, and then analyzed by fluorescence microscopy. We performed automated image analysis using the open source software CellProfiler,(15) based on four 20× fields imaged per well; for each image, we captured three fluorescence channels corresponding to Hoechst nuclear signal, mCherry, and GFP (Figure 1b). For the mCherry and GFP channels, we quantitated punctae-per-cell as a Z-score, relative to the distribution of punctae-per-cell observed in the presence of DMSO alone. Specifically, we scored each well based on the fraction of cells containing punctae in numbers that were at least three standard deviations greater than the mean number of punctae-per-cell observed in DMSO-treated wells.

Because we assessed LC3-associated punctae at a single time point, compounds that induce autophagy could result in increased numbers of autophagosomes, but also potentially decreased autophagosomes (e.g., if autophagosome maturation to autophagolysosomes is rapid). An increase in autolysosomes (mCherry+ GFP-) is suggestive of bona fide activation of autophagy. However, electron microscopy has confirmed that GFP+ punctae can in fact be autolysosomes, imaged presumably before the GFP is quenched.(12) Because of these subtleties in interpretation of the mCherry-GFP-reporter, we analyzed our screen for different image-based endpoints that could indicate enhanced basal autophagy: compounds that increased or decreased the number of GFP+ punctae per cell (autophagosomes or autolysosomes), or that increased numbers of mCherry+ GFP- autolysosomes per cell (autolysosomes).

Compound set enrichment analysis (CSEA) to identify compound classes that induce basal autophagy

We analyzed our small-molecule screening data using compound set enrichment analysis (CSEA), which evaluates whether a set of functionally or structurally related compounds is statistically enriched among top-scoring compounds in an assay.(16) The output of CSEA is a Normalized Enrichment Score (NES) for each compound set; statistical significance of the enrichment can be evaluated by 1000 random permutations of compound set membership. CSEA is statistically analogous to gene set enrichment analysis (GSEA), which is widely used to detect concordant patterns of increased or decreased gene expression among related genes. By analyzing the performance of an entire set of related compounds in the assay, CSEA can improve the robustness of choosing compounds for follow up studies, compared to choosing “hits” in isolation.

Based on CSEA, we selected 16 compound sets that were top-scoring among the following classes: increased GFP+ punctae (positive NES for GFP+ punctae), decreased GFP+ punctae

(negative NES for GFP+ punctae), or increased mCherry+ GFP- punctae (canonical autolysosomes; positive NES for mCherry+ GFP- punctae). Sets were chosen for follow-up based on one or more CSEA criteria: permutation p-value < 0.05; the same set scored well for both GFP+ punctae and canonical autolysosomes; multiple sets that were structurally or functionally related scored well; or all of the set's compounds scored in the same half (upper or lower) of the ranked list of all compounds (Supplementary Figure S1; Supplementary Table S1, Data File S1).

Representative compounds from these sets were further evaluated in autophagic flux assays, consisting of Western blot analysis to detect the phosphatidylethanolamine-conjugated form of LC3 (LC3-II).⁽¹⁴⁾ Each compound was evaluated in the presence and absence of E64D/pepstatin A (which inhibit lysosomal proteases); compounds that induce autophagic flux would be expected to show a further increase in LC3-II by Western blotting in the presence of E64D/pepstatin A, whereas compounds that inhibit autophagosome maturation (e.g., by inhibiting lysosomes) would not. Twelve out of 16 representative compounds evaluated by Western blot were thus confirmed to induce autophagic flux (Supplementary Figure S2; Supplementary Table S1); their CSEA profiles are shown in Figure 2. Autophagy inducers that increase GFP+ punctae include reserpine (from the set *Rauwolfia* alkaloids, NES 1.46, $p = 0.002$), metergoline (prolactin inhibitors, NES = 1.69, $p = 0.005$), and the phenothiazine thioridazine (phenothiazines with piperidine structure, NES 1.47, $p = 0.064$). Of the compound sets that decrease GFP+ punctae, increased autophagic flux was confirmed for digoxin (digitalis glycosides, NES -2.46 , $p < 0.001$), fludrocortisone (mineralocorticoids, NES -1.68 , $p = 0.003$), noscapine (opium alkaloids, NES -1.39 , $p = 0.089$), and clemastine (antihistamines for systemic use, NES -1.29 , $p = 0.061$). (Figure 2; Supplementary Table S1)

Compounds were also associated with increased mCherry+ GFP- punctae and confirmed to increase autophagic flux, especially digoxin (NES 2.26, $p = 0.004$), and two related phenothiazines, chlorpromazine and thioridazine (phenothiazines with aliphatic or piperidine substituents, respectively; NES 1.88, $p < 0.001$ and NES = 1.29, $p = 0.163$). Compound sets including the digitalis glycosides, phenothiazines, and selected subsets of antihistamines scored well by CSEA according to both GFP+ and autolysosome criteria (Figure 2).

Inhibition of IL-1 β production

We next evaluated compounds confirmed to increase autophagic flux for their ability to inhibit IL-1 β production, a phenotype related to innate immunity and inflammation. Bone marrow-derived macrophages (BMDMs) were primed with IFN- γ , and stimulated with LPS (a TLR4 agonist) and muramyl dipeptide (MDP, a peptidoglycan component of bacterial cell walls that activates the NLRP3 inflammasome).⁽¹⁷⁾ The majority of compounds significantly decreased IL-1 β production; the exceptions were some but not all of the digitalis glycosides (Figure 3). At the Bonferroni threshold for significance of 0.0033 (which is overly stringent because the five digitalis glycosides are not independent), all of the digitalis glycosides, cetirizine, and reserpine did not meet statistical significance. Testing at multiple doses confirmed that reductions in IL-1 β production were observed at a dose (5 μ M) that did not decrease cell viability (Supplementary Figure S3). In general, we observed minimal correlation between small molecules' effects on inhibition of IL-1 β release and reactive oxygen species (ROS) production (Supplementary Figure S4).

When BMDMs are stimulated with ATP (which does not act through Nod2), we observe that several compounds significantly inhibit IL-1 β production in response to both ATP or MDP, whereas others inhibit IL-1 β production only in response to MDP (Supplementary Figure S5). Thus, our autophagy inducers may be partitioned into those that inhibit both

Nod2-dependent and Nod2-independent inflammasome-mediated IL-1 β production, and those that inhibit only Nod2-dependent IL-1 β production.

Bacteria-induced autophagy and killing

To evaluate the impact of our autophagy inducers on host defense mechanisms against intracellular bacteria, we quantitated the effect of compounds on co-localization of GFP-LC3-labelled autophagosomes and DsRed-expressing *S. Typhimurium*. The majority of the compounds showed significant, dose-dependent increases in LC3:*Salmonella* colocalization; in contrast, bromperidol, fludrocortisone, noscapine and cetirizine did not reach statistical significance (Figure 4a, 4b; Supplementary Figure S6). At a Bonferroni-corrected threshold (0.0033), proscillaridin (alone among the digitalis glycosides), thioridazine, chlorpromazine, prochlorperazine, reserpine and clemastine caused statistically significant increases in bacterial autophagy without significantly affecting cell viability (Supplementary Figure S6).

We further tested whether co-localization led to increased *Salmonella* killing, by quantitating surviving bacteria using a bacterial bioluminescence assay. In general, bacterial killing showed an excellent correlation with increased LC3-colocalization. All of the compounds that significantly increased LC3:bacterial colocalization also enhanced bacterial killing (Figure 4c). Of the compounds that failed to significantly increase LC3: bacterial colocalization, bromperidol is the only one that increased bacterial killing (this discrepancy may be due simply to the 5 μ M dose selected for testing, as bromperidol caused a significant increase in LC3:*Salmonella* colocalization at 10 μ M; Supplementary Figure S6). This suggests that the autophagosomes induced by our small molecules have a high probability of maturing into autolysosomes, and killing their bacterial cargo. We confirmed that enhanced bacterial killing was not due to direct bactericidal activity of the small molecules in the absence of host cells (Supplementary Figure S7).

We also noted within-class heterogeneity for the digitalis glycosides (Figure 4C); detailed dose titration shows that digoxin and digoxigenin show more modest effects on bacterial killing, while other glycosides (including lanatoside C, strophanthine, and proscillaridin) show greater effects (Supplementary Figure S8).

T cell differentiation

We next sought to define how the autophagy inducers modulated development of T cell subsets from naïve T cells. Compounds were incubated with naïve wild-type murine T cells in the presence of suboptimal concentrations of cytokines that bias differentiation towards T_{reg}, T_H17 or T_H1 subsets; compound effects on each T cell subset were scored on a normalized effect scale ranging from 0 (percentage observed under control conditions) to 1 (percentage observed under maximal stimulation, or positive control, conditions).

Compounds from multiple functional classes caused dose-dependent changes in different T cell subsets (Figure 5; Supplementary Figure S9a-c). For instance, thioridazine, prochlorperazine and proscillaridin selectively increased T_{reg} development. Digoxin, chlorpromazine and bromperidol caused significant dose-dependent decreases in T_H17 development, with minimal effect on other lineages. Fludrocortisone markedly decreased T_H17 and T_H1 lineages concordantly. Noscapine caused an increase in T_{reg} cells as well as a modest increase in T_H17 cells. We confirmed that these effects all occurred at doses without significant cell toxicity or death. By comparison, none of the compounds tested affected development of the TR1 anti-inflammatory cell population (expressing both IFN- γ and IL-10), providing additional evidence that the observed effects on T cell subsets are not non-specific compound effects (Figure 5).

Small molecule effects on BMDMs from *Atg16L1*^{-/-} mice: IL-1 β production

Our findings that many autophagy inducers can also modulate processes associated with innate and adaptive immunity raise the hypothesis that these small molecules may suppress adverse effects in genetically defined models of autophagy and disease. To address this, we examined IL-1 β production in murine BMDMs bearing a floxed allele of the essential autophagy gene *Atg16L1* (i.e., containing one deleted allele and one floxed allele); in the absence of Cre expression, the BMDMs are heterozygous for *Atg16L1* (analogous to hypomorphous *Atg16L1* function), whereas expression of Cre recombinase results in *Atg16L1*-null BMDMs.

In BMDMs heterozygous for *Atg16L1* deletion, the small-molecule effects on IL-1 β production corresponded closely to those in cells wild type for *Atg16L1*; compounds that inhibited IL-1 β production in wild-type cells (Figure 3) also inhibited in *Atg16L1* heterozygous cells, with a high degree of statistical significance (Figure 6a). Thus, our identified autophagy inducers can still significantly inhibit IL-1 β production in cells haploinsufficient for an essential autophagy gene, *Atg16L1*.

For the compounds that inhibited IL-1 β production in wild-type or *Atg16L1* heterozygous cells, complete deletion of *Atg16L1* causes a marked, statistically significant increase in IL-1 β production in the presence of compound, compared to *Atg16L1* heterozygous cells (Figure 6b). This suggests that the compounds' inhibition of IL-1 β production has a significant component that is dependent either upon the process of autophagy, or on *Atg16L1* itself.

Absolute levels of IL-1 β production in *Atg16L1*-null BMDMs are dramatically increased, both at baseline, and following LPS/MDP stimulation (compared to Cre⁺ control cells bearing a functioning *Atg16L1* allele) (Fig. 6b, c). Upon LPS/MDP stimulation, all of the small molecules that previously inhibited IL-1 β production (with the exception of noscapine) also significantly decreased IL-1 β production in *Atg16L1*^{-/-} cells (Fig. 6c), although to a more modest extent compared to in *Atg16L1* heterozygous cells. To the extent that the compounds show modest inhibition of IL-1 β production in the absence of *Atg16L1*, this suggests that certain compounds possess varying degrees of autophagy-independent effects on IL-1 β production as well. A two-way ANOVA for IL-1 β production shows a significant effect of *Atg16L1* genotype ($p < 1 \times 10^{-04}$), a significant effect of the small molecules ($p < 1 \times 10^{-04}$), and a significant interaction between *Atg16L1* genotype and the small molecules ($p < 1 \times 10^{-04}$).

Because the conditional Cre expression does not delete *Atg16L1* in epithelial cells, we used siRNA knockdown to decrease *Atg16L1* expression in HeLa cells by approximately 80% (Supp. Fig. S10a). When anti-*Salmonella* autophagy was examined in these *Atg16L1* knockdown cells, only digoxin and strophantine showed significant decreases in *Salmonella* viability (Supp. Fig. S10b). This implies that the majority of our compounds enhance anti-bacterial autophagy in an autophagy-dependent manner. However, because the cells retain significant *Atg16L1* expression, we cannot exclude the possibility that even digoxin and strophantine also require autophagy for their effects; this question can be further examined in *Atg16L1*-null epithelial cells.

Selective small molecule activity profiles reveal architecture of autophagy and inflammatory and immune pathways—The data presented here improve our understanding of the architecture underlying connections between autophagy and inflammatory and immune-related pathways. We have partitioned small molecules that induce basal autophagy into several distinct functional classes based on their effects on IL-1 β production, intracellular bacterial autophagy and killing, and T cell development.

(Figure 7a) These patterns of small molecule activity profiles define potential genetic entry points into autophagy and related pathways, corresponding to nodes of intersection between autophagy, inflammation and innate and adaptive immunity (Figure 7b). By using Compound Set Enrichment Analysis (CSEA) to prioritize compounds that induce basal autophagy, we identified compounds not previously known to induce autophagy, compounds previously annotated as autophagy inhibitors rather than inducers, as well as previously identified inducers. For instance, chlorpromazine and fludrocortisone, both confirmed to increase autophagic flux here, were previously characterized as autophagy inhibitors based on GFP+ punctae.(18) To our knowledge, bromperidol has not been previously associated with autophagy.

Small-molecule modulators of autophagy (18-24) have largely been studied in individual biological contexts (e.g., cytokine production, pathogen killing, or degradation of intracellular protein aggregates). The extent to which genetic or small molecule perturbation could coordinately or selectively modulate multiple processes was not obvious *a priori*. Our data show convincingly that many of these functional connections appear to be amenable to specific, selective modulation by small molecules. Further studies will help clarify the mechanisms by which our small molecules modulate basal autophagy, IL-1 β production, bacterial autophagy or T cell differentiation, all of which are subject to multiple regulatory inputs.

For instance, one cluster of activities comprises the cardiac glycosides, exemplified by the widely used drug digoxin, and reserpine (an antipsychotic and antihypertensive drug) (Compound structures are shown in Supplementary Figure S11). Despite acting primarily through distinct protein targets (the Na⁺-K⁺ ATPase and vesicular monoamine transporter, respectively) these compounds do not inhibit IL-1 β production, yet significantly enhance autophagy and killing of *Salmonella*. Another cluster includes drugs from different structural groups that share activity against dopamine and serotonin receptors (25): thioridazine, chlorpromazine, bromperidol and metergoline (an ergoline that is also used to treat hyperprolactinemia). (Figure 7a) These compounds show autophagy-dependent inhibition of IL-1 β production, and enhanced *Salmonella*-induced autophagy and killing. Other compounds inhibited IL-1 β production with no effect on *Salmonella* autophagy or killing (noscapine, fludrocortisone). Overall, we note several examples of small molecules that share autophagy-related activity profiles despite acting through distinct targets, suggesting either a shared secondary target, or distinct targets that occupy analogous functional roles (or network connections) in autophagy regulation.

Deletion of the essential autophagy gene *Atg16L1* causes a significant diminution in the ability of most of these compounds to inhibit IL-1 β production, but still reveals modest inhibitory effects of the compounds. This suggests that, while many of our compounds act through mechanisms dependent on the process of autophagy (or the action of *Atg16L1*), we cannot exclude the possibility that some of these phenotypes are mediated by small molecules acting on one or more protein targets independent of autophagy. For instance, digoxin has been reported to inhibit T_H17 differentiation by binding to and inhibiting the transcriptional activity of the retinoic acid-related orphan nuclear receptor γ t (ROR γ t).(26). Furthermore, phenothiazines inhibit growth of *M. tuberculosis*, attributed potentially to inhibition of calmodulin (27) or PI3K/Akt/mTor (28) signaling. While fludrocortisone possesses only minimal glucocorticoid activity, it is possible that the ability of glucocorticoids such as dexamethasone to inhibit IL-1 β expression through post-transcriptional mechanisms (29) could contribute to autophagy-independent effects. Future studies will be necessary to systematically determine the autophagy-independent effects of our compounds on the T cell differentiation pathway as well as on intracellular *Salmonella* killing.

Small molecule activity profiles suggest therapeutic hypotheses—Identifying compounds that selectively modulate basal autophagy, IL-1 β production, as well as additional autophagy-related inflammatory or immune processes constitutes a proof-of-concept for the future development of therapeutics that exploit these pathways. For instance, Crohn's disease (a form of inflammatory bowel disease) susceptibility alleles have been found in essential autophagy genes such as *ATG16L1* and *IRGM*, the bacterial intracellular pattern recognition receptor NOD2 (which can trigger autophagy of intracellular bacteria), and the inflammasome component NLRP3.(30, 31) RNAi knockdown of *ATG16L1* impairs anti-bacterial autophagy.(30, 32) Elevated levels of IL-1 β have been reported in mucosal biopsy specimens from IBD patients,(33-35) and from macrophages from chimeric *Atg16L1* mutant mice that also develop a severe experimental colitis.(7) Crohn's disease is also associated with increased T_H17 and decreased T_{reg} activity.(36) Increased IL-1 β signaling also underlies a group of autoinflammatory syndromes, many of which respond clinically to IL-1 β blockade.(37, 38) These include monogenic conditions such as cryopyrin associated periodic syndrome, as well as genetically complex diseases such as systemic juvenile idiopathic rheumatoid arthritis and Still's disease.

These reports, combined with our data, provide a strong rationale for further evaluation of our FDA-approved autophagy inducers in disease models of autophagy deficiency or IL-1 β excess. Several of the FDA-approved drugs identified here demonstrate a constellation of potentially beneficial effects for controlling pathogen infection, or auto-immune or auto-inflammatory diseases: inducing autophagy, especially of intracellular bacteria; inhibiting IL-1 β production; decreasing T_H17 differentiation; and increasing T_{reg} differentiation. For instance, several small molecules all inhibit IL-1 β production and enhance killing of intracellular bacteria; of these, bromperidol also decreases T_H17 differentiation, thioridazine and prochlorperazine enhance T_{reg} differentiation, and metergoline exerts minimal effects on T cell development. The cardiac glycoside digoxin has favorable effects on bacterial autophagy and killing, and downregulates T_H17 differentiation. Many of our autophagy inducers can inhibit IL-1 β production in cells haploinsufficient or even null for *Atg16L1*. Furthermore, there is an unmet need for therapies for auto-inflammatory diseases such as Crohn's disease that are not associated with opportunistic infections, and broad-based suppression of immune function. The autophagy inducers identified here are approved for a variety of psychiatric, cardiac, endocrine, or allergic indications, and none are associated with generalized immunodeficiency. More generally, these compounds may inform therapeutic development of antibacterial agents, as well as drugs for auto-immune or auto-inflammatory diseases.

In addition to therapeutic trials, our compound classes with distinct activities can clarify the mechanistic contributions of different autophagy-related pathways on cellular phenotypes and mouse disease models. It will be informative to integrate data from chemical biology studies (including this study) with systematic RNAi data for the same inflammatory and immune assays described here to address these questions.(24, 39)

METHODS

Screen for inducers of basal autophagy

HeLa Cells stably expressing mCherry-GFP-LC3 (8000 per well) were plated in Matrical 96-well glass bottom plates overnight in Iscove's Modified Dulbecco's Medium (IMDM) media. Compounds were pinned the following morning (in duplicate) using the CyBIO CyBi Well Vario (96-well pintool). Cells were treated for 8 hours, washed 3X in PBS, fixed in 4% paraformaldehyde (PFA) for 15 minutes, washed 3X in PBS, and stained with Hoechst dye for 15 minutes. Imaging (Molecular Devices ImageXpress Micro) was performed the

following day, with four 20× images taken with DAPI/FITC/TRITC filters (at fixed exposures and brightness throughout).

Data analysis used a CellProfiler module that calculates number of punctae per individual cell across an image. We calculated GFP+ punctae per cell, and mCherry+ GFP- punctae per cell (canonical autolysosomes). For each of these punctae species, we first calculated the average and standard deviation (SD) of punctae per cell for ~160 wells treated only with DMSO. We defined a cutoff number of punctae per cell as the mean + 3 SD punctae for DMSO wells. For each compound (and each of the GFP+ and mCherry+ GFP- punctae species), we calculated the percent of cells with punctae exceeding the cutoff value. Z-Scores were then calculated for each compound and for each punctae species ($Z = (\text{compound mean} - \text{DMSO mean})/\text{DMSO SD}$).

Compound Set Enrichment Analysis (CSEA) of basal autophagy screen

CSEA was performed as described previously (16) to detect three types of enrichment: (i) increased GFP+ punctae per cell (i.e., positive enrichment within the GFP+ punctae distribution); (ii) decreased GFP+ punctae per cell (i.e., negative enrichment within the GFP+ punctae distribution); (iii) increased mCherry+ GFP- punctae per cell.

IL-1 β assay in bone marrow-derived macrophages (BMDMs)

Bone marrow cells from femurs and tibia of C57 BL/6, Atg16L1^{KO/wt} × Tie2-cre, and Atg16L1^{KO/f} × Tie2-cre mice were isolated and cultured at 37°C in RPMI 1640 containing 10% FBS, 100U/ml penicillin/streptomycin, and 25ng/ml M-CSF (Peprotech). On Day 5, 1.5×10⁵ BMDMs/well were plated in 96-well plates (Corning, 3904) and all conditions (including non-stimulated cells) were treated with 100ng/ml IFN γ for 16h. Stimulated BMDMs were then treated with 2ng/ml LPS and 10 μ g/ml MDP for 24h. IFN γ and LPS/MDP treatments were performed in the presence of compound. IL1- β release was measured from cell supernatants by ELISA (BD Biosciences, 559603). Mouse maintenance and cell isolation was performed under protocols approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (SRAC), in conformance with the NIH Guide for the Care and Use of Laboratory Animals.

LC3: *Salmonella* co-localization assay

Bacterial autophagy assays were performed as described, with minor modifications.(30, 32) Details are in Supporting Information.

Bioluminescent *Salmonella* replication assay

1×10⁴ HeLa cells/well were plated in 50 μ l antibiotic-free IMDM in 96-well plates (Corning, 3904) and incubated at 37°C. *S. typhimurium* expressing the *Photobacterium luminescens lux* operon (Xen26, PerkinElmer, 119230) were grown in Luria Broth (LB) with 30 μ g/mL kanamycin for 12-16 hours then diluted (1:30) into fresh LB + kanamycin and incubated for an additional 4 h. The bacterial culture was subsequently diluted (1:40) in antibiotic-free IMDM, and 100 μ l/well was added to HeLa cells. Following infection (37°C for 30 minutes), cells were washed with IMDM containing 20 μ g/ mL gentamicin and incubated with IMDM containing compound and gentamicin. Luminescence was measured with a BioTek Synergy H4 plate reader.

T cell differentiation

Balb/c mice (Jackson Laboratories) were euthanized and splenic CD4⁺CD62L⁺ T cells were magnetically sorted (Miltenyi Biotec, USA) to >95% purity and cultured in DMEM media on plates pre-coated with anti-CD3 and anti-CD28 antibodies (clones 145-2C11 and 37.51,

BioXCell, USA). Media was supplemented with neutralizing antibodies (BioXCell, USA) at 2 μ g/ml as follows: all T_{reg} and T_H17 conditions, anti-IL-4 (clone 11B11), anti-IL-12 (clone C17.8) and anti-IFN γ (clone XMG1.2); all T_H1 conditions, anti-IL-4. Media was also supplemented with cytokines (PeproTech, USA) as follows: T_{reg}^{low} and T_{reg}^{high}, TGF β (2ng/ml and 10ng/ml, respectively); T_H1^{low} and T_H1^{high}, IL-12 (0.05ng/ml and 10ng/ml, respectively); T_H17^{low}, TGF β (0.5ng/ml) and IL-6 (5ng/ml); T_H17^{high}, TGF β (0.5ng/ml), IL-6 (20ng/ml) and IL-1 β (20ng/ml). Small molecules were added at day 0. Cultures were fed with DMEM + IL-2 (10ng/ml, PeproTech) containing small molecules at day 2, split 1:2 into DMEM + IL-2 at day 3 and analyzed at day 4. 5 hours prior to analysis, T_H1 and T_H17 cultures were restimulated using Golgistop (BD Biosciences, USA), PMA and ionomycin (50 and 500 ng/ml respectively, Sigma Aldrich, USA). Flow cytometry quantitation of T cell subsets is described in Supporting Information.

Sytox green assay

(Invitrogen) was performed according to manufacturer instructions.

Statistical analysis

Compound effects were compared with DMSO using two-way student's t-test; Bonferroni corrections for multiple hypothesis testing were also applied. Two-way ANOVA (GraphPad Prism 5) was used to evaluate the combined effects of *Atg16L1* genotype and compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

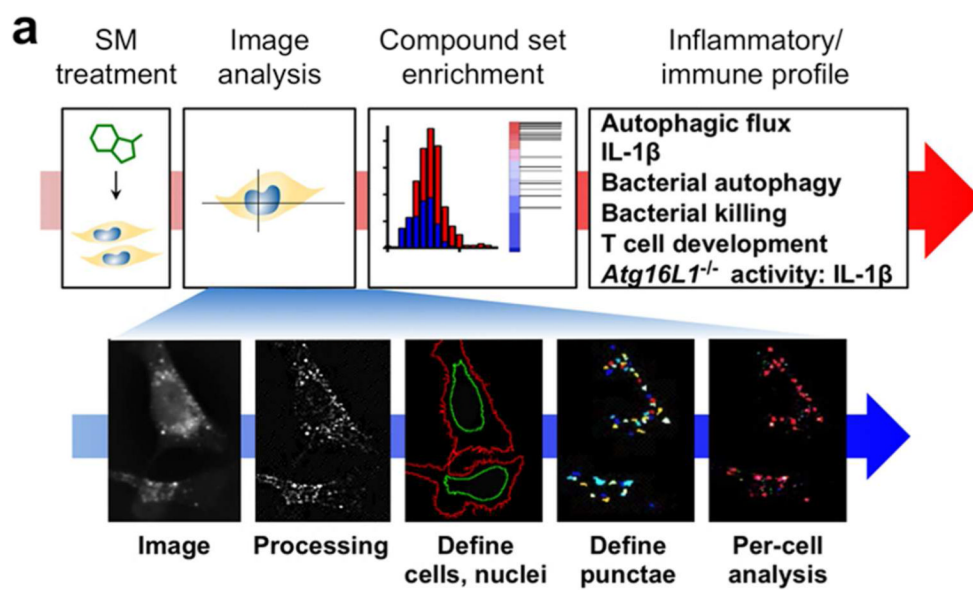
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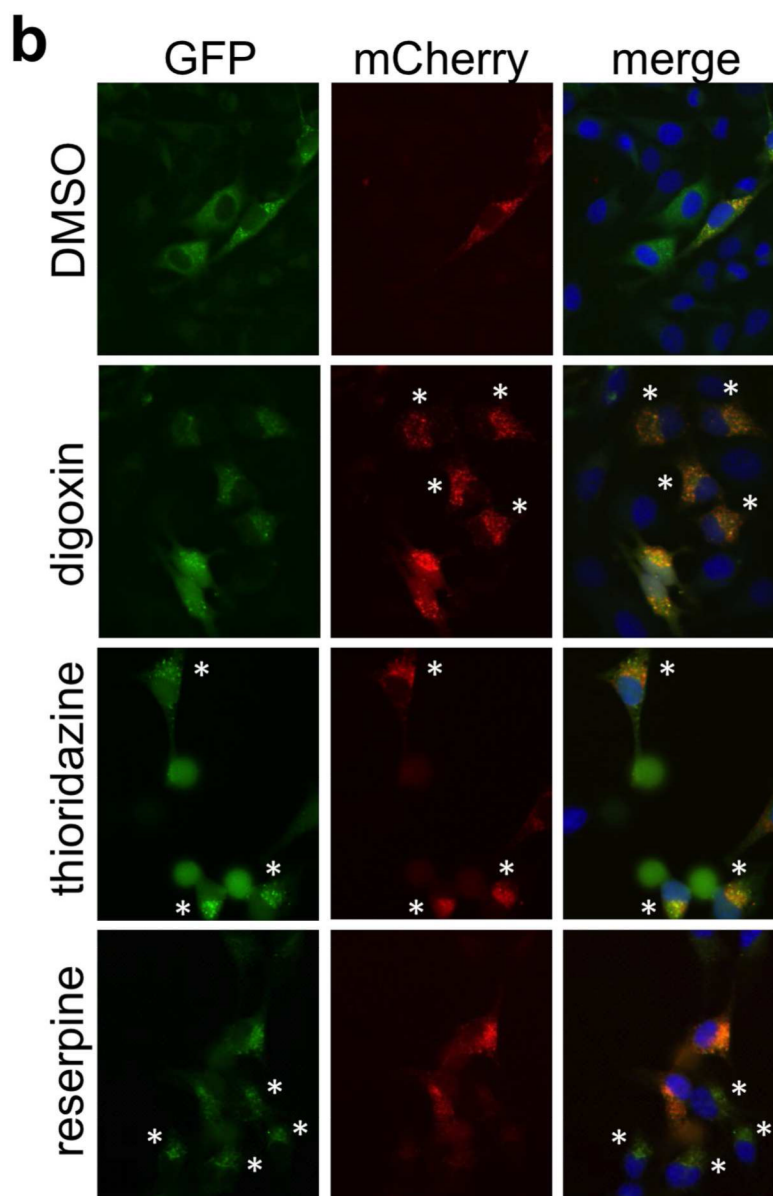


Figure 1. Overview of autophagy screen and inflammatory and immune assays. **a.** Summary of initial small molecule screen, Compound Set Enrichment Analysis, and associated inflammatory and immune assays. **b.** Representative images from small molecule screen. DMSO wells show occasional cells with punctae but also homogeneous cytoplasmic LC3 signal, whereas other compounds show increases in GFP+ punctae, mCherry+ punctae, or both. Cells that exemplify autophagosome or autolysosome enrichment patterns typical of the small molecule class (see Figure 2) are marked by asterisks. (Note images do not correspond directly to CSEA analysis, which analyzed GFP+ and mCherry+ GFP- punctae.)

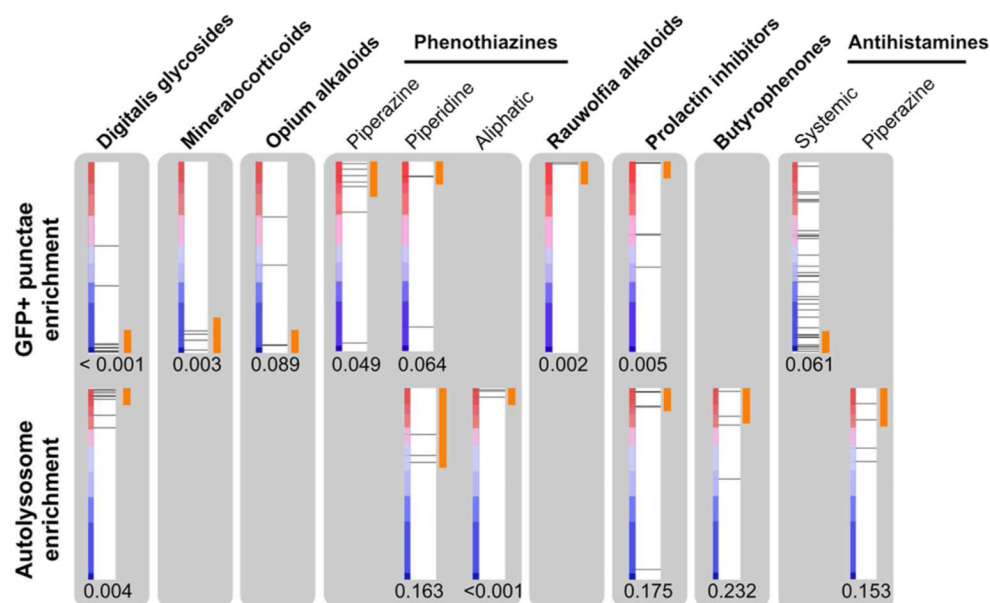


Figure 2. CSEA-identified compound sets enriched for enhancers of basal autophagy. Each red-blue bar depicts the list of screened compounds, ranked according to their score for GFP+ punctae (top) or mCherry+ GFP- autolysosomes (bottom). Horizontal lines show the position within the ranked list of a member of a compound set. Orange segments denote the set members that contributed to enrichment by the CSEA algorithm. The permutation p-value for enrichment is shown below each bar.

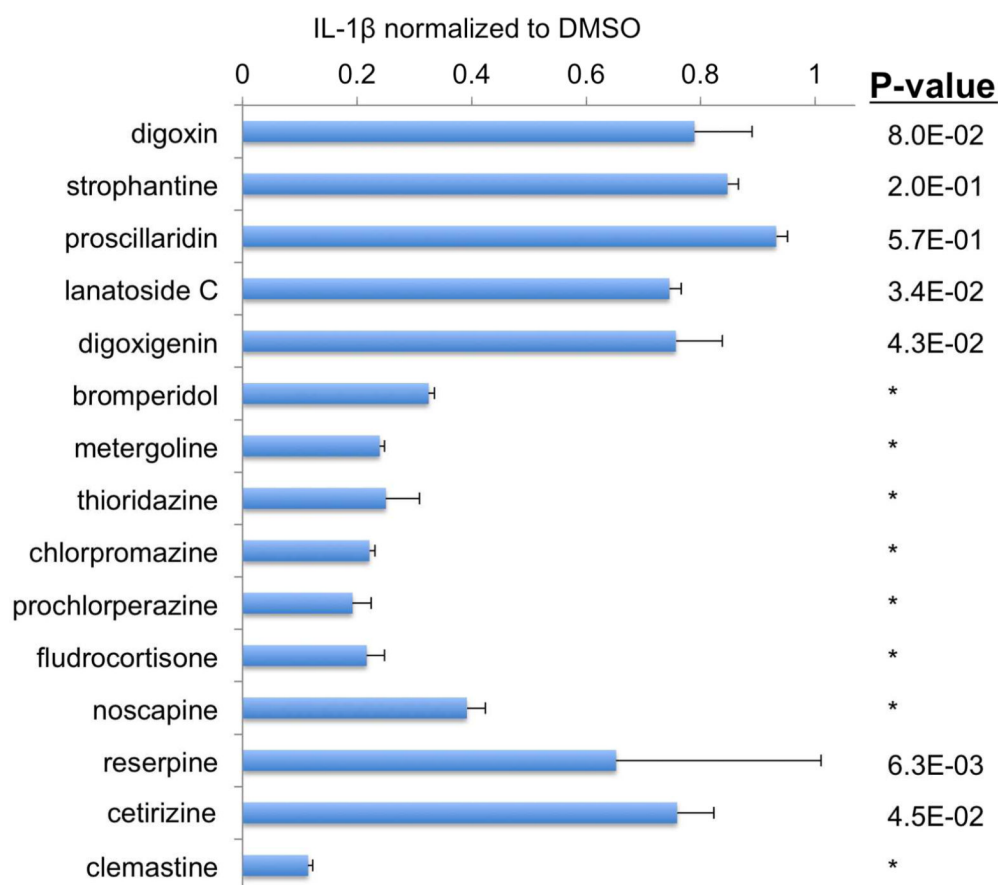


Figure 3. Effects of small-molecule enhancers of autophagy on IL-1 β production from BMDMs. Compounds were all tested at 5 μ M, and IL-1 β production was normalized to stimulated DMSO control (treated with IFN γ , LPS and MDP in the presence of DMSO). P-values are shown for the comparison of each compound with stimulated DMSO control.

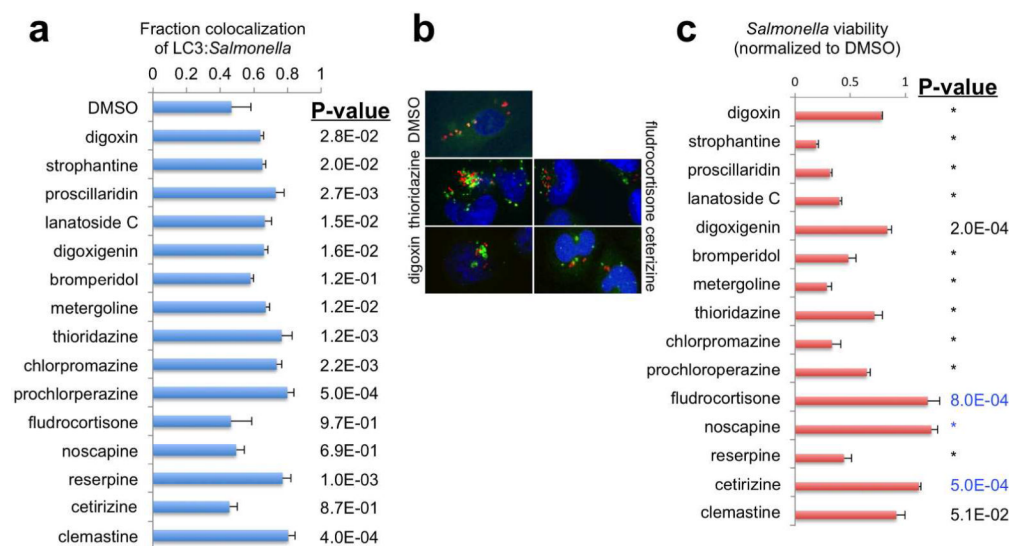


Figure 4. Effects of small-molecule enhancers of autophagy on *S. typhimurium* autophagy and killing. **a.** Fractional co-localization of LC3 punctae with DsRed *S. Typhimurium*. All compounds were evaluated at 5 μ M. **b.** Representative images showing increased (thioridazine, digoxin) or unchanged (fludrocortisone, cetirizine) co-localization of LC3 punctae with DsRed *S. Typhimurium*. **c.** *S. Typhimurium* survival, as assessed by a bacterial bioluminescence assay. P-values in panels **a** and **c** are for the comparison with DMSO control. * denotes $p < 1.0 \times 10^{-4}$. (Blue p-values represent comparisons where compound wells showed a modest increase in *Salmonella* compared to DMSO.)

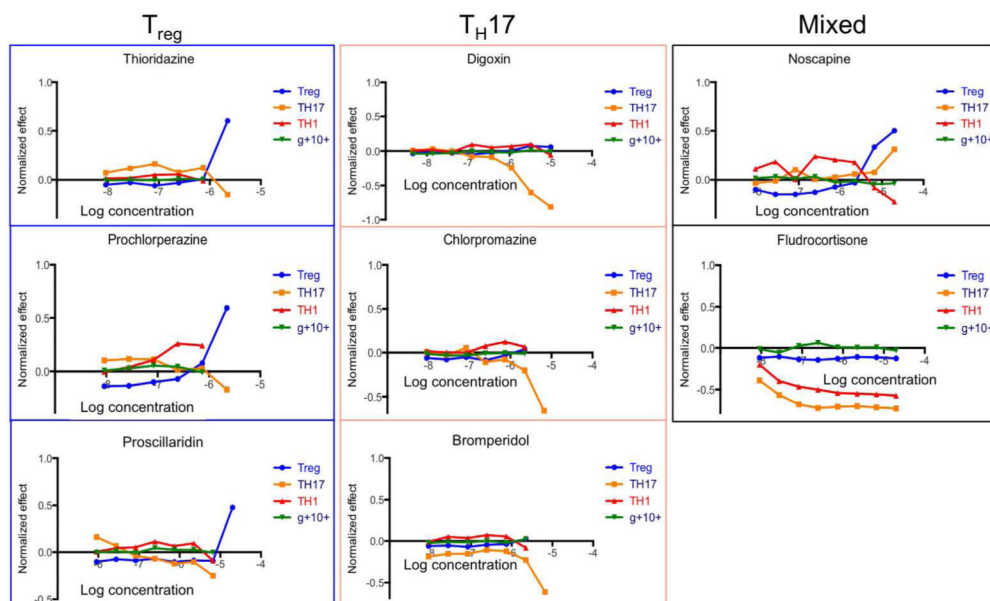
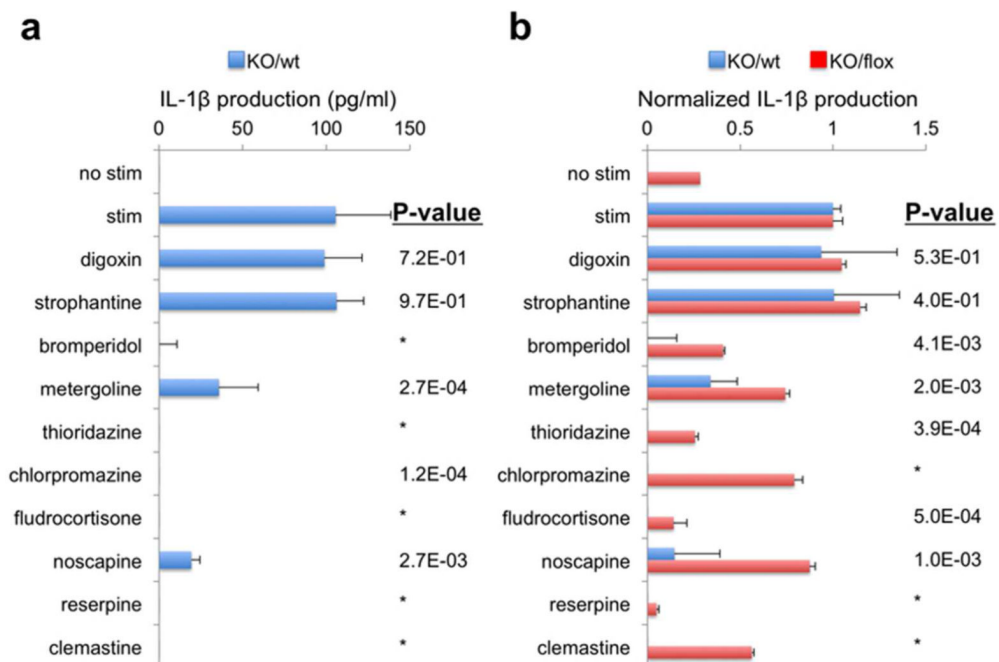


Figure 5. Effects of small-molecule enhancers of autophagy on T cell subset differentiation. Data are presented as normalized fraction of T cell subset prevalence under maximal stimulation. Blue, orange and black outlined panels indicate predominantly T_{reg} , T_{H17} , or mixed effects, respectively. G+ 10+ data points (green) refer to the TR1 anti-inflammatory cell population (expressing both IFN- γ and IL-10).



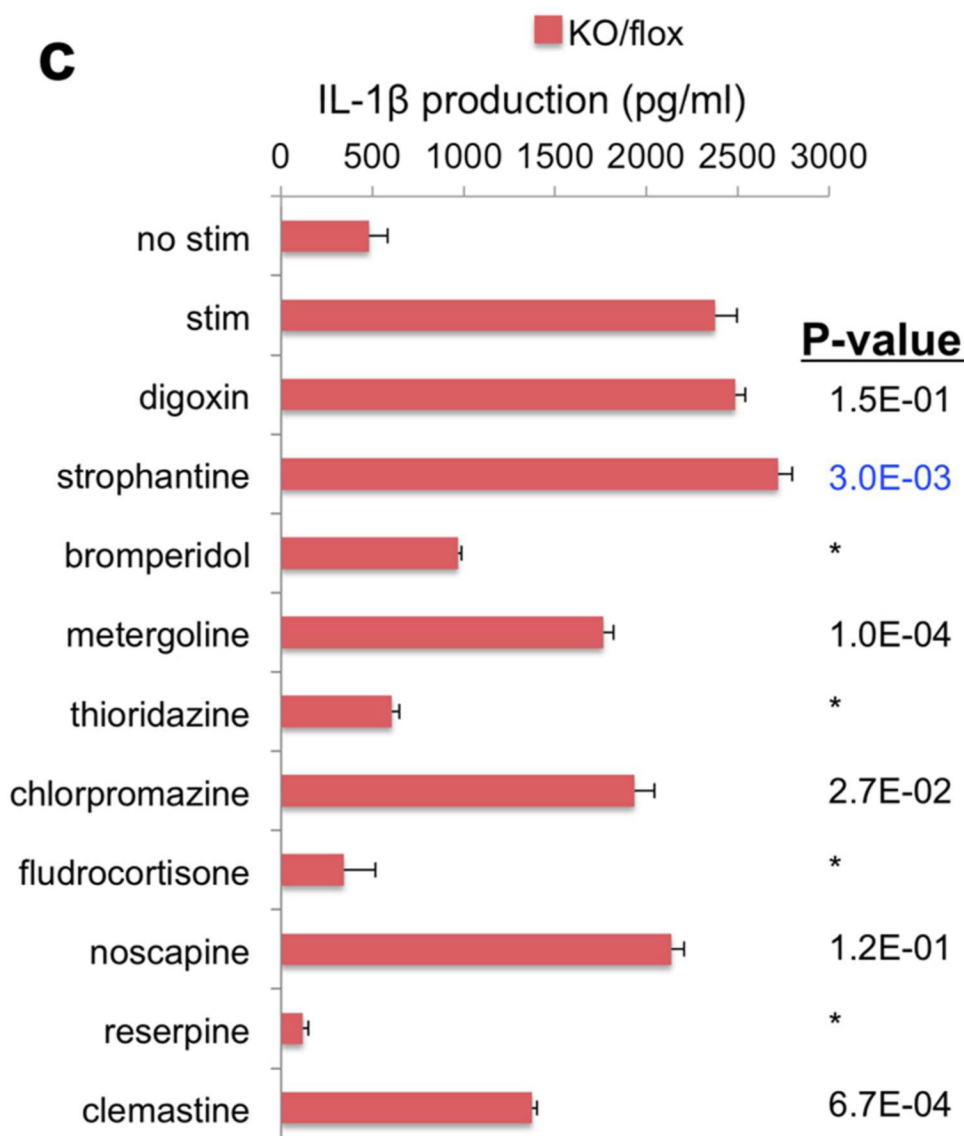


Figure 6. Inhibition of IL-1 β production in BMDMs deficient in *Atg16L1*. **a.** IL-1 β production in BMDMs bearing one functional *Atg16L1* allele (KO/wt). P-values are for the comparison between compound treatment and stimulated DMSO control (“stim” = stimulated control cells treated with IFN γ , LPS and MDP in the presence of DMSO; “no stim” = treated with IFN γ but neither LPS nor MDP; see Methods). **b.** IL-1 β production in BMDMs either heterozygous (KO/wt) or null (KO/flox) for *Atg16L1*, expressed as a normalized ratio of the maximal stimulated value. P-values are for the comparison between the compound's normalized effect in *Atg16L1*-heterozygous vs.-null cells. **c.** Compound effects in *Atg16L1* null cells (KO/flox). P-values are for the comparison between compound and stimulated DMSO control (“stim”). P-values in blue denote IL-1 β values that are mildly increased vs. control. * denotes $p < 1.0 \times 10^{-4}$. Absolute levels of IL-1 β production for KO/wt or KO/flox cells are shown in panels a and c, respectively, whereas normalized IL-1 β levels are shown in panel b to facilitate comparison between KO/wt and KO/flox cells.

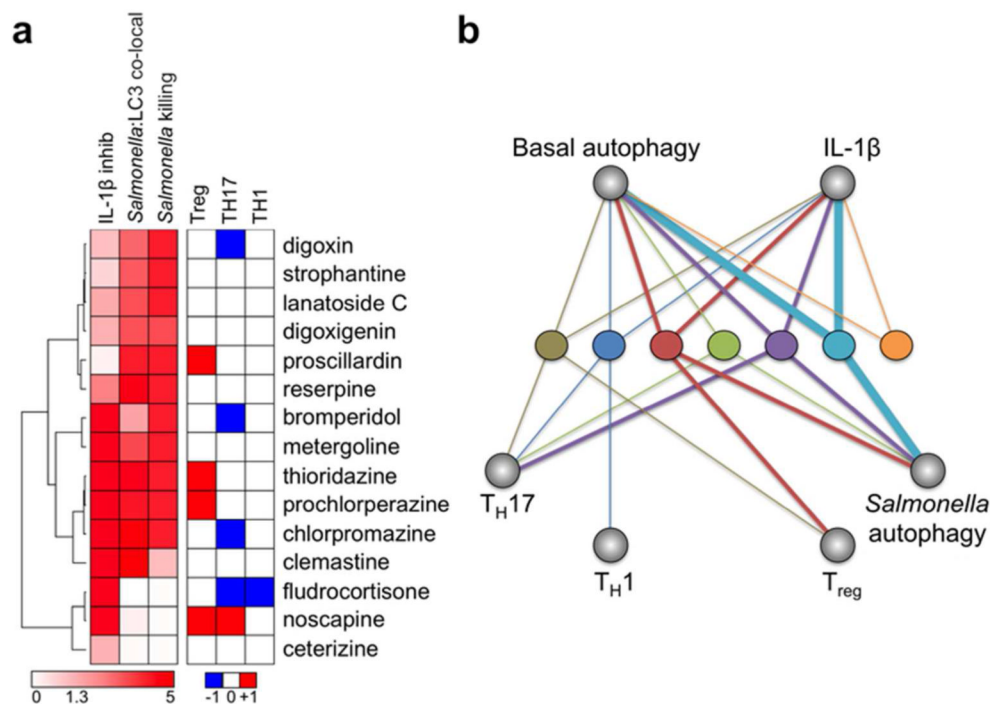


Figure 7.

Activity profiles of small molecule autophagy enhancers. **a.** Heatmap for IL-1 β inhibition, enhanced *Salmonella*:LC3 co-localization, and increased *Salmonella* killing, depicting the negative log of the P-value for the comparison of the compound with DMSO control. Because small molecule effects could result in either direction of effect for T cell subsets, effects were noted as +1 (increased), 0 (unchanged), or -1 (decreased) if a compound altered T cell subset development at any concentration. **b.** Diagram of activity profiles of small molecules (colored circles) across autophagy and associated assays (grey circles). The thickness of the edges reflects the number of compounds showing these relationships; note digoxin is the only cardiac glycoside depicted for clarity.