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Autophagy inhibition dysregulates TBK1 signaling and promotes pancreatic inflammation

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Abstract

Autophagy promotes tumor progression downstream of oncogenic KRAS, yet also restrains inflammation and dysplasia through mechanisms that remain incompletely characterized. Understanding the basis of this paradox has important implications for the optimal targeting of autophagy in cancer. Using a mouse model of cerulein-induced pancreatitis, we found that loss of autophagy by deletion of *Atg5* enhanced activation of the I κ B kinase (IKK) related kinase TBK1 *in vivo*, associated with increased neutrophil and T cell infiltration and PD-L1 upregulation.

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Consistent with this observation, pharmacologic or genetic inhibition of autophagy in pancreatic ductal adenocarcinoma (PDAC) cells, including suppression of the autophagy receptors *NDP52* or *p62*, prolonged TBK1 activation and increased expression of CCL5, IL6, and several other T-cell and neutrophil chemotactic cytokines *in vitro*. Defective autophagy also promoted PD-L1 upregulation, which is particularly pronounced downstream of IFN γ signaling and involves JAK pathway activation. Treatment with the TBK1/IKK ϵ /JAK inhibitor CYT387 (also known as momelotinib) not only inhibits autophagy, but also suppresses this feedback inflammation and reduces PD-L1 expression, limiting *KRAS*-driven pancreatic dysplasia. These findings could contribute to the dual role of autophagy in oncogenesis and have important consequences for its therapeutic targeting.

Keywords

TBK1; autophagy; pancreatitis; PD-L1; JAK

Introduction

Macroautophagy (herein termed autophagy) involves the degradation of ubiquitinated pathogens or recycling of cellular components, typically in a selective manner to maintain homeostasis (^{1,2}). Autophagy also regulates major histocompatibility class II antigen presentation, and thus also plays an important cell extrinsic role in immune recognition (³). Engagement of autophagy downstream of oncogenic *KRAS* counteracts cellular stress and promotes tumor progression, in part by maintaining mitochondrial integrity, detoxifying reactive oxygen species, and altering cellular metabolism (^{4–8}). Thus, therapeutic strategies that target autophagy may be an important component in attaining long-term control of aggressive *KRAS*-driven malignancies.

Yet autophagy is also tumor suppressive, and autophagy inhibition enhances tumor initiation via a mechanism that is incompletely understood (^{9,10}). Concurrent p53 deletion may further limit the efficacy of autophagy inhibition in *Kras*-driven pancreatic (¹¹) and lung cancer (^{12,13}). Even in PDAC models that depend on autophagy in the setting of stochastic *Trp53* LOH (⁶), *Atg5*^{−/−} mice exhibited markedly increased PanINs. Thus, autophagy inhibition clearly predisposes to an environment conducive to dysplasia.

Several studies have suggested that restriction of tumor-promoting inflammation by autophagy may contribute to this relationship (^{12–14}). In murine oncogenic *Kras*-induced lung cancer, *Atg7* deficiency upregulated multiple cytokines (¹²), and *Atg5* loss resulted in Treg accumulation (¹³). Pancreatic *Atg5* inactivation itself increased inflammation and acinar-to-ductal metaplasia (ADM), causing atrophic chronic pancreatitis (¹⁵). Proteomic analyses in PDAC cell lines following autophagy inhibition also identified upregulation of Tank-binding kinase 1 (TBK1) and interferon gamma receptor 1 (IFNGR1), among other inflammatory signaling components (^{16,17}). Thus, enhanced inflammation following autophagy inhibition may at least initially fuel tumor formation downstream of *KRAS*, although the underlying mechanism remains poorly characterized.

TBK1 has emerged as a novel regulator of pathogen xenophagy⁽¹⁸⁾ and KRAS-induced basal autophagy⁽¹⁹⁾. TBK1 promotes selective autophagy by phosphorylating p62^(20,21), NDP52⁽²²⁾, and optineurin⁽²³⁾. TBK1 and its homologue IKK ϵ are also established regulators of cytokine expression during innate immunity⁽²⁴⁾ and promote tumorigenesis through a feedforward circuit involving the protumorigenic cytokines CCL5 and IL6^(25,26). We therefore considered the interplay between autophagy and TBK1 signaling in well characterized pancreatic models, given the implications for targeting these pathways in KRAS-induced dysplasia.

Materials and Methods

Cell culture

PA-TU-8988T was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig), other cell lines were from the American Type Culture Collection (ATCC). PA-TU-8988T, PANC-1, MCF7, H460, PL45, and MIA CaPa-2 were obtained in 2012 from the Kimmelman laboratory, A549 and H1437 cells were obtained in 2011 from the Broad Institute, where we authenticated all cell lines by STR genotyping. Jurkat T cells were obtained in 2015, and were authenticated by TCR sequencing. HPDE cells were obtained in 2013 and RAW 264.7 cells were obtained in 2011, and have remained authenticated by visual inspection and their unique growth requirements. All cells were derived from frozen stocks that had undergone fewer than 4 passages prior to use in the experiments reported here. For details see Supplementary Information.

Inhibitors, cytokines and autophagy assays

CYT387 was synthesized and purchased from Shanghai Haoyuan Chemexpress Co. Ltd. Chloroquine (CQ) was obtained from Sigma. Assessment of phosphorylated TBK1 (pTBK1) levels downstream of inflammatory stimuli was conducted using IL1 β (25 ng/ml) pulse treatment, followed by washout. For autophagy flux measurement in 8988T-LC3-GFP cells, were fixed, and imaged by ImageXpress Micro Screening System and then analyzed by CellProfiler as described⁽²⁷⁾. Additional details are provided in Supplementary Information.

Antibodies, Immunoblotting, and ELISA

Immunoblotting and CCL5 and IL6 ELISAs (R&D) were performed as described⁽²⁵⁾. For details regarding antibodies please see Supplementary Information

Immunofluorescence staining and microscopy

8988T-LC3-GFP cells were pretreated $\pm$ CQ, then pulse stimulated with IL1 β $\pm$ CQ, followed by fixation and indirect immunofluorescence. For details see Supplementary Information.

Lentiviral shRNA/sgRNA production/infection

Lentiviral infection of 8988T cells was performed as described⁽²⁵⁾. Short hairpin (sh)RNA experiments followed 48h puromycin selection, single guide (sg)RNA experiments involved

clonal selection for 1 month. See Supplementary Information for shRNA/sgRNA sequences (Supplemental Table S1) and details.

Quantitative Real-Time PCR

Quantitative real-time polymerase chain reaction (PCR) was performed using LightCycler® 480 SYBR Green I Master (Roche) and the Light Cycler 480 II real-time PCR system (Roche). See Supplementary Information for RT-PCR primer sequences (Supplemental Table S2) and details.

Mouse Treatment/Study Approval

All mouse experiments were conducted in accord with a Dana-Farber Cancer Institute or University of Michigan Cancer Center Institutional Animal Care and Use Committee (IACUC) approved protocol. For details of cerulein administration, drug treatment, and immunohistochemistry see Supplementary Information. pTBK1 and CCL5 expression were evaluated by two pathologists (Y.L. and Z.R.Q) who were blinded to other data. Acinar to ductal metaplasia and PanIN lesions in mice were quantified by grade in a blinded manner (28).

Statistics

Statistical analyses were performed using the Student *t*-test. *P*-values < 0.05 (two-tailed) were considered statistically significant.

Results

Atg5 deletion enhances pancreatitis, TBK1 activation, and PD-L1 expression

We first analyzed autophagy-deficient pancreatic tissue from *Pdx1*-Cre-expressing *Atg5^{L/L}* mice versus *Atg5^{L/+}* mice that retain functional autophagy. In consonance with prior work, *Atg5* deletion resulted in increased pancreatic inflammation (Fig. 1A and B). To study this relationship further, we induced acute pancreatitis by treating mice with cerulein, which hyperstimulates the pancreas and drives IL1 signaling (29). Whereas we observed modest pancreatitis in *Atg5^{L/+}* mice one day after treatment, treatment of *Atg5^{L/L}* mice resulted in severe pancreatitis associated with marked disruption of tissue architecture and substantially greater inflammatory cell infiltration (Fig. 1A and B). Histologic characterization and CD3ε staining confirmed increased recruitment of neutrophils and T cells following cerulein treatment in *Atg5^{L/L}* mice (Supplementary Fig. S1A and B). These results confirm that autophagy restrains pancreatic inflammation *in vivo*, especially during cerulein-induced pancreatitis.

To study TBK1 activation in this context, we performed immunohistochemistry (IHC) in these tissue sections for activation loop-phosphorylated TBK1 (S172 pTBK1) and TBK1-regulated chemokine CCL5. pTBK1 and CCL5 were modestly elevated at baseline in *Atg5^{L/L}* mice relative to *Atg5^{L/+}* mice (Fig. 1C), while strongly enhanced by cerulein treatment of *Atg5^{L/L}* mice compared with *Atg5^{L/+}* mice (Fig. 1D). Thus, autophagy restrains TBK1 activation, particularly following an inflammatory stimulus. Despite this accentuated inflammation, it eventually resolved by day 7 in both *Atg5^{L/+}* and *Atg5^{L/L}* mice, with

reduction of pTBK1 levels back to baseline (Supplementary Fig. S1C and D), suggesting immune checkpoint activation. Indeed, PD-L1 upregulation coincided with elevated pTBK1 and CCL5, especially in cerulein-treated *Atg5^{Δ/Δ}* mice (Fig. 1C and D). Thus, a self-limited increase in inflammation after autophagy inhibition is associated with excessive TBK1 activation and PD-L1 upregulation.

Pharmacologic autophagy inhibition results in pTBK1 accumulation in PDAC cells

To study this further we used 8988T cells, a *KRAS* mutant PDAC line with elevated basal autophagy⁽⁴⁾. First, we enhanced autophagy by acute starvation (HBSS) or inhibited it with chloroquine (CQ) treatment. The amount of pTBK1 decreased after HBSS and was increased by CQ treatment, whereas total TBK1 was unaffected (Fig. 2A). Inhibition of basal or starvation-induced autophagy by CQ also increased pTBK1 across multiple cell types, including RAW macrophages and A549 lung cancer cells (Supplementary Fig. S2A). We observed similar results in HBSS-treated 8988T cells following lysosomal inhibition by bafilomycin A (BFA) or PI3K inhibition by wortmannin (Supplementary Fig. S2B). These findings were consistent with what we observed *in vivo*, and suggested that activated pTBK1 concentrations might be preferentially controlled by autophagosomal degradation.

Since cerulein treatment enhanced this response, we next examined the consequences of pulse treatment with IL1 β , given its role downstream of cerulein and in feed-forward TBK1 cytokine signaling in cancer^(25,30). Whereas in 8988T cells pTBK1 was modestly increased by a 10 min IL1 β pulse, peaking at 60 min then returning to baseline, we observed pronounced accumulation of pTBK1, but not total TBK1, upon cotreatment with CQ (Fig. 2B). We further examined pTBK1 in cell lines with little basal autophagy, such as primary human ductal pancreatic epithelial (HPDE) cells, as compared with 8988T and other PDAC cell lines characterized by active basal autophagy⁽⁴⁾. Though baseline pTBK1 was elevated in 8988T cells relative to HPDE cells, consistent with its activation downstream of oncogenic *KRAS*⁽²⁵⁾, continuous treatment with IL1 β for 60 min or evaluation 60 min after a 30 min IL1 β pulse revealed a substantially greater increase in pTBK1 in HPDE cells compared with 8988T cells (Supplementary Fig. S3A). Continuous or pulse IL1 β treatment of multiple *KRAS* driven PDAC cell lines with elevated basal autophagy (8988T, PANC1, MiaCapa, PL45) induced minimal pTBK1, compared with strong pTBK1 induction in autophagy-low MCF7 cells⁽⁴⁾ or NSCLC cell lines such as A549, H460, or H1437 cells with reduced basal autophagy from STK11/LKB1 inactivation⁽³¹⁾ (Supplementary Fig. S3B). Consistent with these results, we also observed increased CCL5 or IL6 production following IL1 β treatment of A549 cells as compared with 8988T cells, correlating with their enhanced pTBK1 induction and reduced basal autophagy (Supplementary Fig. S3C and D).

To determine whether these findings could be an indirect effect of CQ, or the actual accumulation of pTBK1 with autophagosomes, we examined pTBK1 localization in GFP-LC3 expressing 8988T cells. In contrast to control IL1 β treatment alone, cotreatment with IL1 β and CQ for 60 min resulted in the formation of discrete foci of pTBK1, which overlapped directly with GFP-LC3 labeled autophagosomes (Fig. 2C). In contrast, analysis of total TBK1 revealed more diffuse cellular localization irrespective of IL1 β treatment, consistent with its overall lack of regulation by autophagy (Supplementary Fig. S4). Since

pTBK1 specifically promotes selective autophagy at autophagosomes (²³), these findings suggested potential counter-regulation by selective autophagy.

TBK1 activity and cytokine expression is restrained by selective autophagy

We next tested whether genetic suppression of autophagy machinery components recapitulated this phenomenon, since CQ treatment has pleiotropic effects. First, we expressed control or multiple validated *ATG3*, *ATG7*, or *Beclin1* shRNAs (⁴) in 8988T cells, treated cells with a 30 min IL1 β pulse, and measured pTBK1 over time after chasing with media (Fig. 3A and B). While the maximum degree of pTBK1 induction by this pulse was unaffected by genetic autophagy inhibition (0 min following IL1 β pulse), and levels eventually returned to baseline, the decay of pTBK1 amounts was prolonged. In particular, 60 min following this IL1 β pulse, pTBK1 remained elevated following suppression of *ATG* family members and *Beclin1* with multiple different shRNAs as compared to control shRNA vectors (Fig. 3A and B). Consistent with this transient prolongation in pTBK1 levels, suppression of *ATG3*, *ATG7*, or *Beclin1* also resulted in elevated *CCL5* mRNA expression, which peaked at 6 hours and then returned to baseline (Fig. 3C). To further verify these results, we performed stable CRISPR/Cas9 mediated genetic deletion of *ATG5* or *Beclin1* in 8988T cells, and observed that pTBK1 was just as active 60 min following IL1 β stimulation, and was also higher at baseline in this setting, compared to the Cas9 control (Fig. 3D).

To assess directly whether pTBK1 levels might be restrained by selective autophagy, we next performed a focused shRNA screen in 8988T cells directed against a panel of autophagy receptors or adaptors, using this same assay. Suppression of *NBR1*, *HDAC6*, or *OPTN* failed to affect IL1 β -induced pTBK1 levels relative to control, whereas *NDP52* or *RAB7* suppression prolonged pTBK1 activation (Supplementary Fig. S5A). *NDP52* suppression also increased expression of multiple TBK1-regulated cytokines including *CCL5*, *IL6*, and *CXCL10*, in contrast to *IFN γ* , which is TBK1 independent (Supplementary Fig. S5B). Since the effects of *p62* shRNAs on pTBK1 prolongation were borderline, but associated with incomplete target suppression (Supplementary Fig. 5A), we also generated 8988T cells with *p62* CRISPR-mediated deletion, which was just as effective as *ATG5* deletion at enhancing pTBK1 levels 60 min post IL1 β (Fig. 3D). Taken together, these results confirmed the observations following CQ treatment and reveal counter-regulation of pTBK1 and inflammatory cytokine production by selective autophagy.

TBK1 inhibition impairs autophagy in oncogenic KRAS-driven PDAC cells

TBK1 also regulates autophagy downstream of oncogenic KRAS signaling in lung adenocarcinoma cells (¹⁹). We therefore measured basal autophagy in GFP-LC3-labeled 8988T cells following treatment with the TBK1/IKK ϵ inhibitor, MRT67307 (³²). We used automated imaging to quantify GFP-LC3 foci in the absence or presence chloroquine (CQ) as a measure of autophagy flux, and found that MRT67307 treatment suppressed CQ-induced GFP-LC3 accumulation compared with DMSO control (Fig. 4A and B). We confirmed a direct role for TBK1 in regulating basal autophagy in 8988T cells, since *TBK1* suppression with three different shRNAs also impaired autophagy flux in these cells (Fig. 4C). Because of its clinical utility and more effective disruption of a cytokine signaling circuit, we also examined the consequences of treatment with CYT387, a multitargeted

TBK1/IKK ϵ /JAK inhibitor (²⁵). In 8988T cells, treatment with CYT387 was even more potent than MRT67307 at inhibiting basal autophagy, as measured by its suppression of CQ induced LC3-GFP foci (Fig. 4B). Using a salmonella clearance assay designed to identify small molecule modulators of autophagy (²⁷), both CYT387 and MRT67307 also increased salmonella burden compared with DMSO control (Supplementary Fig. S6). Together, these results established that CYT387 behaves similarly to other TBK1 inhibitors and disrupts the high basal autophagy in KRAS-driven PDAC cells. This suggests the involvement of TBK1 as a rheostat of autophagy control and points of therapeutic intervention distinct from CQ.

CYT387 treatment inhibits CCL5 and PD-L1 expression in PDAC cells

We considered the possibility that CYT387, by suppressing TBK1 and JAK signaling, might inhibit autophagy yet prevent feedback cytokine and PD-L1 expression in 8988T cells. Indeed, heightened CCL5 production in autophagy defective 8988T-sgATG5 cells (Fig. 5A) was ablated by CYT387 treatment (Fig. 5B), which required both TBK1 and JAK inhibition, since the selective JAK1/2 inhibitor Ruxolitinib only marginally reduced CCL5 expression (Fig. 5B). We also noted increased cell surface PD-L1 expression in 8988T-sgATG5 cells compared with Cas9 control (Supplementary Fig S7A), consistent with our findings *in vivo*. Although CCL5 is associated with increased PD-L1 expression in melanoma (³³), treatment of 8988T cells with CCL5 failed to increase PD-L1 compared with IFN γ (Supplementary Fig. S7B). Yet CYT387 treatment also prevented IFN γ -induced PD-L1 expression in 8988T-sgATG5 cells (Supplementary Fig. 7C), consistent with its JAK-specific activity since TBK1 is not activated downstream of IFN γ (²⁵).

To assess more broadly what other cytokines might influence T cell or other inflammatory cell migration, we performed luminex profiling from 72-h conditioned media from 8988T-Cas9 or 8988T-sgATG5 cells cultured as spheroids in collagen, using a microfluidic 3D culture system to better recapitulate the tumor microenvironment (²⁵) (Fig. 5C). Compared to Cas9 control, 8988T-sgATG5 spheroids also produced much more IL6, IL8, CXCL1, and CXCL5 (Fig. 5D), in contrast to MIF, which was highly expressed irrespective of autophagy status (Supplementary Fig. S7D). In addition to attracting T cells, IL6 and IL8 in particular have well-established roles in neutrophil recruitment and angiogenesis, consistent with the increased neutrophils we observed during cerulein induced pancreatitis (Supplementary Fig. S1A and B), and have protumorigenic roles in cancer.

We wondered if the elevated PD-L1 *in vivo* might instead be an indirect consequence of enhanced T cell or other inflammatory cell recruitment by these cytokines. Indeed, exogenous treatment with CCL5 at increasing concentrations recruited Jurkat T cells into collagen in the device (Supplementary Fig. S8A), consistent with the enhanced T-cell influx we observed during cerulein-induced pancreatitis. We next utilized this 3D culture system to co-culture Jurkat T cells with 8988T-Cas9 cells or 8988T-sgATG5 cells themselves (Fig. 5C). We first embedded 8988T spheroids in into the central collagen matrix, incubated them in media for 72 h to establish autocrine cytokine production, then added Jurkat T cells and measured their egress into collagen over the next 24 h. Compared with the Cas9 control, we observed markedly greater migration of T cells into the collagen towards 8988T-sgATG5 cells (Fig. 5C, Supplementary Fig. S8B). Together, these findings support a direct role for

defective autophagy and dysregulated cytokine activation in fueling an inflammatory state that could promote increased dysplasia by oncogenes such as KRAS.

CYT387 treatment inhibits pancreatic inflammation and oncogenic KRAS-induced dysplasia

Pharmacologic inhibition of autophagy in pancreatic cancer has largely relied upon CQ, which is effective in mouse models (⁶), but fails to account for this feedback inflammatory response. Given the *in vitro* activities of CYT387, we considered the possibility that this drug could uniquely counteract this inflammatory feedback response and limit dysplasia *in vivo*. First, we examined the effects of CYT387 treatment on murine cerulein induced pancreatitis in a *Kras*^{WT} background. In order to ensure steady state concentration of drug, we pretreated mice with vehicle or CYT387 (50 mg/kg daily) by oral gavage for 2 days, induced acute pancreatitis with cerulein, then measured pancreatic inflammation on day 1 post-cerulein exposure. Consistent with what we observed *in vitro*, CYT387 treatment suppressed CCL5 and PD-L1 expression and resulted in a significant reduction in the influx of inflammatory cells following cerulein exposure (Fig. 6A and B). We confirmed that CYT387 inhibited autophagy *in vivo* by measuring p62, which accumulated specifically after CYT387 treatment (Fig. 6A). Thus, CYT387 treatment both inhibits autophagy and effectively impairs inflammation during cerulein-induced pancreatitis.

Incorporation of inducible *Kras*^{G12D} expression (iKras* model) with cerulein treatment promotes feed-forward cytokine signaling and pancreatic dysplasia (^{28,34}). Indeed, in contrast to *Kras* WT mice (Supplementary Fig. S1C and D), we observed persistent elevation of pTBK1 and CCL5 day 7 post-cerulein in the iKras* model (²⁸) (Supplementary Fig. S9A). Using doses comparable to prior studies in *Kras*-driven murine lung cancer (²⁵), we pretreated mice with CYT387 (100 mg/kg) by daily oral gavage concurrent with pancreas-specific doxycycline-inducible *Kras*^{G12D} expression, and then induced pancreatitis with transient cerulein exposure (Fig. 6C). Prolonged CCL5 production at day 7 was inhibited by CYT387 treatment in this model, confirming effective disruption of this feed-forward cytokine signaling (Supplementary Fig. 9B). Compared with vehicle treated animals, CYT387 treatment suppressed the protracted inflammation induced by KRAS-TBK1 signaling and preserved pancreatic acinar architecture, limiting acinar to ductal metaplasia (ADM) and PanIN formation at day 7 (Fig. 6D and E). CYT387 also directly inhibited 3D proliferation of iKras* PDAC cells *in vitro* (Supplementary Fig. S9C). Thus, inhibiting both autophagy and cytokine signaling by CYT387 treatment limits KRAS-induced pancreatic dysplasia, with potential therapeutic implications for KRAS-driven PDAC.

Discussion

Although well described, the role of autophagy during KRAS-driven tumorigenesis remains complex and incompletely defined. Autophagy suppresses tumor initiation yet enhances tumor progression (⁹). In addition, feed-forward cytokine signaling promotes KRAS oncogenicity (^{25,30,34}), but how excess inflammation is restrained is unclear. Our findings begin to illuminate the signaling mechanism that maintains homeostasis and explains this

apparent paradox (Fig. 7). The observation that TBK1 promotes basal autophagy in PDAC cells adds to a growing literature that RALB signaling downstream of KRAS and IL1 engages this stress response pathway (19,35,36). At the same time, the degradation of pTBK1 by autophagy limits the degree of TBK1 signaling, which not only prevents excessive activation of autophagy by TBK1, but also limits the production of pro-inflammatory cytokines, recruitment of neutrophils and T cells. These data have important implications for the particular approach to autophagy inhibition in cancer, given these immune effects.

Negative feedback inhibition of TBK1-induced cytokine signaling by autophagy was also described downstream of STING (37) and RIG1-like receptor (RLR) engagement (17). Exposure to cytoplasmic DNA and cyclic dinucleotide activates STING to deliver TBK1 to endosomal/lysosomal compartments whereby IRF3 and NF- κ B signaling is activated, but then subsequently restrained by ULK1-dependent phosphorylation and inhibition of STING (37). *STING*^{-/-} mice also were found to be strongly resistant to DMBA-induced skin carcinogenesis, suggesting a role for TBK1 regulated cytokines in tumor initiation (38). In the case of RLR engagement, genetic ablation of autophagy in the context of oncogenic HRAS or KRAS signaling also promoted excessive TBK1 and cytokine activation (17), though exposure of cells to poly-IC resulted in a strong IFN β response that favored apoptosis and necroptosis. In contrast, our findings reveal that stimuli such as cerulein that induce IL1 activation favor the production of cytokines such as CCL5 and IL6, which promote tumorigenesis. Thus, the consequences of excessive TBK1 activation after inhibition of autophagy are likely stimulus- and context-dependent. The T cell recruitment observed following autophagy inhibition can be pro-tumorigenic (13), but it is also possible that antitumorigenic T-cell subpopulations may exist, and that inflammatory cell recruitment could actually be harnessed to stimulate an anticancer immune response.

Our work specifically identified a role for selective autophagy involving NDP52, p62, and RAB7 in negative feedback regulation of TBK1 activity. NDP52 and p62 have been implicated as cargo receptors that associate with TBK1 across multiple studies (19,20,22,39). Although both NDP52 and p62 are direct targets of TBK1 activity that promote selective autophagy, our findings indicate that NDP52 and p62 may also promote autophagy of pTBK1 complexes themselves. Consistent with this observation, NDP52 has been implicated in the negative feedback control of inflammation. Upon silencing of the ubiquitin-editing enzyme A20, NDP52 activity suppresses poly-I:C-induced pro-inflammatory gene expression, ensuring prevention of excessive inflammation (40). On the other hand, p62 also a component of TRAF6 complexes and promotes NF- κ B activation (41), suggesting a more complex interplay between its role in autophagy and inflammation. Although further work is necessary to determine how NDP52 and p62, as well as RAB7, regulate pTBK1, these findings support a previously unappreciated bidirectional relationship.

This data also show that PD-L1 expression is upregulated following ATG5 deletion *in vivo* and *in vitro*, suggesting that excessive TBK1 activation occurs concomitantly with engagement of the PD-1 immune checkpoint. Although CCL5 production does not directly induce PD-L1 in this context, we found that IFN γ , which is produced by T cells that are

recruited by CCL5, can fuel PD-L1 upregulation. We also observed upregulation of several other cytokines, such as IL6, that promote a T cell suppressive immune environment via neutrophil recruitment. In lung cancer, inactivation of STK11/LKB together with oncogenic KRAS mutation, upregulates a similar set of cytokines and fuels tumorigenesis through neutrophil infiltration (⁴²), which may be related to our findings since STK11/LKB1 deletion impairs autophagy (³¹). CCL5 and IL6 also foster dysplasia directly by promoting PDAC epithelial cell proliferation and survival, as in KRAS-dependent lung cancer cells (²⁵). Regardless, the observation that CYT387, a multiple-target JAK/TBK1/IKK ϵ inhibitor, not only suppresses TBK1-mediated autophagy and feedback CCL5 activation, but also JAK-driven contributions to cytokine signaling and PD-L1 expression, further highlights its fortuitous ability to disrupt multiple pro-tumorigenic events.

Current clinical attempts to inhibit autophagy in cancer have relied upon hydroxychloroquine, which acts primarily at the lysosome and has shown inconsistent activity (⁴³). Because pharmacologic inhibition of autophagy with CYT387 acts in a unique manner to suppress feedback cytokine activation and inflammation, these findings could have important consequences for the optimal targeting of autophagy in KRAS-dependent cancers. Indeed, CYT387 is currently under evaluation in human clinical trials in combination with chemotherapy for advanced PDAC (NCT02101021 and NCT02244489), and with MEK inhibition in KRAS-mutated lung adenocarcinoma (NCT02258607) (²⁵). More generally, strategies that impair both the cytoprotective effects of autophagy and pro-tumorigenic cytokine signaling or PD-L1 may represent key components of treating established KRAS tumors or preventing their formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Autophagy restrains pancreatic inflammation and expression of pTBK1, CCL5, and PD-L1 *in vivo*. A, Representative low and high magnification images of H&E staining of pancreatic tissue obtained from mice with heterozygous *Atg5* pancreatic deletion (*Atg5*^{L/+}) or parallel mice with homozygous *Atg5* pancreatic deletion (*Atg5*^{L/L}) at baseline or on d1 following cerulein treatment. B, Mononuclear inflammatory cell infiltration scoring from three randomly selected areas of each mouse pancreas (200x magnification). A total of 18 mice, 18 × 3 = 54 spots were measured; bars show mean ± SEM. *P* values by Student *t* test. C, Immunohistochemistry (IHC) for S172 pTBK1, CCL5, and PD-L1 in representative sections of *Atg5*^{L/+} (upper panel) or *Atg5*^{L/L} (lower panel) mouse pancreatic tissue at baseline. D, IHC for S172 pTBK1, CCL5, and PD-L1 in representative sections of *Atg5*^{L/+} (upper panel) or *Atg5*^{L/L} (lower panel) mouse pancreatic tissue on d1 following cerulein treatment.

Figure 2. pTBK1 levels are controlled by lysosomal degradation in cultured KRAS-driven PDAC cells. A, Immunoblot of pTBK1, total TBK1, and β -actin in 8988T cells in media or HBSS $\pm$ 30 μ M CQ. B, pTBK1, total TBK1, and β -actin immunoblot in 8988T cells pretreated $\pm$ CQ for 60 min, then 10 min IL1 β pulse (25 ng/ml), then chased $\pm$ CQ as indicated. C, pTBK1 indirect immunofluorescence in 8988T-LC3-GFP cells pretreated $\pm$ CQ for 60 min, then 10 min IL1 β pulse/chase, in the absence (upper panels) or presence (lower panels) of CQ. Autophagosomes (green) and pTBK1 (red) colocalize in the cytoplasm (yellow), particularly pronounced with CQ. Blue = DAPI nuclear stain. Quantification of overlap was performed across 16 different fields for each condition, mean number of colocalized foci per cell $\pm$ SD shown. $P < 0.0001$ by the Student t test.

Figure 3.

Inhibition of autophagy prolongs inducible TBK1 activation. A, pTBK1, total TBK1, ATG3, and β -actin immunoblot in 8988T cells expressing control or ATG3-specific shRNAs, treated with a 30 min IL1 β pulse (25 ng/ml), then chased in media for the indicated times. Ctrl = no IL1 β stimulation. Time represents minutes following chase with media. B, Immunoblot of pTBK1, total TBK1, ATG7, Beclin1, and β -actin in 8988T cells expressing control or 2 different ATG7 or Beclin1 shRNAs, pulsed with IL1 β for 30 min, then chased in media for the indicated times. C, *CCL5* mRNA in 8988T cells expressing control, ATG3, ATG7, or Beclin1 shRNAs and pulsed with IL1 β for 30 min, followed by chase for the indicated times. Mean and SEM of triplicate samples shown. D, Upper panel shows immunoblot of pTBK1, total TBK1, ATG5, Beclin1, and β -actin in 8988T cells expressing the Cas9 control alone or the indicated sgRNAs. Lower panel shows immunoblot of pTBK1, total TBK1, ATG5, p62, and β -actin in 8988T cells expressing Cas9 alone or the indicated sgRNAs.

Figure 4.

TBK1 signaling also promotes basal autophagy in 8988T cells. A, Images of 8988T-LC3-GFP cells pretreated $\pm$ 30 μ M chloroquine (CQ) for 60 min, then DMSO control or 5 μ M MRT67307 for another 60 min, followed by fixation. B, Mean vesicle quantification in 8988T-LC3-GFP cells treated in triplicate with DMSO, MRT67307 (5 μ M), or CYT387 (5 μ M) $\pm$ 30 μ M CQ. Cells were fixed, imaged by ImageXpress Micro Screening System then analyzed by Cellprofiler for vesicle count and area per cell from 6 independent areas per well. Red = autophagy flux. C, 8988T-LC3-GFP cells were stably infected with three different TBK1 shRNAs then treated $\pm$ CQ for 120 min in triplicate. Mean vesicle count and area per cell were quantified by Cellprofiler. TBK1 suppression in these cells was confirmed by immunoblot of TBK1 and β -actin loading control, shown in the insert.

Figure 5. TBK1/JAK inhibition by CYT387 suppresses chemotactic cytokine production. A, Immunoblot shows LC3-II and β -actin in 8988T cells expressing Cas9 alone or sgATG5, ELISA detecting CCL5 in 8988T-Cas9 or 8988T-sgATG5 cells stimulated with IL1 β for the indicated pulses and chased in media for an additional 24 h, mean and SD of duplicate samples shown, $P < 0.0001$ for each comparison between Cas9 and sgATG5. B, CCL5 ELISA in 8988T-sgATG5 cells 24 h after a 60 min IL1 β pulse $\pm$ 2.5 μ M CYT387 or ruxolitinib, mean and SD of duplicate samples shown. ** P value = 0.0004 for comparison to IL1 β alone, n.s = not significant compared to IL1 β alone. C, Left, schematic of 3D microfluidic culture device, Jurkat T cells (left channel) loaded 72 h after 8988T spheroids embedded in central collagen matrix. Middle, 10x phase contrast images of Jurkat migration towards 8988T-Cas9 or 8988T-sgATG5 spheroids 24 h later. Right, quantification of unmigrated Jurkat T cells, mean and SD from 4 different fields. D, Luminex profiling of cytokines from 72 h conditioned media in the device.

Figure 6.

CYT387 treatment suppresses pancreatitis and oncogenic Kras-induced pancreatic dysplasia. A, H&E staining and IHC for CCL5, p62 or PD-L1 in pancreatic tissue harvested from C57/BL6 mice pretreated with vehicle control or daily CYT387 (50 mg/kg) day 1 post-cerulein. B, Inflammatory cell infiltration in pancreatic tissue from 3 random sites (200x) following vehicle ($n = 4$) or CYT387 ($n = 2$) treatment. Mean and SEM shown, P value calculated by Student t -test. C, Schematic of CYT387 therapy in iKras* model. D, Pancreatic tissue histology 1 wk after iKRAS induction in daily vehicle or CYT387 (100 mg/kg) treated animals. E, Blinded quantification of ADM and PanINs from 5 random images (20x) including 50 total acinar or ductal clusters per pancreas in vehicle ($n = 2$) or CYT387 ($n = 3$) treated mice. Mean and SEM shown.

Figure 7.

Rheostat regulation of pancreatic inflammation and dysplasia. TBK1 activation downstream of factors such as oncogenic KRAS and IL1 signaling induces both autophagy and cytokine signaling. Autophagy feeds back to inhibit pTBK1; thus, its inhibition may result in inflammatory signaling, promoting dysplasia. In contrast, CYT387 treatment impairs autophagy at the level of this rheostat and suppresses proinflammatory cytokines that fuel neutrophil recruitment, PD-L1 and dysplasia.