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 IκB kinase (IKK)-related kinase TBK1 in vivo, associated with increased neutrophil and T-cell infiltration and PD-L1 upregulation. Consistent with this observation, pharmacologic or genetic inhibition of autophagy in pancreatic ductal adenocarcinoma 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 cytokines in vitro. Defective autophagy also promoted PD-L1 upregulation, which is particularly pronounced downstream of IFNγ signaling and involves JAK pathway activation. Treatment with the TBK1/IKKε/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. Cancer Immunol Res; 4(6); 520–30. ©2016 AACR.

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 pancreatic ductal adenocarcinoma (PDAC) models that depend on autophagy in the setting of stochastic Trp53 LOH (6), Atg5−/− mice exhibited markedly increased PanNgs. 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 regulatory T cell (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 homolog IKKε are also
established regulators of cytokine expression during innate immunity (24) and promote tumorigenesis through a feed-forward 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), other cell lines were from the ATCC, PA-TU-8988T, PANC-1, MCF7, H460, PL45, and Mia CaPa-2 cells 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 short tandem repeat (STR) genotyping. Jurkat T cells were obtained in 2011, and have remained authenticated by visual inspection and their unique growth requirements. All cells were obtained in 2011, and have remained authenticated by T-cell receptor (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 four passages prior to use in the experiments reported here. For details see Supplementary Information.

Inhibitors, cytokines, and autophagy assays
CVT387 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 IL1β (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 Systems) were performed as described (25). For details regarding antibodies, please see Supplementary Information.

Immunofluorescence staining and microscopy
8988T-LC3-GFP cells were pretreated with or without CQ, then pulse stimulated with IL1β ± CQ, followed by fixation and indirect immunofluorescence. For details see Supplementary Information.

Lentiviral shRNA/siRNA production/infection
Lentiviral infection of 8988T cells was performed as described (25). Short hairpin (sh)RNA experiments followed 48-hour puromycin selection, single guide (sg)RNA experiments involved clonal selection for 1 month. See Supplementary Information for shRNA/siRNA sequences (Supplementary Table S1) and details.

Quantitative real-time PCR
Quantitative real-time 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 (Supplementary Table S2) and details.

Mouse treatment/study approval
All mouse experiments were conducted in accordance 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. Imamura and Z. Qian) who were blinded to other data. AciNar-to-ductal metaplasia (ADM) and PanIN lesions in mice were quantified by grade in a blinded manner (28).

Statistical analyses
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 Atg5fl/fl mice versus Atg5+/+ 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 Atg5fl/fl mice 1 day after treatment, treatment of Atg5fl/fl 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 Atg5fl/fl mice (Supplementary Fig. S1A and S1B). 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 Atg5fl/fl mice relative to Atg5+/+ mice (Fig. 1C), while strongly enhanced by cerulein treatment of Atg5fl/fl mice compared with Atg5+/+ 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 Atg5fl/fl and Atg5+/+ mice, with reduction of pTBK1 levels back to baseline (Supplementary Fig. S1C and S1D), suggesting immune checkpoint activation. Indeed, PD-L1 upregulation coincided with elevated pTBK1 and CCL5, especially in cerulein-treated Atg5fl/fl 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 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 wortmanin (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.

Because cerulein treatment enhanced this response, we next examined the consequences of pulse treatment with IL1β, given its role downstream of cerulein in feed-forward TBK1 cytokine signaling in cancer (25, 30). Whereas in 8988T cells pTBK1 was modestly increased by a 10-minute IL1β pulse, peaking at 60 minutes, and 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). Although baseline pTBK1 was elevated in 8988T cells relative to HPDE cells, consistent with its activation downstream of oncogenic KRAS (25), continuous treatment with IL1β for 60 minutes or evaluation 60 minutes after a 30-minute IL1β pulse revealed a substantially greater increase in pTBK1 in HPDE cells compared with 8988T cells (Supplementary Fig. S3A). Continuous or pulse IL1β treatment of multiple KRAS-driven PDAC cell lines with elevated basal autophagy (8988T, PANC1, MiaCapa, and PL45) induced minimal pTBK1, compared with strong pTBK1 induction in autophagy-low MCF7 cells (4) or non–small cell lung carcinoma cell lines such as A549, H460, or H1437 cells with reduced basal autophagy from STK11/LKB1 inactivation (ref. 31; Supplementary Fig. S3B). Consistent with these results, we also observed increased CCL5 or IL6 production following IL1β treatment of A549 cells as compared with 8988T cells, correlating with their
enhanced pTBK1 induction and reduced basal autophagy (Supplementary Fig. S3C and S3D).

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 IL1β treatment alone, cotreatment with IL1β and CQ for 60 minutes 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 IL1β treatment, consistent with its overall lack of regulation by autophagy (Supplementary Fig. S4). Because pTBK1 specifically promotes selective autophagy at autophagosomes (23), these findings suggested potential counterregulation 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, because 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-minute IL1β 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 (6 minute following IL1β pulse), and levels eventually returned to baseline, the decay of pTBK1 amounts was prolonged. In particular, 60 minutes following this IL1β pulse, pTBK1 remained elevated following suppression of ATG family members and Beclin1 with multiple different shRNAs as compared with 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 ATG3 or Beclin1 in 8988T cells, and observed that pTBK1 was just as active 60 minutes following IL1β stimulation and was higher at baseline in this setting, compared with 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 NDP52 or RAB7 failed to affect IL1β-induced pTBK1 levels relative to control, whereas NBR1, HDAC6, or OPTN failed to affect IL1β-induced pTBK1 levels relative to control, whereas NBR1, HDAC6, or OPTN failed to affect IL1β-induced pTBK1 levels relative to control.
activation (Supplementary Fig. S5A). NDP52 suppression also increased expression of multiple TBK1-regulated cytokines, including CCL5, IL6, and CXCL10, in contrast to IFNg, which is TBK1 independent (Supplementary Fig. S5B). Because the effects of p62 shRNAs on pTBK1 prolongation were borderline, but associated with incomplete target suppression (Supplementary Fig. S5A), we also generated 8988T cells with p62 CRISPR–mediated deletion, which was just as effective as ATG5 deletion at enhancing pTBK1 levels 60 minutes after IL1β (Fig. 3D). Taken together, these results confirmed the observations following CQ treatment and reveal counterregulation 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 of 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, because TBK1 suppression with three different
PDAC cells. Thisitors and disrupts the high basal autophagy in KRAS-driven DMSO control (Supplementary Fig. S6). Together, these results MRT67307 also increased molecule modulators of autophagy (27), both CYT387 and Salmonella Using a sured by its suppression of CQ-induced LC3-GFP foci (Fig. 4B). TBK1 signaling also promotes basal autophagy in 8988T cells. A, images of 8988T-LC3-GFP cells pretreated with CYT387 treatment inhibits CCL5 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, because 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 IFNγ (Supplementary Fig. S7B). Yet, CYT387 treatment also prevented IFNγ-induced PD-L1 expression in 8988T-sgATG5 cells (Supplementary Fig. S7C), consistent with its JAK-specific activity because TBK1 is not activated downstream of IFNγ (25).

To assess more broadly what other cytokines might influence T-cell or other inflammatory cell migration, we performed luminex profiling from 72-hour 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 (ref. 25; Fig. 5C). Compared with Cas9 control, 8988T-sgATG5 spheroids also produced much more IL6, IL8, CXCL1, and CXCL5 (Fig. 5D), in contrast with MIF, which was

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/IKKe/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 finding 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, 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 IFNγ (Supplementary Fig. S7B). Yet, CYT387 treatment also prevented IFNγ-induced PD-L1 expression in 8988T-sgATG5 cells (Supplementary Fig. S7C), consistent with its JAK-specific activity because TBK1 is not activated downstream of IFNγ (25).

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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 IL1β for the indicated pulses and chased in media for an additional 24 hours; mean and SD of duplicate samples are shown; P < 0.0001 for each comparison between Cas9 and sgATG5. B, CCL5 ELISA in 8988T-sgATG5 cells 24 hours after a 60-minute IL1β pulse/C6.25 μmol/L CYT387 or ruxolitinib (Ruxo); mean and SD of duplicate samples are shown. **P = 0.0004 for comparison with IL1β alone; n.s., not statistically significant compared with IL1β alone. C (left), schematic of 3D microfluidic culture device, Jurkat T cells (left channel) loaded 72 hours after 8988T spheroids embedded in central collagen matrix. Middle, ×10 phase contrast images of Jurkat migration toward 8988T-Cas9 or 8988T-sgATG5 spheroids 24 hours later. Right, quantification of unmigrated Jurkat T cells; mean and SD from four different fields. D, luminex profiling of cytokines from 72-hour conditioned media in the device.
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 used this 3D culture system to coculture Jurkat T cells with 8988T-Cas9 cells or 8988T-sgATG5 cells themselves (Fig. 5C). We first embedded 8988T spheroids into the central collagen matrix, incubated them in media for 72 hours to establish autocrine cytokine production, and then added Jurkat T cells and measured their egress into collagen over the next 24 hours. Compared with the Cas9 control, we observed markedly greater migration of T cells into the collagen toward 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 KrasWT background. In order to ensure steady-state concentration of the drug, we pretreated mice with vehicle or CYT387 (50 mg/kg daily) by oral gavage for 2 days, induced acute pancreatitis with cerulein, and then measured pancreatic inflammation on day 1 after 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 KrasG12D expression (iKras model) with cerulein treatment promotes feed-forward cytokine signaling and pancreatic dysplasia (28, 34). Indeed, in contrast with Kras WT mice (Supplementary Fig. S1C and S1D), we observed persistent elevation of pTBK1 and CCL5 day 7 after cerulein in the iKras model (ref. 28; Supplementary Fig. S9A). Using doses comparable with those of 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 KrasG12D 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. S9B). Compared with vehicle-treated animals, CYT387 treatment suppressed the protracted inflammation induced by KRAS–TBK1 signaling and preserved pancreatic acinar architecture, limiting 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 RBAL 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 proinflammatory cytokines and 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/or cyclic dinucleotides activates STING to deliver TBK1 to endosomal/lysosomal compartments whereby IRF3 and NF-kB signaling is activated, but then subsequently restrained by ULK1-dependent phosphorylation and inhibition of STING (37). STING−/− mice were also 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), although exposure of cells to poly-I:C resulted in a strong IFNβ 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. In addition, the T-cell recruitment observed following autophagy inhibition can be protumorigenic (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 are associated with TBK1 across multiple studies (19, 20, 22, 39). Although both NDP52 and p62 are direct targets of TBK1 activity. NDP52 and p62 have been implicated as cargo receptors that are associated with TBK1 across multiple studies (19, 20, 22, 39). Although both NDP52 and p62 are direct targets of TBK1 activity, NDP52 and p62 may also promote autophagy of TBK1 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 proinflammatory gene expression, ensuring prevention of excessive inflammation (40). On the other hand, p62, also a component of TRAF6 complexes, promotes NF-kB 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.

These 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 IFNγ, 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/LKB1, 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 because 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/TKB1/IKKε 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 protumorigenic 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; ref. 25). More generally, strategies that impair both the cytoprotective effects of autophagy and protumorigenic cytokine signaling or PD-L1 may represent key components of treating established KRAS tumors or preventing their formation.

Disclosure of Potential Conflicts of Interest

A.C. Kimmelman reports receiving speakers bureau honoraria from Agios and is a consultant/advisory board member for Astellas, Forma Therapeutics, and Gilead. D.A. Barbie reports serving as a consultant/advisory board member for N of One. No potential conflicts of interest were disclosed by the other authors.

References


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Autophagy Inhibition Dysregulates TBK1 Signaling and Promotes Pancreatic Inflammation

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