Autophagosomal IκBα Degradation Plays a Role in the Long Term Control of Tumor Necrosis Factor-α-induced Nuclear Factor-κB (NF-κB) Activity*§

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Transcription factor NF-κB is persistently activated in many chronic inflammatory diseases and cancers. The short term regulation of NF-κB is well understood, but little is known about the mechanisms of its long term activation. We studied the effect of a single application of TNF-α on NF-κB activity for up to 48 h in intestinal epithelial cells. Results show that NF-κB remained persistently activated up to 48 h after TNF-α and that the long term activation of NF-κB was accompanied by a biphasic degradation of IκBα. The first phase of IκBα degradation was proteasome-dependent, but the second was not. Further investigation showed that TNF-α stimulated formation of autophagosomes in intestinal epithelial cells and that IκBα colocalized with autophagosomal vesicles. Pharmacological or genetic blockade of autophagosome formation or the inhibition of lysosomal proteases decreased TNF-α-induced degradation of IκBα and lowered NF-κB target gene expression. Together, these findings indicate a role of autophagy in the control of long term NF-κB activity. Because abnormalities in autophagy have been linked to ineffective innate immunity, we propose that alterations in NF-κB may mediate this effect.

Nuclear factor-κB (NF-κB) constitutes a family of transcription factors pivotal in the control of immune responses. NF-κB acts downstream of a series of cellular receptors, including certain Toll-like receptors, cytokine receptors, and lymphocyte antigen receptors, to induce the expression of genes that promote immunity and inhibit the apoptosis of those cells (reviewed in Ref. 1). Through these actions, NF-κB protects against infections and tissue damage (2, 3). However, significant evidence also indicates that excessive or prolonged NF-κB activity can be harmful. For example, active NF-κB has been observed in many chronic inflammatory diseases, such as inflammatory bowel disease (4, 5), and in many cancers, including colon cancer (6). Moreover, specific blockade of NF-κB activity in animal models of those diseases appears to lower disease severity, indicating that NF-κB plays a pathogenic role in the disease process (7). Deeper understanding of the molecular regulation of NF-κB in chronic disease states may lead to novel therapeutic strategies.

Under “non-stress” conditions, most cells sequester NF-κB proteins in the cytoplasm, where they are inactive, via interaction with members of a family of inhibitors termed IκBs. NF-κB is rapidly activated upon the induction of cellular “stress” (e.g. by exposure to the proinflammatory cytokine tumor necrosis factor (TNF)-α). TNF-α stimulates the serine phosphorylation and subsequent degradation of IκB proteins, allowing NF-κB to translocate to the nucleus (8, 9). IκBα is the most abundant NF-κB inhibitor in most cell types, and it is targeted for proteasomal degradation by serine phosphorylation mediated by the IKK complex (10). Nuclear NF-κB proteins up-regulate the de novo expression of IκBα, which decreases NF-κB activity in a negative feedback loop (11). This model of rapid stimulus-induced NF-κB activity, followed by its termination, has been extensively characterized in many cell types and serves to explain well the acute up-regulation and subsequent termination of NF-κB activity over periods of up to 4 h. However, the usefulness of this model to explain the persistent NF-κB activity that is observed in human diseases, such as inflammatory bowel disease and cancer, is more limited because little is known about how IκBα levels are chronically suppressed to allow NF-κB to remain active.

In this work, we explored the regulation of NF-κB activity over longer periods of time that more closely mimic in vivo situations of disease. We used intestinal epithelial cells that were stimulated with TNF-α to study this process because this cytokine is a pivotal factor in chronic inflammatory bowel diseases. Our results have revealed that IκBα is targeted for autophagosomal degradation at later phases following stimulation with TNF-α, leading to persistently active NF-κB.

EXPERIMENTAL PROCEDURES

Reagents and Media—McCoy’s 5A, Dulbecco’s modified Eagle’s medium, and fetal calf serum were obtained from Invitrogen. TNF-α was obtained from Bioscience; IL-1β, E64d, triciribine, and wortmannin were from Calbiochem. All other chemicals were purchased from Sigma. All antibodies were from Cell Signaling Technology unless otherwise stated.

Cell Lines—HT29 and HCT116 cell lines were from the American Type Culture Collection (Manassas, VA). HT29 cells were cultured in Dulbecco’s modified Eagle’s medium (low glu-
cose) supplemented with 10% serum, and HCT116 cells were cultured in McCoy’s 5A medium supplemented with 10% serum, 1% L-glutamine, 1% penicillin/streptomycin. In some experiments, HT29 cells were conditioned with interferon-γ (10 ng/ml) for 40 h. NIH3T3 wild type and LAMP2A−/− mouse fibroblastic cells were a kind gift from Dr. A. M. Cuervo (Albert Einstein College of Medicine, New York), and ATG5+/− and ATG5−/− mouse embryonic fibroblasts were obtained from RIKEN, Japan. Cells were grown in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% serum, 1% L-glutamine, and 1% penicillin/streptomycin.

**Immunoblotting**—Whole cell or cytoplasmic and nuclear proteins were extracted using lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% SDS, 150 mM NaCl, 1 mM EDTA, with protease and phosphatase inhibitors) or the NE-PER nuclear extraction kit (Pierce), respectively. Samples containing 20 μg of whole cell, 30 μg of cytoplasmic, or 10 μg of nuclear protein extract were resolved on 10% SDS-polyacrylamide gels and transferred to PVDF membrane. Membranes were probed overnight with the indicated antibodies and visualized using a G:BOX Chemi system (Syngene).

**NF-κB-dependent Reporter Gene Assay**—The reporter plasmid pNF-κB-luc, negative control plasmid pCis-CK (lacking NF-κB enhancer), and internal transfection rate control plasmid (Renilla, pRLTK) were obtained from Stratagene. Transfection was carried out using Fugene 6 transfection reagent (Roche Applied Science). The Dual Glo luciferase assay (Promega) was used to measure luciferase activity. NF-κB firefly luminescence readings were normalized for Renilla values and reported relative to an unstimulated calibrator sample.

**Measurement of NF-κB DNA Binding**—Activated transcription factor p65 in the nuclear extract was measured with an ELISA kit (TransAM™ NF-κB p65, Active Motif) according to the manufacturer’s instructions.

**IL-8 ELISA**—A human IL-8 ELISA kit (R&D Systems) was used to quantify IL-8 protein in cell culture supernatant.

**Quantitative Real-time RT-PCR**—RNA was extracted from cells using the Qiagen RNaseasy kit (Qiagen). cDNA was prepared from total RNA using Superscript II (Invitrogen). Primer sequences were designed using the Primer 3 algorithm and are provided in supplemental Table 1. Quantitative real-time RT-PCR was performed using Power SYBR Green master mix on a 7500 real-time PCR system (Applied Biosystems). GAPDH was used as internal control, and relative mRNA expression levels were calculated using the ΔΔCT method.

**Proteasome Activity Assay**—Cells were harvested, lysed, and enriched for proteasomal proteins by cycles of freeze-thaw (−80°C) and centrifugation. Extracts were incubated with fluorescent proteasome substrates, N-succinyl-LLVY-AMC,3 benzoyloxycarbonyl-ARR-AMC, and benzoyloxycarbonyl-LEL-AMC (Molecular Probes) for chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing activity, respectively, in substrate buffer (5 mM ATP and 0.5 mM DTT). Fluorescence was measured in a microtiter plate fluorimeter (excitation, 395 ± 25 nm; emission, 460 ± 40 nm). Proteasome activity values are shown as enzyme activity in nmol of AMC released/min/mg of protein.

**Immunofluorescence Analysis**—Cells were fixed in 4% formaldehyde and permeabilized in Triton X-100, blocked in 5% goat serum, and incubated with the appropriate primary and secondary antibodies. DAPI (25 μg/ml) was used to stain nuclei. Images were acquired with a confocal fluorescence microscope (Andor Revolutions Spinning Disk with Olympus IX81 motorized inverted microscope) and subjected to deconvolution with the manufacturer’s software. Images were assessed for co-localization by overlaying signals for appropriate wavelengths (DAPI 401 nm (blue), IκBα 488 nm (green), and LC3 561 nm (red)) with the manufacturer’s software, where co-localized signal is yellow. Images were taken at ×40 magnification with oil.

**Electron Microscopy**—Cells were initially fixed in a fixative buffer (2% glutaraldehyde (Serva) and 2% formaldehyde, 0.1 M sodium cacodylate/HCl) and postfixed with osmium tetroxide, dehydrated with graded alcohols and propylene oxide, and embedded in Epon-based resin (Agar Scientific). Thin (1-μm) sections were cut and stained with toluidine blue as cell “scout” sections to determine regions of dense cells. Ultra-thin (90-nm) sections were cut and stained with uranyl acetate (Leica) and lead citrate (Leica) and examined using a transmission electron microscope (Hitachi H7000). The term “autophagic compartments” was used to describe all lysosome-like double membrane vesicle structures of correct approximate size and contents. The relative volume densities (Vv) of autophagic compartments and or intestinal epithelial cells (cytoplasm and nuclei) samples were estimated by point counting. The point sample intercept method was used to estimate nuclear volume; this was combined with the fractional volumes to obtain absolute parameters (12).

**Statistical Analysis**—All experiments were conducted at least three times, and representative data are shown. Data generated in luciferase reporter assays, DNA binding assays, proteasome activity assays, and ELISAs were analyzed by one-way ANOVA and expressed as mean ± S.E. for independent experiments. Statistically significant changes in two sample groups were analyzed using Student’s t test, and error bars are expressed as mean ± S.E. for independent experiments. The differences are considered to be significant if p is <0.05 (indicated by an asterisk); those at p < 0.01 are indicated by double asterisks; and those at p < 0.001 are indicated by triple asterisks.

**RESULTS**

**A Single Stimulation of TNF-α Results in Prolonged NF-κB Activation in Intestinal Epithelial Cells**—As an initial step to investigate the effects of TNF-α on long term NF-κB activity, we examined the DNA binding capacity, p65/RelA protein localization, and NF-κB promoter reporter gene activity for up to 48 h after a single application of TNF-α. We found that TNF-α rapidly induced NF-κB DNA binding activity in both HT29 and HCT116 cells. This induction persisted for up to 24 h, with 3–4-fold increases over base-line NF-κB DNA binding activity remaining evident at that time point (Fig. 1A). Similar results were found in other intestinal epithelial cell lines, HCT15, HCA7, SW48, RKO, and HCT8 (supplemental Fig. 1).

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3The abbreviations used are: AMC, 7-amido-4-methylcoumarin; 3-MA, 3-methyladenine.
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Little p65/RelA was evident in nuclear protein extracts from resting cells, but TNF-α resulted in rapid nuclear translocation and phosphorylation of p65/RelA (Fig. 1B). Both nuclear translocation and phosphorylation, which are markers of active NF-κB, persisted for up to 24 h following the application of TNF-α. TNF-α also rapidly induced NF-κB luciferase reporter gene activity. Notably, the amount of luciferase activity progressively increased for up to 48 h following TNF-α application, which indicates that NF-κB was persistently activated at least up to this time (Fig. 1C).

To determine if prolonged NF-κB activity was accompanied by native gene transcription, we assessed the mRNA expression of a panel of established NF-κB target genes. Both HT29 and HCT116 cells responded to TNF-α by increasing the abundance of mRNA of most of the genes (Fig. 1D). The magnitude of the induction of expression of individual genes by TNF-α varied, indicating heterogeneity in the responsiveness of gene promoters to NF-κB. Elevated gene expression was detected both at 1 h and at 24 h following stimulation. Some genes had greater abundance of mRNA expression at 1 h, whereas others had the greatest expression after 24 h, possibly reflecting different kinetics of mRNA transcription and degradation. In addition to mRNA expression, IL-8 secretion by TNF-α-stimulated HT29 cells was significantly increased for up to 24 h following TNF-α stimulation (Fig. 1E). Taken together, these data indicate that a single stimulation of TNF-α results in the prolonged activation of NF-κB, which results in elevated target gene expression for at least 24 h.

TNF-α Stimulation Results in a Biphasic Pattern of IkBα Protein Expression—In intestinal epithelial cells, NF-κB activity is controlled primarily by IkBα (13, 14). Consistent with established paradigms of stimulus-induced degradation of IkBα followed by its reaccumulation, TNF-α resulted in the rapid loss of IkBα expression in cells, which was followed by its re-expression 1–2 h later (Fig. 2A). Notably, when we monitored expression at later time points, IkBα levels fell for a second time and remained suppressed relative to baseline for up to 72 h. This biphasic pattern of protein expression was also seen in HCT15, HCT8, HCA7 RKO, and SW48 intestinal epithelial cells (supplemental Fig. 2A). Serine 32/36-phosphorylated IkBα was not detected at the base-line condition but was observed following re-expression of total IkBα at the 1 h time point (Fig. 2B). Levels of phosphorylated IkBα gradually declined at later time points, coincidental with loss of total IkBα. Toll-like receptor ligands lipopolysaccharide and flagellin did not produce robust IkBα responses, despite preconditioning HT29 cells with interferon-γ, which increases responsiveness of those cells to such ligands (supplemental Fig. 2B). We also assessed the expression levels of other proteins involved in the TNF-α-NF-κB pathway but found no evidence of biphasic expression patterns for those proteins (Fig. 2C). The low levels of IkBα expression observed at the later time points following stimulation with TNF-α are consistent with the prolonged induction of NF-κB activity.

We next assessed the expression levels of IkBa protein following TNF-α stimulation to determine if the second phase of suppression of IkBa protein might be due to low mRNA levels. Results showed that expression of IkBa mRNA was persistently up-regulated following TNF-α stimulation. By 24 h poststimulation, IkBa mRNA levels remained 14–26-fold above baseline levels in HT29 and HCT116 cells (Fig. 3). Similar findings were obtained in HCT15, HCT8, HCA7 RKO, and SW48 intestinal epithelial cells (supplemental Fig. 3). Therefore, although IkBa protein levels are low at the later time points after TNF-α
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Inhibiting Alternative Mechanisms for IkBα Proteolysis Does Not Restore Protein Expression—In addition to the proteasome, prior studies had provided evidence for alternative pathways to IkBα degradation, including calpain (16), casein kinase II (17, 18), and caspases (19). We therefore tested the effects of pharmacological inhibitors of those enzymes on short and long term responses of HCT116 and HT29 cells to TNF-α. Results revealed that those inhibitors failed to block either the first or second phase of TNF-α-induced IkBα degradation (supplemental Fig. 6).

TNF-α Induces Autophagy in HT29 Cells—In continued efforts to explain the second phase of IkBα degradation, we turned to an alternative mechanism of protein loss, autophagosomal proteolysis. Autophagy, a process of cellular self-digestion can be induced by a variety of cellular stresses (reviewed in Ref. 20), so we initially assessed the possibility that TNF-α might induce this process of cellular self-digestion in HT29 cells, as was previously reported in sarcoma cells (21). We used transmission electron microscopy to monitor the accumulation of autophagosomal compartments in HT29 cells following stimulation with TNF-α. Autophagosomal compartments were identified as double membrane vacuoles containing cytoplasm or cytoplasmic organelles (22). In unstimulated cells, few autophagosomal compartments are seen, but with TNF-α stimulation, a progressive accumulation of autophagosomal compartments occurs over 24 h (Fig. 5A). Notably, the time course of autophagosomal compartment appearance following TNF-α coincides with that of IkBα disappearance. We also examined the cytosolic abundance of LC3, a specific marker of macroautophagy, which is modified from cytosolic LC3-I to membrane-bound LC3-II upon activation of autophagy (23). TNF-α stimulation resulted in an increase in LC3-II, especially in the presence of the lysosome inhibitors pepstatin A and E64d (Fig. 5B). In addition, punctate immunofluorescent staining of LC3, another indicator of autophagosome formation, was up-regulated following TNF-α stimulation (Fig. 5C). The phosphoinositol 3-kinase (PI3K) pathway has been implicated as a key signaling pathway in stress-induced autophagy (24). Therefore, we examined the effect of inhibitors of this pathway on TNF-α-induced autophagy in HT29 cells. Results showed that treatment with the PI3K inhibitors 3-methyladenine (3-MA) and wortmannin or with the lysosome inhibitors pepstatin A and E64d (supplemental Fig. 7) blocked TNF-α-induced autophagosomal compartment formation (Fig. 5D). Together, these findings indicate that HT29 cells respond to TNF-α stimulation by activation of the PI3K pathway, resulting in the induction of lysosome-dependent autophagy.

Evidence for Autophagosomal Degradation of IkBα—To assess the possibility that IkBα degradation was linked to autophagy, we monitored the localization of IkBα with the...
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autophagosome-associated protein, LC3, using confocal co-immunofluorescence (Fig. 6A). In unstimulated cells, expression of IκBα (green) and LC3 (red) showed little co-localization (yellow). However, by 8 and 24 h following stimulation, there was a significant increase in punctate yellow immunofluorescence. The TNF-α-induced punctate co-localization of IκBα with LC3 supported the notion that IκBα might be regulated by autophagy. Therefore, we next assessed if lysosomal proteases had any role in the loss of IκBα expression following TNF-α stimulation. Pretreatment of HT29 cells with leupeptin and ammonium chloride or with pepstatin A and E64d, which inhibit lysosomal protein degradation, partially blocked both the early and the late phases of IκBα degradation (Fig. 6B). The PI3K inhibitors LY294002 and wortmannin and the Akt inhibitor triciribine partially blocked the early phase of TNF-α-stimulated IκBα degradation but almost completely blocked the second phase of IκBα disappearance (Fig. 6C). More strikingly, the type III PI3K inhibitor 3-MA (25) had no effect on the early phase of IκBα loss but completely blocked the second phase of its degradation.

Additional support for the concept of autophagosomal IκBα degradation was obtained in experiments using mouse fibroblasts stably expressing shRNA targeting LAMP2A, a critical lysosomal protein involved in chaperone-mediated autophagy. LAMP2A(−) fibroblasts have been reported to exhibit enhanced macroautophagy (26). Wild-type fibroblasts exhibited a biphasic pattern of IκBα expression following exposure to TNF-α that is similar to that observed in HT29 cells (Fig. 7A). In contrast, LAMP2A(−) cells had very low levels of IκBα expression irrespective of stimulation, indicating that the cells with enhanced autophagy have constitutively low levels of IκBα. Next, we utilized embryonic fibroblasts obtained from ATG5

FIGURE 5. TNF-α induces autophagy in HT29 cells. A, transmission electron micrography of HT29 cells stimulated with TNF-α (10 ng/ml) for the indicated times. Mean cell volume and autophagic compartment volume were assessed using stereology. B, HT29 cells were stimulated with TNF-α (10 ng/ml) or left unstimulated following a 24-h preincubation with lysosome inhibitors (L) pepstatin A and E64d. Protein extracts were immunoblotted for LC3 and β-actin. C, confocal immunofluorescence analysis of LC3 in HT29 cells left unstimulated or stimulated with TNF-α (10 ng/ml), with or without a 24-h preincubation with lysosome inhibitors pepstatin A and E64d. D, transmission electron micrography of HT29 cells pretreated with 3-MA (10 mM) or wortmannin (Wm; 100 mM) and stimulated with TNF-α (10 ng/ml) for 24 h.

FIGURE 6. Evidence for autophagosomal degradation of IκBα in HT29 cells. A, cells were left untreated or were stimulated with TNF-α (10 ng/ml) for the indicated times. Confocal microscopy was used to assess localization of IκBα (green) and LC3 (red) and merge (yellow). The autothreshold function was used in capturing these images. B, HT29 cells were pretreated with lysosomal inhibitors leupeptin (100 μM) and ammonium chloride (20 mM) (L&N) or pepstatin A and E64d (10 ng/ml) (P&E), followed by stimulation with TNF-α for the indicated times (h). Cellular extracts were immunoblotted for IκBα protein and β-actin. C, HT29 cells were pretreated with PI3K/AKT inhibitor 3-MA (10 mM), triciribine (20 μM) (TRI), wortmannin (100 mM) (Wm), or LY294002 (20 μM) (LY) prior to TNF-α (10 ng/ml) stimulation for the indicated times (h). Cellular extracts were immunoblotted for IκBα and β-actin.
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knock-out mice to determine if a genetically mediated loss of autophagy affected IκBα expression (27). Similar to intestinal epithelial cells, Western blotting revealed a biphasic pattern of IκBα expression in control wild-type mouse embryonic fibroblasts following TNF-α stimulation. However, ATG5−/− cells displayed the first phase of TNF-α-stimulated IκBα degradation but failed to completely degrade IκBα during the second phase (Fig. 7B). Taken together, these pharmacological and genetic data demonstrate that TNF-α results in the activation of autophagy via a pathway involving PI3K and Akt. IκBα tracks to autophagosomes, which appear to promote its lysosomal protease-mediated degradation, resulting in lower expression levels.

Finally, to assess the functional consequences of autophagy up-regulation in HT29 cells in response to TNF-α, we assessed the effects of the type III PI3K inhibitor 3-MA on TNF-α-induced expression of NF-κB target genes and IL-8 protein secretion. Results show that, in the presence of 3-MA, up-regulated mRNA expression of NF-κB target genes by TNF-α was reduced both at the early 1 h time point and more strikingly at the 24 h time point (Fig. 8A). In addition, TNF-α-induced IL-8 secretion was repressed in the presence of 3-MA (Fig. 8B). Similar results were obtained with other PI3K inhibitors and lysosome inhibitors (supplemental Fig. 8).

DISCUSSION

Our work has revealed a previously unrecognized mechanism by which NF-κB can be maintained in a persistently activated state through the degradation of IκBα by autophagy. In contrast to the early, short term activation of NF-κB that is mediated by proteosomal degradation of IκBα, the induction of autophagy by TNF-α appears to cause a delayed but more prolonged reduction of IκBα levels. This leads to the long term up-regulation of NF-κB target gene expression, magnifying the inflammatory response. In this way, autophagy may promote innate immunity by promoting long lasting NF-κB activity.

Studies of biospecimens obtained from chronic human inflammatory diseases and cancers and from animal models of diseases have shown NF-κB activity in numerous cell types. Many of those studies have documented the nuclear localization of NF-κB proteins (4, 5) as well as the up-regulated expression of nuclear factor target genes (28). Because those diseases have natural histories that extend over months to years, it appears that NF-κB is persistently active for that length of time. That long time frame is in marked contrast to the short time within which a framework exists for understanding the regulation of NF-κB activity, which extends only to about 4 h. Extensive work has documented a paradigm for cell surface receptor-initiated signaling cascades that lead to activation of the IκB kinase complex that targets IκBα for proteosomal degradation, leading to the nuclear translocation of NF-κB (1). IκBα resynthesis under the control of NF-κB shuts off the signaling pathway. Multiple layers of additional complexity in this paradigm have been documented, including post-translational modifications of NF-κB proteins (29, 30), shuttling of NF-κB and IκB proteins between cytoplasm and nucleus (31), and short term oscillations in nuclear factor activity controlled by different IκB proteins (32–34). Nevertheless, little is known about the control of NF-κB over time periods longer than about 4 h.

Our work has shown that a single application of TNF-α to cultured cells causes a persistent activation of NF-κB that persists for up to 48 h. During the second phase of IκBα protein loss that permits persistent NF-κB activity in this experimental system, IκBα mRNA expression remains about 20-fold above base-line values. The biphasic IκBα protein response was not observed with lipopolysaccharide or flagellin, two Toll-like receptor ligands, or with interleukin-1 (data not shown), probably reflecting the relative hypersensitivity of intestinal epithelial cells to such ligands. We searched for proteolytic mechanisms that could explain the second phase loss of IκBα expression following TNF-α and could exclude most of the processes described previously for the degradation of IκBα from involvement in the second phase. However, our results have pointed toward the involvement of autophagosomes and their lysosomal proteases in the second phase degradation of IκBα.

Autophagy is a process of cellular self-digestion in which cytoplasmic organelles or proteins are sequestered into double-membrane vesicles and digested by lysosomal proteases, allowing the “recycling” of biomolecules under conditions of starva-
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In this context, autophagy is viewed as a physiological cell survival response. A recent wave of interest in autophagy has characterized numerous alternative inducers, including inflammatory factors, such as interferon-γ (35), lipopolysaccharide (36), and the NOD2 ligand peptidoglycans (37, 38). Our results show that exposure to TNF-α also results in the induction of autophagy in intestinal epithelial cells, lending further support to the notion that an interplay exists between immunity and autophagy.

Our work with PI3K inhibitors indicated that TNF-α-induced autophagy depended on the signaling pathways mediated by this family of kinases. In particular, the type III PI3K inhibitor 3-MA exhibited the strongest and most selective blockade of the second phase of IκBα degradation, a finding that is consistent with the established role of 3-MA as an inhibitor of autophagy (25). We found that PI3K inhibitors, including the non-selective ones wortmannin and LY294002 as well as 3-MA, lowered both the early and the later phases of TNF-α-induced NF-κB gene expression. This observation could reflect a role for PI3K in TNF-α-induced NF-κB activity, a possibility that has been proposed previously (39) and which is supported by the partial inhibition of the early phase of IκBα degradation, a finding that is consistent with the established role of 3-MA as an inhibitor of autophagy (25). Alternatively, it is possible that proteasomal and autophagosomal IκBα degradation proceed simultaneously following TNF-α stimulation but that proteasomal degradation of IκBα predominantly occurs during the first phase and that autophagosomal degradation of IκBα occurs during the second phase. This possibility is supported by the inhibitory effects we observed of the type III PI3K inhibitor 3-MA and the lysosomal protease inhibitors on early and late NF-κB target gene induction.

Several recent papers have explored cross-talk between the NF-κB pathway and autophagy. One key observation has been that the RelA/p65 NF-κB protein induces the expression of Beclin1, a protein required for the induction of apoptosis (40). A role for the NF-κB pathway in autophagy was further supported by the finding that the IKK complex was necessary for the stimulation of autophagy by several factors, including starvation, rapamycin, or pifithrin-α. Intriguingly, the role of IKK in stimulating autophagy did not appear to involve NF-κB (41). However, in contrast to these data are several reports that NF-κB appears to inhibit autophagy under certain experimental conditions, including TNF-α-induced cell death (21). Other recent studies have reported evidence of a role for autophagy in the control of NF-κB pathway proteins (42). More work is needed to clarify stimulus- and cell type-specific regulation of NF-κB by autophagy and vice versa.

Genome-wide association studies have identified polymorphisms in two autophagy-related genes, ATG16L and IRGM, that are linked to the presence of Crohn disease (43), a chronic inflammatory disorder of the intestines. Prior to the discovery of this genetic association, autophagy was not known to be linked to intestinal inflammation, but recent work has identified a potential mechanism by which defective function of ATG16L could be linked to Crohn disease. It was found that the secretion of antimicrobial defense proteins by intestinal Paneth cells was defective in the absence of functional ATG16L, possibly reflecting the role of this protein in controlling intracellular membrane trafficking and dynamics (44, 45). Other recent work has linked NOD2 and ATG16L by showing that NOD2 ligands induce autophagy, which is required for normal intracellular bacterial killing (37). Our findings present an alternative hypothesis to explain the link between ATG16L and Crohn disease. In Crohn disease patients with low activity alleles of autophagy genes, the ability of cells to degrade IκBα and mount robust prolonged NF-κB activation and up-regulated innate immune responses could be dampened. Impaired NF-κB-dependent innate immunity has already been linked to Crohn disease pathogenesis in the case of NOD2 mutations (reviewed in Ref. 46). Thus, Crohn disease may be more likely in individuals with a reduced ability to persistently activate NF-κB whether due to NOD2 or autophagy gene variants.

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