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## The Role of Autophagy in the Degradation of Misfolded HLA-B27 Heavy Chains

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### Abstract

**Objective.**—To determine whether autophagy is involved in the degradation of misfolded HLA-B27 in experimental spondyloarthritis.

**Methods.**—Bone marrow derived macrophages from HLA-B27 and human  $\beta_2m$  ( $h\beta_2m$ ) transgenic rats were examined with and without IFN $\gamma$  and proteasome or autophagy inhibitors. Immunoprecipitation, western blotting, and immunofluorescence were used to measure HLA-B27 heavy chains and autophagy. Autophagy was induced using rapamycin. HLA-B7/ $h\beta_2m$  transgenic and wild type rat macrophages were used as controls.

**Results.**—HLA-B27-expressing macrophages showed similar LC3B-II levels compared to both controls, before and after manipulating autophagy. Blocking autophagic flux with bafilomycin resulted in the accumulation of misfolded HLA-B27 dimers and oligomers as well as monomers, comparable to blocking endoplasmic reticulum-associated degradation (ERAD) with the proteasome inhibitor bortezomib. HLA-B7 monomers also accumulated after blocking each degradation pathway. The proportion of ubiquitinated heavy chains was ~3-fold lower for HLA-B27 compared to HLA-B7. Activation of autophagy with rapamycin rapidly eliminated ~50% of misfolded HLA-B27, while folded HLA-B27 or HLA-B7 monomeric heavy chains were minimally affected.

**Conclusion.**—These results demonstrate for the first time that both autophagy and ERAD play a role in the elimination of excess HLA class I heavy chains expressed in transgenic rats. We found no evidence that HLA-B27 expression modulated the autophagy pathway. Our results suggest that impaired ubiquitination of HLA-B27 may play a role in the accumulation of misfolded disulfide-linked dimers, whose elimination can be enhanced by activation of autophagy. Manipulation of the autophagy pathway should be further investigated as a potential therapeutic avenue in spondyloarthritis.

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#### AUTHOR CONTRIBUTIONS

F.N. and R.A.C. designed the experiments and F.N., G.L-S., K.A.S., and A.C. performed the experiments. Data were analyzed by F.N., R.A.C., G.L-S., and K.A.S.; F.N. and R.A.C. wrote the paper, and G.L-S., K.A.S., and A.C. provided editing.

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## Keywords

HLA-B27; spondyloarthritis; ER quality control; ER-associated degradation; autophagy

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## INTRODUCTION

HLA-B27 is strongly associated with the development of spondyloarthritis (SpA), and in particular axial disease that progresses to ankylosing spondylitis (1). While the pathogenic mechanisms remain incompletely understood, abnormal properties of the HLA-B27 heavy chain have been implicated (2, 3). HLA-B27 exhibits a propensity to misfold and dimerize in the endoplasmic reticulum (ER) (4–6), and to form  $\beta_2m$ -free homodimers on the cell surface (2). HLA-B27 has also been implicated in dendritic cell dysfunction (7, 8). Each of these properties has been mechanistically linked to the IL-23/IL-17 axis where several additional risk genes are located (9), supporting a central role for this axis in pathogenesis (10). Indeed, when HLA-B27 is expressed in rats along with human  $\beta_2m$  (h $\beta_2m$ ), a SpA-like disease that is dependent on gut microbiota develops spontaneously (11), with striking activation of the IL-23/IL-17 axis in the inflamed gut and involved joints (12, 13). Interleukin-17A (IL-17A) has recently been established as a therapeutic target in ankylosing spondylitis (14), and there is evidence that blocking IL-23/IL-12 may be beneficial (15), underscoring the importance of this axis in human disease, and the relevance of this animal model for dissecting mechanisms.

The abnormal behavior of HLA-B27 in the endoplasmic reticulum (ER) involves the formation of disulfide-linked dimers by newly synthesized heavy chains (5, 16–18). These misfolded heavy chains accumulate bound to the ER chaperone BiP (also known as Grp78; encoded by *Hspa5*), and under certain circumstances can activate an ER stress response that alters immune signaling pathways (17, 19). There are ER quality control pathways that orchestrate the elimination of misfolded proteins, usually mitigating the threat they pose to ER homeostasis (20). HLA-B27 has been shown to undergo ER associated degradation (ERAD) (4, 21, 22), which involves dislocation of target proteins from the ER membrane, with retrotranslocation and ubiquitination in the cytosol where they are degraded by proteasomes (23). While the machinery differs for different proteins, ERAD of HLA class I and HLA-B27 is dependent on the E3 ubiquitin ligase, HRD1 (3-hydroxy-3-methylglutaryl-coenzyme A reductase degradation protein 1) (also known as synoviolin; encoded by *SYVN1*), and the E2 ubiquitin conjugating enzyme UBE2J1 (21, 22). Depletion of HRD1 as well as other components of the ERAD pathway (e.g. XBP1, derlin-1, derlin-2) promotes the accumulation of misfolded HLA-B27 heavy chain dimers in HeLa cells. Moreover, forced overexpression of EDEM1 (ER degradation enhancing  $\alpha$ -mannosidase-like protein 1), which targets unsalvageable ER glycoproteins for ERAD, or upregulation of the ERAD pathway, enhances the elimination of HLA-B27 dimers (22). Despite ERAD serving a role in ER quality control (ERQC) for HLA-B27, it is insufficient to prevent the accumulation of misfolded dimers in cells expressing this HLA class I molecule (5, 17).

Autophagy delivers cytoplasmic organelles and macromolecules to lysosomes for destruction (24), and is important for cellular survival during short-term starvation, recycling

of damaged organelles, differentiation of several cell types, and for the clearance of intracellular microbes (24–27). In macroautophagy (generally referred to as autophagy), a double membrane is formed around targeted organelles or aggregated proteins creating an autophagosome, that subsequently fuses with lysosomes (28). Autophagy is important for the elimination of terminally misfolded cytosolic and ER luminal proteins that are resistant to proteasomal degradation (29, 30), and more recently was shown to degrade non-aggregated membrane proteins that are resistant to ERAD (31). This prompted us to ask whether autophagy is also involved in the degradation of misfolded forms of HLA-B27.

Here, we show that autophagy as well as ERAD contributes to the elimination of misfolded disulfide-linked HLA-B27 heavy chains. Moreover, activation of autophagy with rapamycin can be used to promote degradation of misfolded HLA-B27. We also show that HLA-B27 dimers are poorly ubiquitinated relative to the control substrate HLA-B7, suggesting that the formation of disulfide linked dimers may be an impediment to efficient ERAD. These results raise the possibility that the autophagy pathway might be exploited for therapeutic benefit.

## MATERIALS and METHODS

### Animals.

Hemizygous HLA-B\*27:05 and human  $\beta_2m$ -transgenic (B27-Tg) Lewis rats carrying 55 copies of HLA-B27 and 66 copies of human  $\beta_2m$  in the 33–3 locus on the Lewis background were used as described previously (32). The HLA-B\*07:02 and human  $\beta_2m$ -transgenic (B7-Tg) rat line (Lewis, 120–4 transgene locus) was kindly provided by Joel Taurog (University of Texas Southwestern Medical Center, Dallas, TX). Homozygous B7-Tg rats containing 52 copies of HLA-B7 and 10 copies of human  $\beta_2m$  were used, as were wild type (WT) Lewis transgene negative littermates. Rats were bred and housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities on the Bethesda campus of the National Institutes of Health. All animal experiments were preapproved by the Animal Care and Use Committee at the National Institute of Arthritis and Musculoskeletal and Skin Disease.

### Isolation of bone marrow-derived macrophages.

Bone marrow was obtained from the tibias and femurs of 8–12 week old Lewis rats following euthanasia. To derive macrophages, non-adherent bone marrow derived monocytes were incubated at  $5 \times 10^6$ /mL in high-glucose Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal calf serum (Thermo Fisher Scientific), 100 U/mL Penicillin, 100 ug/ml Streptomycin (Thermo Fisher Scientific), and 20 ng/mL macrophage colony-stimulating factor (M-CSF; Peprotech, Rocky Hill, NJ). Cells were cultured at 37°C in humidified atmosphere containing 5% CO<sub>2</sub>. Media was changed every other day. Cells were used for experiments at day 7.

### Reagents and antibodies.

IFN $\gamma$  was used at 50 ng/ml for all experiments. For autophagy modulation the following reagents were used: Rapamycin (InvivoGen, San Diego, CA), Bafilomycin A1 (Bafilomycin) (InvivoGen) and Chloroquine diphosphate salt (Sigma-Aldrich, St. Louis,

MO). Bortezomib (Cell Signaling, Danvers, MA) was used to block proteasomal degradation of proteins and hence ERAD. For western blotting and immunoprecipitation the following antibodies were used: rabbit anti-LC3B antibody (Sigma-Aldrich), rabbit anti- $\beta$  Tubulin antibody (Abcam, Cambridge, UK), goat anti-Grp78/Hspa5 antibody (R&D Systems, Minneapolis, MN), mouse HC10 antibody (33) and rat 3B10.7 (5), recognizing HLA class I. For flow cytometry, the ME1 antibody was used. ME1 recognizes HLA-B27 and other B alleles, but not rat class I. As secondary antibodies for western blotting, horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-goat, anti-mouse (R&D Systems) and anti-rat (SouthernBiotech, Birmingham, AL) were used. For immunofluorescence rabbit anti-LC3B antibody (Cell Signaling) and Alexa Fluor 488 conjugated donkey anti-rabbit secondary antibody (Life Technologies, Carlsbad, California) were used.

### **Immunofluorescence.**

For immunofluorescence experiments, macrophages from WT, B7-Tg or B27-Tg rats were incubated with different autophagy modulators for 2 hours. Cells were then fixed and permeabilized with ice-cold methanol and incubated with 5% normal goat serum (Life Technologies) for one hour at room temperature, followed by overnight incubation at 4°C with the primary antibody (anti-LC3B, Cell Signaling). After washing with PBS, cells were incubated with the fluorescent-labeled secondary antibody (anti-rabbit Alexa 488) for 2 hours at room temperature. The nucleus was visualized with DAPI (Sigma-Aldrich). Immunofluorescence was evaluated by using the confocal fluorescence microscope Leica DMI6000 inverted microscope SP5 NLO (Leica Microsystems, Wetzlar, Germany) at 63x magnification.

### **Immunoprecipitation and immunoblotting.**

To prevent spontaneous sulfhydryl bond formation and breakdown, at the end of each experiment and immediately prior to harvest, cells were treated for 20 min with 10 mM methyl methanethiosulfonate (MMTS, Thermo Scientific) in PBS on ice. Cells were then lysed with lysis buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol; pH 7.4; Thermo Fisher Scientific) containing complete, EDTA-free proteinase inhibitor cocktail tablets (Roche, Indianapolis, IN) for 20 min on ice. After centrifugation at 13,000 g for 10 min to remove nuclei, supernatants were used for western blot analysis or immunoprecipitation. For western blotting, lysates were diluted with non-reducing 5x sample buffer (Thermo Fisher Scientific) and boiled for 5 min at 95°C. In order to reduce samples, 4x sample buffer containing dithiothreitol (Sigma-Aldrich) was used. Proteins were separated on a 4–20% Tris/Glycine gel (BioRad, Hercules, CA). After immunoblotting, the PVDF membrane was blocked with 5% milk in PBS containing 0.02% Tween (PBS-T) buffer for 1 hour and incubated overnight at 4°C in 5% BSA/PBS-T with the following first antibodies: 3B10.7 to detect HLA class I heavy chains, goat anti-BiP (R&D Systems), rabbit anti-LC3B (Sigma-Aldrich), rabbit anti-Ubiquitin (Cell Signaling) and rabbit anti- $\beta$ Tubulin (Abcam). After three washes the membrane was incubated with the respective HRP-conjugated the secondary antibody (R&D Systems) for 1 hour at room temperature. Proteins were visualized using the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Prior to immunoprecipitation, sample protein concentration was measured using the BioRad DC Protein Assay (BioRad). Equal amounts of protein were used for immunoprecipitations.

Immunoprecipitation with the HC10 antibody was performed using the Pierce Crosslink IP kit (Thermo Fisher Scientific) according to manufacturers guidelines. To immunoprecipitate polyubiquitin protein conjugates, the Pierce Ubiquitin Enrichment kit (Thermo Fisher Scientific) was used.

### Flow cytometry.

To measure cell surface expression of HLA-B27, macrophages were washed and incubated with 5% BSA (Sigma-Aldrich) in PBS at 4°C for 30 min, and then incubated with allophycocyanin (APC)-labeled HC10 antibody or Pacific Blue-labeled ME1 antibody for 30 min at 4°C. Cells were washed and analyzed by FACS using a Beckman Coulter CyAn ADP (Beckman Coulter).

### Statistical analysis.

Statistical analysis was performed using Student's *t* test. Differences between means with *p* < 0.05 were considered statistically significant using the GraphPad Prism Version 6.0b (LaJolla, CA). Unless otherwise stated, the figures show three independent experiments.

## RESULTS

### Autophagy and ERAD contribute to the degradation of HLA-B27.

To determine whether autophagy plays a role in the degradation of HLA-B27 we assessed bone marrow-derived macrophages (BMM) from HLA-B27 Tg rats. BMM were derived *in vitro* in the absence of pro-inflammatory cytokines, and under these conditions express low levels of  $\beta_2m$ -free HLA-B27 heavy chains (17, 34). To determine the relative contribution of autophagy and ERAD to the elimination of HLA-B27, BMM were incubated without or with IFN $\gamma$  for 21 hours to upregulate HLA-B27 expression, and then bafilomycin A1 (bafilomycin) or bortezomib were added for the final 3 hours. Bafilomycin inhibits the vacuolar H<sup>+</sup> ATPase, which prevents the maturation of autophagic vacuoles and autophagic degradation of proteins (35). Bortezomib binds to the active sites of the  $\beta_5$  and  $\beta_1$  subunits of the proteasome (36) and inhibits proteasomal degradation of proteins, which is essential for ERAD (37). Cells were lysed and HLA-B27 heavy chains immunoprecipitated (IP) from equal amounts of protein with the HC10 antibody, separated by non-reducing SDS-PAGE, and visualized by immunoblotting with the anti-HLA class I antibody 3B10.7 (Fig. 1). HC10 recognizes HLA-B (and HLA-C) heavy chains that are not associated with  $\beta_2m$  (38), including unfolded and misfolded forms that exist as monomers, dimers, or oligomers (5). In rat cells that lack other HLA-B/C alleles, only HLA class I expressed from the transgene is recognized. Co-precipitating BiP bound to HLA-B27 heavy chain was also visualized by immunoblotting after running an aliquot of the same HC10 IP on a separate gel.

In the absence of IFN $\gamma$ , relatively little  $\beta_2m$ -free HLA-B27 heavy chain is detected in IPs from macrophages (Fig. 1A). HLA-B27 is strongly upregulated by IFN $\gamma$  resulting in the accumulation of heterogeneous disulfide-linked dimers and oligomers of HLA-B27 (also referred to as misfolded forms), as well as monomers (also referred to as free heavy chains) (Fig. 1A). Proteasome inhibition with bortezomib (Bor) resulted in a 1.5 to 1.7-fold increase in dimeric and oligomeric forms of HLA-B27, respectively. This confirms previous results

demonstrating that misfolded HLA-B27 undergoes ERAD in human cells (4, 21, 22), and extends these findings to HLA-B27 Tg rats. Blockade of autophagic flux with bafilomycin (Baf) induced a similar (1.3 to 1.4-fold) accumulation of HLA-B27 dimers and oligomers, with effects of proteasome inhibition being slightly more prominent. Bafilomycin and bortezomib also led to a 1.2–1.3-fold increase in the accumulation of monomers. Inhibition of autophagy and ERAD also resulted in the accumulation of BiP in HLA-B27 heavy chain immunoprecipitates (2 to 3-fold). BiP accumulates bound to misfolded HLA-B27 in the ER (17), and has been shown to preferentially accumulate with HLA-B27 dimers (16). Treatment with each inhibitor alone in cells not treated with IFN $\gamma$  did not lead to accumulation of HLA-B27, indicating that neither autophagy nor ERAD are contributing significantly to rapid heavy chain degradation at baseline. Similar experiments were performed with wortmannin and LY294002, both of which inhibit autophagosome formation by blocking phosphatidylinositol 3-kinase. We obtained results similar to bafilomycin, supporting involvement of the autophagy pathway (unpublished observations).

We next asked whether transfected cells that constitutively express HLA-B27 (e.g. C1R.B27) in the absence of pro-inflammatory cytokines also use autophagy to degrade misfolded heavy chains. We have shown previously that C1R.B27 cells eliminate a proportion of HLA-B27 through the ERAD pathway, with deglycosylated heavy chains accumulating in the cytosol after 3 hours of proteasome inhibition (4). We treated C1R.B27 cells for 3 hours with bortezomib or bafilomycin, then assessed accumulation of various forms of HLA-B27 heavy chain (Fig. 1B). Both bafilomycin and bortezomib increased the accumulation of HLA-B27, with the effect on dimers being most prominent, followed by oligomers and monomers. Co-precipitating BiP also increased. Bafilomycin was more effective than bortezomib at inhibiting HLA-B27 degradation and causing accumulation of heavy chains bound to BiP in C1R.B27 cells. To demonstrate that both inhibitors were blocking the destruction of proteins targeted for degradation, we examined ubiquitinated complexes in whole cell lysates. In both rat BMM and C1R cells, incubation with bafilomycin or bortezomib for 3 hours led to accumulation of ubiquitinated proteins compared to untreated cells, or cells treated with IFN $\gamma$  alone (Suppl. Fig. S1). Only bafilomycin caused an increase in LC3B-II, a marker of autophagy (35, 39), indicating that bortezomib was not affecting autophagic flux under these conditions (Suppl. Fig. S1). Taken together these results show that in addition to ERAD, rat BMM as well as C1R cells use the autophagy pathway to degrade misfolded dimers and oligomers, as well as some monomers of HLA-B27.

### **Autophagy is not altered by HLA-B27 expression in rat bone marrow macrophages.**

The involvement of autophagy in HLA-B27 degradation raised the possibility that this pathway might be altered in HLA-B27-expressing rat cells. To test this, BMM from WT, B27-Tg and B7-Tg rats were incubated with bafilomycin or chloroquine to block autophagic flux, or with rapamycin, which activates autophagy by inhibiting mTOR. Changes in LC3B-II level were monitored as a marker for autophagic flux (39). The LC3B protein (encoded by *Map1lc3b*) is cleaved at the C-terminus forming LC3B-I, which is localized in the cytosol. During the initiation of autophagy, LC3B-I is conjugated to phosphatidylethanolamine (PE) to form LC3B-II, which becomes embedded in the autophagosome membrane. Hence, an

increase in LC3B-II is widely used as a measure of autophagy as it reflects the number of autophagosomes. The antibody used for western blotting reveals two bands representing LC3B-I (upper) and LC3B-II (lower) (e.g. Fig. 2A; LC3B-II migrates faster due to the PE moiety). Increased accumulation of LC3B-II can reflect activation of autophagy (until autophagosomes are destroyed and steady state is reached), or inhibition of later stages of autophagy, which leads to accumulation of autophagosomes. Therefore, inhibitors (chloroquine and bafilomycin) as well as activators of autophagy (rapamycin) both lead to increases in LC3B-II under these conditions (13). No differences in LC3B-II levels were detected between untreated WT, B27-Tg, and B7-Tg macrophages by immunoblotting (Fig. 2A, NT lanes). Within each group, chloroquine, bafilomycin and rapamycin all increased LC3B-II accumulation as expected (Fig. 2A, lanes C, Baf, and Ra), but there were no differences between genotypes, indicating that the autophagy pathway is not altered by baseline (unstimulated) HLA-B27 or HLA-B7 expression. This was further confirmed by immunofluorescence staining of LC3B-II in macrophages. While chloroquine, bafilomycin, and rapamycin all increase vesicular staining for LC3B-II, there were no apparent differences between genotypes (Fig. 2B). We also asked whether treatment of cells with IFN $\gamma$  would expose differences between genotypes due to either HLA-B27 or HLA-B7 upregulation. Again, there were no differences between WT and HLA class I-expressing cells incubated with bafilomycin alone, IFN $\gamma$  alone, or both (Fig. 2C). It should be noted that activating autophagy in macrophages requires high concentrations of rapamycin that could be toxic (40). Therefore, we confirmed that the dose we used (25  $\mu$ M) did not affect macrophage viability after 2–4 hours of incubation (unpublished observations). These data indicate that the autophagy pathway is not grossly altered by HLA-B27 or HLA-B7 expression in rat macrophages.

#### **HLA-B27 heavy chains are poorly ubiquitinated relative to HLA-B7.**

Many proteins that misfold in the ER are efficiently degraded by ERAD, while others (e.g. HLA-B27) can accumulate and result in cellular dysfunction (30, 41). Resistance to ERAD is incompletely understood, but one contributing factor is the formation of aberrant intermolecular disulfide bonds that result in dimerization or oligomerization (42). Since ERAD requires ubiquitination, we asked whether HLA-B27 heavy chains were ubiquitinated using HLA-B7 for comparison. Homozygous HLA-B7 Tg rats were used so that comparable amounts of HLA class I heavy chain (to HLA-B27) would be expressed and upregulated in response to IFN $\gamma$  (34). In experiments similar to those shown in Figure 1, we noted that HLA-B7 heavy chain monomers accumulate in IFN $\gamma$ -treated cells when autophagy or ERAD are inhibited (Fig. 3A), indicating that excess HLA-B7 heavy chains are also being eliminated by quality control pathways. Next, aliquots of the same HC10 immunoprecipitates shown in Fig. 3A were subjected to SDS-PAGE and probed for ubiquitin. Fig. 3B shows that highly ubiquitinated HLA class I heavy chains accumulate when ERAD is inhibited, and to a lesser extent when autophagy is blocked. We calculated the ratio of ubiquitinated material in the HC10 immunoprecipitates (e.g. Fig 3B) to total heavy chain in the same immunoprecipitates (e.g. Fig. 3A, reduced band) from five experiments, and plotted the ubiquitin to heavy chain ratio (Ub:HC) for both HLA class I molecules (Fig. 3C). These quantitative data reveal several important points. First, the Ub:HC ratio is 2–3 fold greater for HLA-B7 than HLA-B27. This must reflect a greater

number of ubiquitinated heavy chains, since the alternative of more ubiquitin per heavy chain, is not reflected in the patterns shown in Fig. 3B (i.e. no shift toward higher molecular weight complexes for HLA-B7). Second, the Ub:HC ratio increases with blockade of autophagy or ERAD, but is most pronounced when ERAD is inhibited. This is consistent with ERAD being the primary pathway for degradation of ubiquitinated heavy chains. Third, even without blocking degradation (i.e. IFN $\gamma$  upregulation alone) ubiquitinated heavy chains are more abundant for HLA-B7 than HLA-B27 (Fig. 3B,C IFN $\gamma$  lanes). Note that the lower recovery of  $\beta_2$ m-free HLA-B7 heavy chains compared to HLA-B27 (Fig. 3A, reduced panel) is due to more efficient folding and loss of HC10-reactivity as described previously (34).

To gain more insight into the predominant form of heavy chain that is ubiquitinated, we used an anti-ubiquitin antibody to perform immunoprecipitations, and then blotted for HLA class I heavy chains with HC10. Significantly more HLA-B7 is recovered than HLA-B27 (Fig. 3D), consistent with the increased Ub:HC ratio for HLA-B7 (Fig. 3C). Furthermore, the predominant form of HLA-B27 that is recovered is dimers, whereas a single band is recovered for HLA-B7. Taken together, these data indicate that there are major differences in how HLA-B7 and HLA-B27 heavy chains are handled by quality control pathways in rat BMM. Notably, HLA-B27 heavy chain dimers (and monomers) are inefficiently ubiquitinated compared to HLA-B7.

#### **Activation of autophagy eliminates misfolded and unfolded forms of HLA-B27.**

We next asked whether activating autophagy would enhance the degradation of HLA-B27. BMM were treated with IFN $\gamma$  for 6 hours to upregulate class I expression, washed to remove IFN $\gamma$ , and then incubated for an additional 2 hours without or with rapamycin to activate autophagy (Suppl. Fig. S2). HLA-B27 heavy chains were then immunoprecipitated with HC10, and visualized by immunoblotting. Oligomers and dimers of HLA-B27 that accumulate with IFN $\gamma$  treatment were reduced at least 50%, whereas monomers were not significantly changed (Fig. 4). Rapamycin also reduced about 40% of BiP-bound HLA-B27 heavy chains (Fig. 4). Rapamycin is also effective at reducing dimers and oligomers of HLA-B27 in cells treated with IFN $\gamma$  for 20–24 hours (unpublished observations).

Cell surface  $\beta_2$ m-free HLA-B27 heavy chains have been implicated in triggering killer immunoglobulin receptors (KIRs) on CD4<sup>+</sup> Th17 T cells (2). To assess the effect of autophagy on cell surface HLA-B27, macrophages were incubated with IFN $\gamma$  for 24 hours, with rapamycin added for the final 2–4 hours to activate autophagy. Cells were then washed and stained with either HC10 to detect free HLA-B27 heavy chains, or ME1 to measure folded HLA-B27 complexes, and analyzed by flow cytometry. Rapamycin treatment led to a rapid, time-dependent reduction in free HLA-B27 heavy chains on the cell surface of IFN $\gamma$ -treated cells (Fig. 5A). In contrast, there was no significant effect on folded HLA-B27 complexes (Fig. 5B). These data indicate that activating autophagy can promote selective elimination of dimeric and oligomeric HLA-B27 heavy chains that accumulate with IFN $\gamma$  treatment, and indicate that activating this pathway might be a beneficial strategy to ameliorate effects of HLA-B27 misfolding.



## DISCUSSION

Here, we investigated whether autophagy plays a role in the degradation of misfolded HLA-B27 heavy chains. Our results show that blocking autophagy for 3 hours led to accumulation of disulfide-linked HLA-B27 dimers and oligomers, as well as monomers, in primary cells from HLA-B27-Tg rats after HLA-B27 upregulation, and in transfected cells (C1R.B27) that continually express HLA-B27 heavy chains. Furthermore, a substantial proportion of accumulating HLA-B27 heavy chains are bound to the ER chaperone BiP, which has been shown to accumulate primarily with dimers of HLA-B27 (16). Importantly, we showed that activating autophagy with rapamycin led to a substantial and rapid reduction (50% in 2 hours) in the amount of misfolded HLA-B27 dimers and oligomers, including BiP-bound HLA-B27 heavy chains. Activation imposed by the exogenous stimulus (rapamycin) was more selective for the degradation of misfolded HLA-B27 (dimers and oligomers) than for monomers (Fig. 4). However, it should be noted that in some experiments, we have seen a reduction in HC10-reactive monomers along with more striking decreases (>50%) in dimers and oligomers, suggesting that autophagy is not entirely selective for misfolded forms (unpublished observations). Experimental variation in the strength of autophagy activation may account for these differences. Taken together, these results implicate autophagy as an ER quality control (ERQC) pathway involved in the elimination of misfolded and BiP-bound forms of HLA-B27. Rapamycin also reduced free HLA-B27 heavy chains on the cell surface (Fig. 5), which is not expected to be a consequence of ERQC. However, free class I heavy chains recycle through endosomes (2), and recycling endosomes contribute to autophagosome formation (43), suggesting a plausible mechanism for enhanced removal of free HLA-B27 heavy chains from the cell surface.

ERAD of HLA class I was first demonstrated for heavy chains synthesized when  $\beta_2m$  or peptide is limited (44). HLA-B27 heavy chains were shown to undergo ERAD even in the presence of  $\beta_2m$  and peptide, providing evidence for misfolding under normal physiologic conditions (4). HLA-B27 misfolding is a consequence of delayed folding imparted by polymorphic amino acids that also reduce its affinity for peptides and promote dimerization in the ER (4–6). Since these early studies, much more has been learned about ERAD of HLA class I heavy chains in general, and HLA-B27 in particular. EDEM1, together with the E3 ubiquitin ligase HRD1, and the ubiquitin conjugating enzyme UBE2J1, orchestrate the recognition, dislocation and ubiquitination of misfolded heavy chains including HLA-B27 dimers (21, 22). Furthermore, manipulation of the ERAD pathway, either by overexpressing EDEM1, or by pre-conditioning the ER with agents that activate the unfolded protein response, enhances the disposal of HLA-B27 dimers (22). Our studies confirm and extend previous results from transfected cells, by showing that ERAD plays a role in quality control of HLA-B27 after upregulation in primary rat macrophages. In rat macrophages blocking ERAD was slightly more effective at forcing the accumulation of HLA-B27 heavy chains than blocking autophagy (Fig. 1A). However, the opposite was seen in C1R cells expressing HLA-B27 (Fig. 1B). Barring differences in the sensitivity of primary macrophages and C1R (B lymphoblastoid) cells to these inhibitors, this suggests that there is a difference in the trafficking of misfolded HLA-B27 through these two pathways. This could be due to a number of factors, including cell type-specific differences in ERQC components, or perhaps

differences in baseline expression of HLA-B27. The C1R.B27 cells continually express a protein that misfolds, which can be detrimental to survival. In contrast, the rat macrophages are derived from bone marrow progenitors *in vitro* in the absence of exogenous stimuli that upregulate HLA class I, and thus HLA-B27 expression is relatively low until the cells exposed to IFN $\gamma$ . It will be of interest to compare the relative contributions of ERAD and autophagy to ERQC of HLA-B27 in other cells derived from HLA-B27 Tg rats.

HLA-B27 dimers form immediately after heavy chains are synthesized in the ER (5), exhibit a half-life exceeding 8 hours (22), and can be found in many cells at steady state (5, 18, 22) suggesting they are not efficiently eliminated and may be resistant to ERAD. This is in sharp contrast to many proteins that misfold in the ER and are efficiently degraded by ERAD, including MHC class I heavy chains produced in the absence of  $\beta_2m$  or peptide (21, 44). This raises the question of why HLA-B27 heavy chain dimers and oligomers are not efficiently degraded. By comparing how rat cells handle HLA-B7 heavy chains, our results demonstrate that dimers of HLA-B27 are poorly ubiquitinated relative to HLA-B7 (monomers) (Fig. 3). This is even apparent in IFN $\gamma$ -treated cells in the absence of autophagy or ERAD inhibitors (Fig. 3D). There is precedent for this as mutations in the T-cell receptor- $\alpha$  (TCR $\alpha$ ) that increase the formation of disulfide-linked homo-oligomers also prevent it from being degraded by ERAD (45). Thus, we hypothesize rapid dimerization of HLA-B27 heavy chains is an impediment to efficient ERAD, and contributes to accumulation of misfolded forms.

Autophagy has been implicated in ER quality control of several proteins, including mutant  $\alpha_1$ -antitrypsin (AT) Z (ATZ). AT is normally produced and secreted in large amounts from the ER of hepatocytes, and functions as a serine protease inhibitor that limits proteolytic damage to tissues particularly during inflammation (30). A single point mutation in ATZ causes it to misfold and polymerize, forming insoluble aggregates in the extended ER. These insoluble aggregates are resistant to dislocation from the ER and thus escape ERAD, but are degraded by autophagy. A second ERQC autophagy pathway has been described for non-aggregated, ERAD-resistant membrane proteins (31), with gonadotropin-releasing hormone receptor type I (GnRHR) mutants as model substrates. Two different GnRHR mutants are subjected to ERQC, but degradation of one is blocked by proteasome inhibitors while the other is blocked by inhibitors of autophagy. Misfolded proteins that cannot be cleared by the proteosomal pathway can activate the autophagy pathway for degradation (46).

In summary, our studies implicate autophagy as well as ERAD in ERQC of misfolded HLA-B27. Nevertheless, neither the autophagy nor the proteasome pathway was sufficient under these conditions to prevent misfolded HLA-B27 from accumulating. In contrast to the proteasome pathway, the autophagy pathway can be further stimulated by starvation or the use of chemicals that inhibit the mTOR pathway (28). Several studies used successfully the mTOR inhibitor rapamycin in neurodegenerative diseases like Huntington or Alzheimer disease to partially resolve the misfolding proteins like tau by further activating autophagy (29, 47, 48), and it is widely used as an immunosuppressant to prevent transplant rejection. Hence, we treated cells with rapamycin for 2 h during upregulation of HLA-B27 or HLA-B7. Interestingly, mainly dimers and oligomers of HLA-B27 were reduced by activating autophagy, including heavy chains bound to BiP. Moreover, rapamycin was able to eliminate

a significant proportion of cell surface free HLA-B27 heavy chains, which represent aberrant forms of HLA-B27 implicated in triggering CD4+ Th17 T cells to produce IL-17 (2). Therefore, these results raise the possibility that rapamycin could be tested therapeutically, although this remains a challenge as autophagy is involved in multiple homeostatic pathways inside and outside of the immune system.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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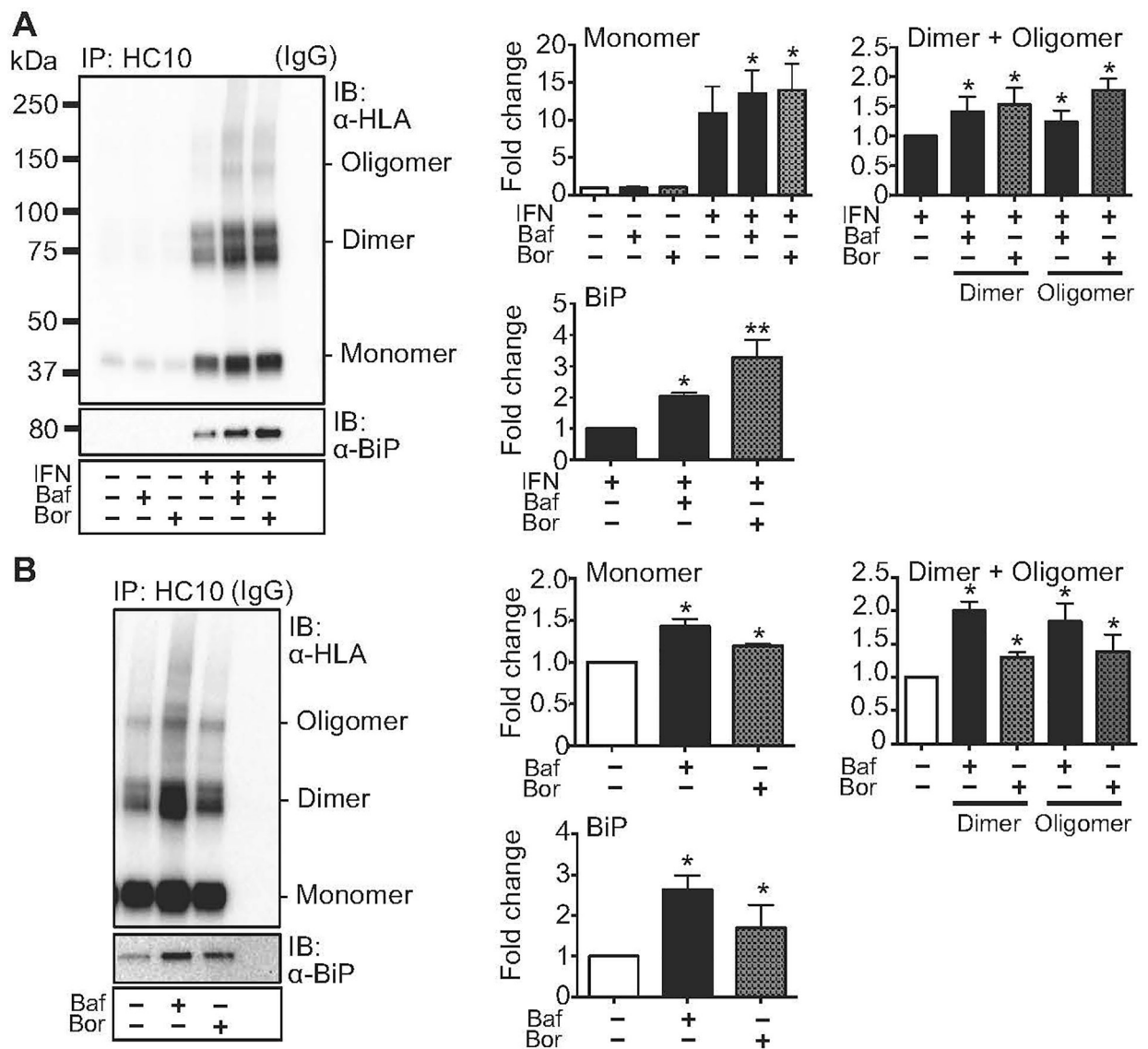
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**Figure 1.** Accumulation of HLA-B27 with inhibition of autophagy or ERAD. **(A)** BM-derived macrophages were left untreated or stimulated with IFN $\gamma$  (IFN) for 21 hours, then treated for 3 hours with bafilomycin (100 nM) (Baf) to block the autophagy pathway, or with bortezomib (10 nM) (Bor) to block ERAD. Untreated cells and cells only treated with bafilomycin or bortezomib were used as controls. Immunoprecipitation (IP) and immunoblotting (IB) with HC10 and 3B10.7 ( $\alpha$ -HLA) or  $\alpha$ -BiP, were as indicated and described in Materials and Methods. Dimers and oligomers are visualized on non-reduced samples electrophoresed on non-reducing gels, while BiP is analyzed under reducing conditions. For quantification, samples were normalized to untreated or IFN $\gamma$ -treated controls, as indicated, and expressed as fold change. For all figures, representative immunoblots are shown. Quantitative data represent mean  $\pm$  SEM of five independent experiments. (\*,  $p < 0.05$ ) **(B)** HLA-B27 expressing C1R cells were stimulated for 3 hours with bafilomycin (Baf) or bortezomib (Bor), prior to immunoprecipitation and

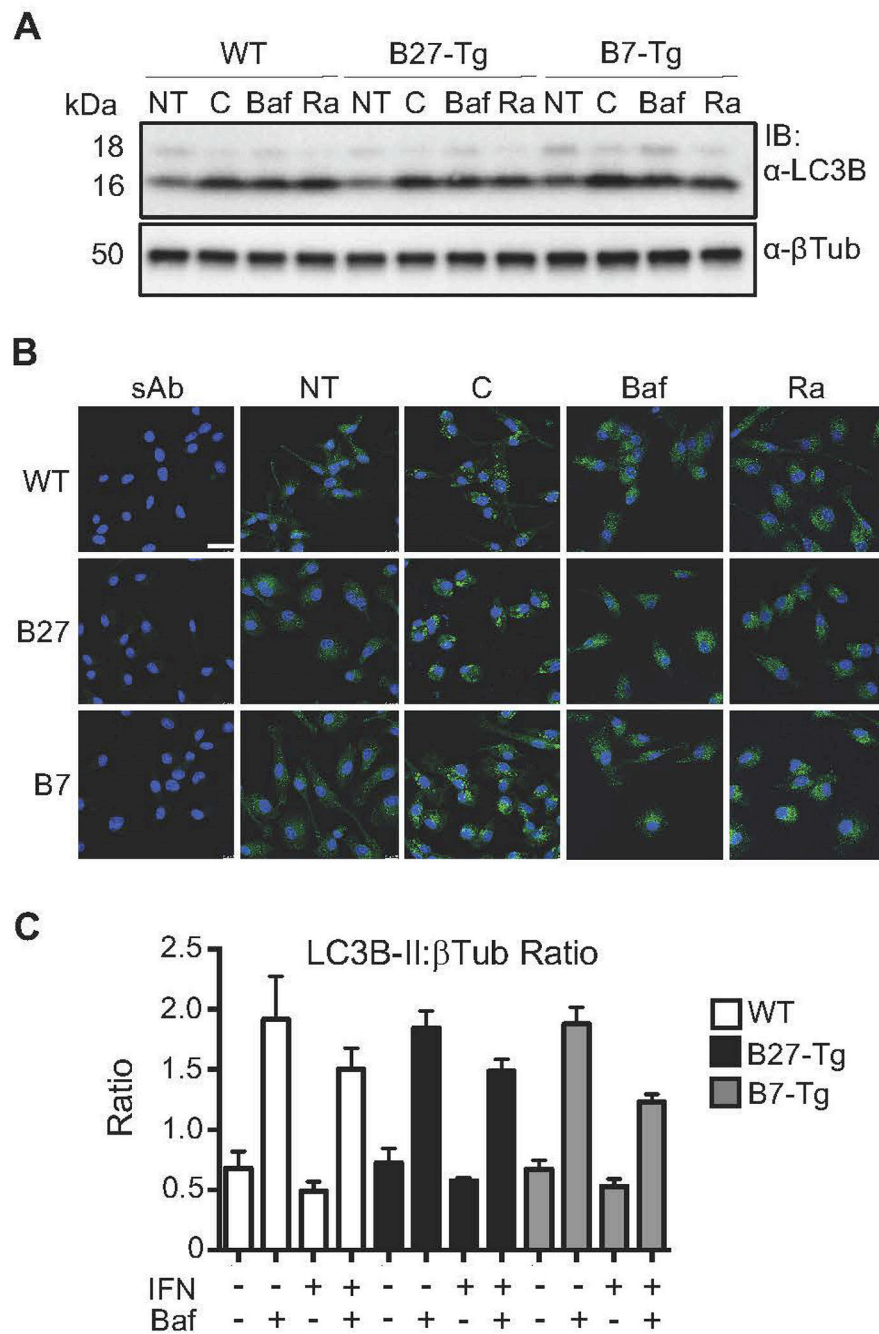
immunoblotting. Quantitative data represent mean  $\pm$  SEM of six experiments with the same cell line. (\*,  $p < 0.05$ ). IgG lanes show background when IP was performed with non-specific IgG alone.

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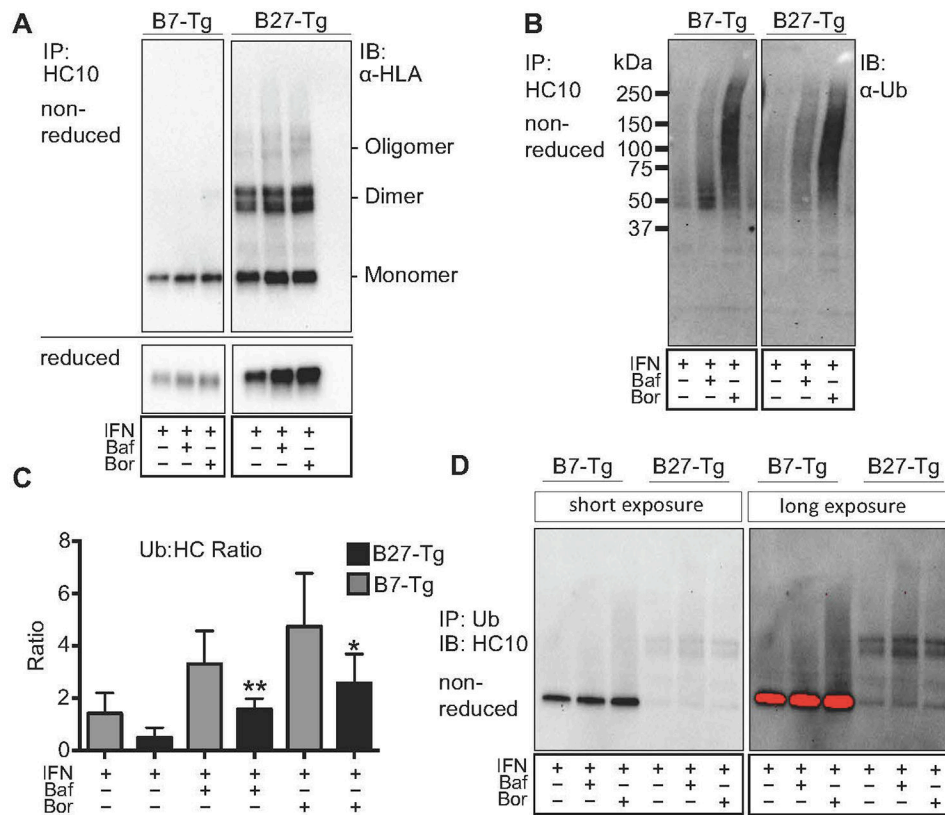
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**Figure 2.**

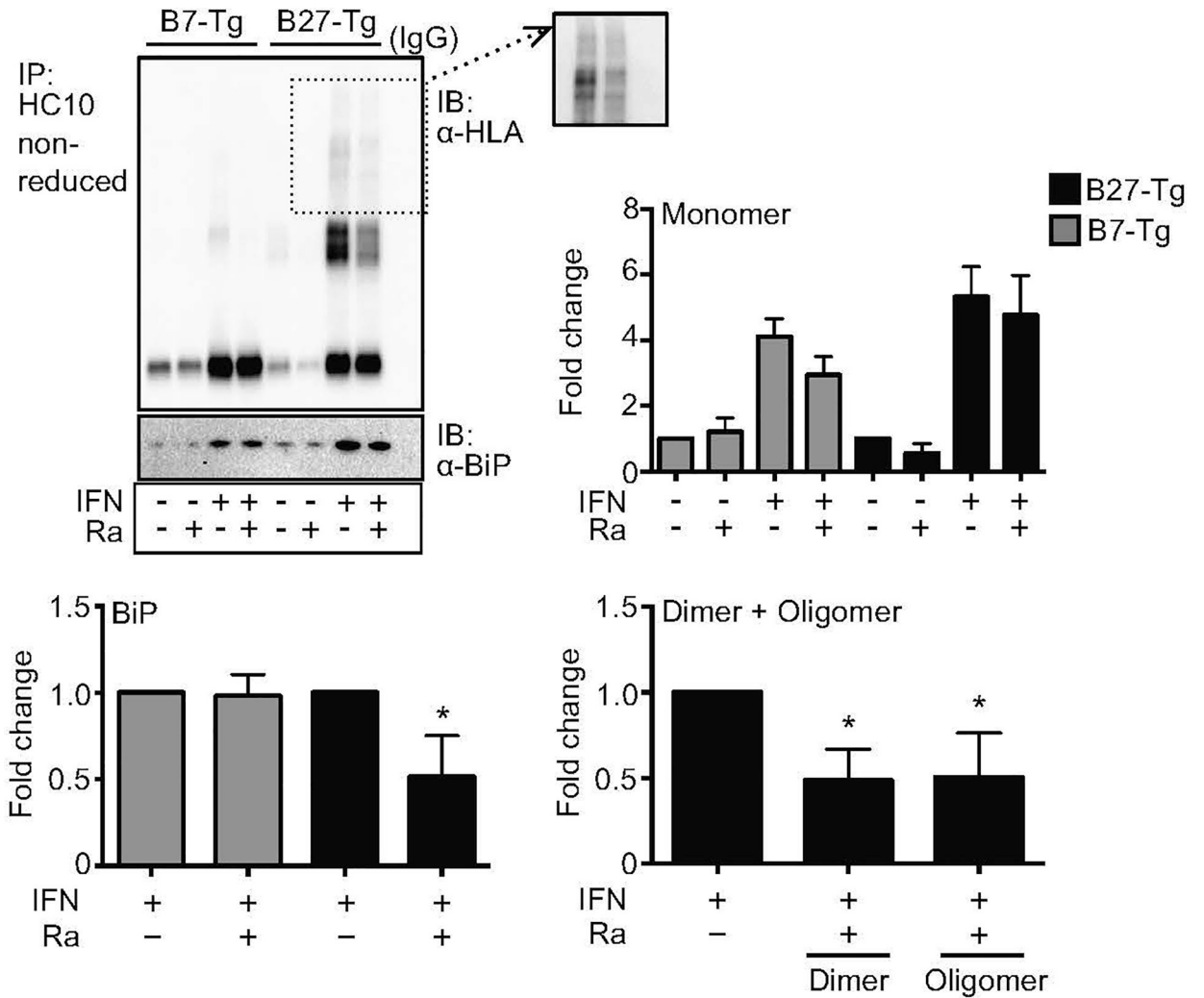
Autophagy in rat macrophages expressing HLA class I transgenes. **(A)** BM macrophages from WT, B27-Tg, and B7-Tg rats were left untreated or stimulated with rapamycin (25  $\mu$ M) (Ra) to induce autophagy, chloroquine (50  $\mu$ M) (C) or bafilomycin (400 nM) (Baf) to block autophagic flux, and then collected 2 hours later and lysed for immunoblotting using anti-LC3B ( $\alpha$ -LC3B), with anti- $\beta$ Tubulin ( $\alpha$ - $\beta$ Tub) used as a loading control. Immunoblot shown is representative of three independent experiments. **(B)** Macrophages treated as described in **(A)** (NT, untreated; sAb, secondary Ab only) were analyzed by



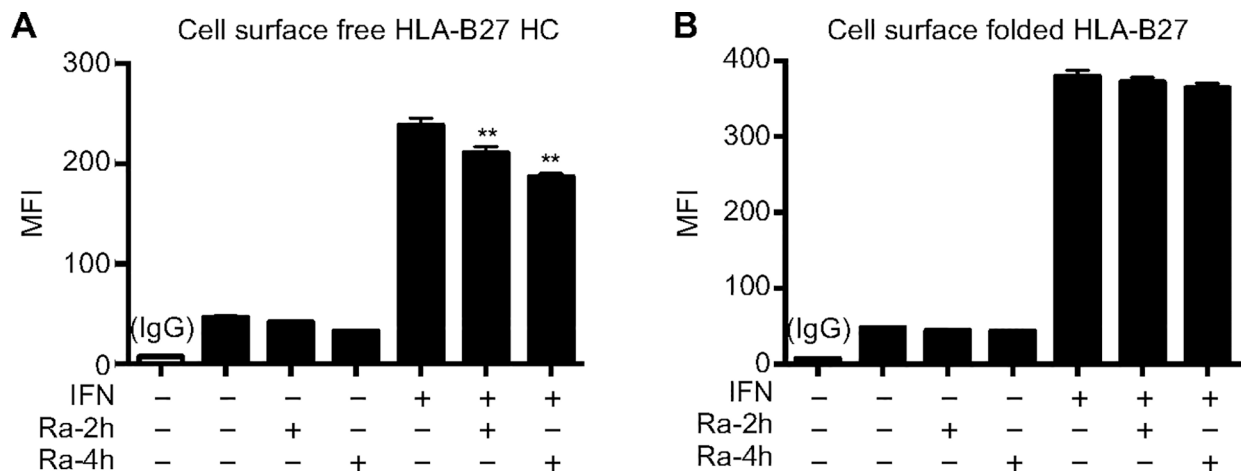
immunofluorescence with anti-LC3B (green), which has a stronger affinity for LC3B-II. Nuclei are visualized by DAPI staining (blue). Immunofluorescence was evaluated using confocal imaging with results shown at 63x magnification. Images are representative of three independent experiments. (C) BM macrophages from WT, B27-Tg, and B7-Tg rats were incubated without or with IFN $\gamma$  (50 ng/ml) for 24 hours, followed by another 24 hours with bafilomycin (10 nM). LC3B-II expression was determined by immunoblotting (as for experiment shown in A), and is expressed as a ratio of LC3B-II: $\beta$ Tub. Results are representative of three independent experiments.



**Figure 3.** Differential ubiquitination of HLA-B7 and HLA-B27 heavy chains. (A) BM macrophages expressing HLA-B27 and HLA-B7 were treated for 21 hours with IFN $\gamma$ , and then an additional 3 hours without or with bafilomycin (100 nM) (Baf) or bortezomib (10 nM) (Bor). HLA class I heavy chains immunoprecipitated with HC10 and analyzed under non-reducing or reducing conditions as indicated, using 3B10.7. (B) Samples from experiment described in (A) were immunoblotted with anti-ubiquitin antibody ( $\alpha$ -Ub). (C) The ratio of ubiquitinated material (B) (from entire lane) to immunoprecipitated total heavy chain quantitated under non-reducing conditions (A, reduced) is plotted for HLA-B27 and HLA-B7 under each experimental condition. Results represent mean  $\pm$  SEM (error bars) from five experiments. (\*,  $p < 0.05$ ) (D) Cells treated as described in (A) were used to immunoprecipitate all ubiquitinated material, and then blotted with HC10 to visualize heavy chains. In the long exposure image the red color indicates saturation.



**Figure 4.** Activation of autophagy by rapamycin reduces misfolded HLA-B27. (A) BM macrophages were incubated without or with IFN $\gamma$  (50 ng/ml) for 6 hours, washed, and treated without or with rapamycin (25  $\mu$ M) (Ra) for 2 hours. HC10 was used to immunoprecipitate unfolded/misfolded heavy chains, followed by immunoblotting with 3B10.7 ( $\alpha$ -HLA). Co-precipitating BiP was measured using an aliquot of the immunoprecipitate run on a separate reducing gel. Immunoblots depict representative results. Quantitative results are mean  $\pm$  SEM from six independent experiments, showing monomers, dimers, oligomers, and co-precipitating BiP. Inset shows longer exposure of oligomers. Dimers, oligomers, and BiP were quantitated only in IFN $\gamma$ -treated samples. (\*,  $p < 0.05$ )



**Figure 5.**

Activation of autophagy by rapamycin reduces free HLA-B27 heavy chains on the cell surface. BM macrophages were incubated without or with IFN $\gamma$  (50 ng/ml) for 24 hours, and treated without or with rapamycin (25  $\mu$ M) for the last 2 (Ra-2h) or 4 hours (Ra-4h). (A) Cells were then stained with APC-conjugated HC10 to detect free heavy chains by flow cytometry. (B) For the detection of folded HLA-B27, cells were stained with Pacific Blue-conjugated ME1. Quantitative results are mean  $\pm$  SEM from four independent experiments (\*\*,  $p < 0.005$ ).