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β-Glucuronidase, a Regulator of Lyme Arthritis Severity, Modulates Lysosomal Trafficking and MMP-9 Secretion in Response to Inflammatory Stimuli

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The lysosomal enzyme β-glucuronidase (Gusb) is a key regulator of Lyme-associated and K/B×N-induced arthritis severity. The luminal enzymes present in lysosomes provide essential catabolic functions for the homeostatic degradation of a variety of macromolecules. In addition to this essential catabolic function, lysosomes play important roles in the inflammatory response following infection. Secretory lysosomes and related vesicles can participate in the inflammatory response through fusion with the plasma membrane and release of bioactive contents into the extracellular milieu. In this study, we show that GUSB hypomorphism potentiates lysosomal exocytosis following inflammatory stimulation. This leads to elevated secretion of lysosomal contents, including glycosaminoglycans, lysosomal hydrolases, and matrix metalloproteinase 9, a known modulator of Lyme arthritis severity. This mechanistic insight led us to test the efficacy of rapamycin, a drug known to suppress lysosomal exocytosis. Both Lyme and K/B×N-associated arthritis were suppressed by this treatment concurrent with reduced lysosomal release. The Journal of Immunology, 2015, 195: 1647–1656.

Lyme disease is the most prevalent arthropod-borne illness in the United States with >300,000 estimated cases per year (1). Lyme disease infection occurs following the bite of a tick infected with the spirochete Borrelia burgdorferi (2). In a majority of cases, infection leads to the development of an erythema migrans bull’s-eye rash at the site of the tick bite, which radiates outward along with dissemination of bacteria. Patients experience a wide spectrum of disease symptoms following infection, which are thought to reflect differences in the infectious bacteria as well as in the patients’ own unique genetic risk factors. Lyme arthritis is the most prevalent symptom of disseminated infection, observed in 30–60% of untreated patients (3). Although early treatment with appropriate antibiotic therapy often effectively prevents the manifestation of later symptoms associated with disseminated infection, up to 10% of treated patients experience recurrence arthritis symptoms that may persist for months or years (4).

Wild mice and other rodents serve a key role in the enzootic cycle of B. burgdorferi in nature (2). Barthold et al. (5) made the pivotal observation that inbred strains of laboratory mice exhibit consistent genetic differences in Lyme arthritis severity. They observed that C3H/HeN (C3H) mice develop severe Lyme arthritis, whereas C57BL/6N (B6) mice experience milder inflammation and pathology, representing opposite ends of the disease spectrum. This observation allowed the influence of underlying host genetic differences to be investigated in a systematic way. Through a forward genetic approach, we recently demonstrated that the lysosomal enzyme β-glucuronidase (Gusb) is a key regulator of Lyme arthritis severity (6). This result was extended to the K/B×N serum transfer model of rheumatoid arthritis, indicating a common role for Gusb in these two models of arthritis.

The Gusb gene in C3H mice contains a single nucleotide polymorphism that encodes a T87I amino acid substitution and leads to a partial reduction in GUSB enzymatic activity. GUSB is a critical enzyme in the lysosomal pathway used to degrade and recycle glycosaminoglycans (GAGs), and individuals with very severe GUSB deficiencies exhibit spontaneous lysosomal storage of partially degraded GAGs and severe joint/skeletal abnormalities (7). C3H mice do not develop spontaneous lysosome storage symptoms until advanced age, but GUSB hypomorphism does drive substantial arthritogenesis in young mice following challenge with an inflammatory trigger (6). The cellular and molecular mechanisms underlying the observed joint pathogenesis are not fully understood, but the subcellular localization of GUSB to the lysosome and its indispensable enzymatic role implicate alterations in lysosomal function or trafficking as strong candidates for additional investigation.

Materials and Methods

Mice

All mice used in this study were maintained in a pathogen-free facility and cared for in accordance with protocols approved by the University of Utah Institutional Animal Care and Use Committee. B6 and C3H mice were obtained from the National Cancer Institute and Charles River Laboratories, respectively. Gusb<sup>het<sup> homozygous and heterozygous (Gusb<sup>het</sup>) mice on a B6 genetic background were originally obtained from The Jackson Laboratory, as described (6). B6.C3H-Gusb<sup>het</sup> (B6-Gusb<sup>het</sup>) congenic mice were developed as previously described (6) and isolate a C3H-derived locus on Chromosome 5 from 129.0 to 130.5 Mb, containing only one...
coding polymorphism that is located in the Gash gene, on an otherwise uniform B6 genetic background.

**Microscopy**

Alcian blue–stained sections were visualized on an Olympus BX41 clinical microscope (Olympus America) using x4, x10, or x40 total magnification. Images were recorded with an Olympus DP72 camera and prepared using Olympus cellSens digital imaging software (Olympus America). Electron microscopy images were obtained using a JEOL JEM-1400 Plus Transmission Electron Microscope (JEOL).

**LAMP-1 flow cytometry assays**

Unelicited resident peritoneal cells were harvested from B6, C3H, and Gashnull littersmates as described (8). Cells contained in this naive peritoneal exudate were resuspended at a concentration of 5 × 10^6/ml in serum-replacement medium (RPMI 1640 medium [Invitrogen] supplemented with 1% l-glutamine and 1% Nutridoma SP [Roche]) (Control) or serum-replacement medium supplemented with 3% Brewer modified thiglycollate medium (3% Thio; Difco). A total of 2 ml cell suspension was aliquoted into 12 × 75 mm Falcon tubes (BD Biosciences), capped, and incubated in a 37°C water bath for 2 h. Samples were then washed twice with FACS buffer and stained with 7-aminoclonazimycin D for viability discrimination and LAMP-1 PE-conjugated Ab (clone 1D4B). For lineage discrimination, peritoneal exudate was stained with DAPI for viability discrimination and a mixture of Abs against LAMP-1 Alexa Fluor 488 (clone 1D4B), FITC APC (clone BMB), and CD19 BV605 (clone 6D5). All Abs were purchased from eBioscience and used at a 1:200 dilution. Sample data were collected on an FACSCanto II (BD Biosciences) and analyzed using FlowJo v. 9.4.11 software (Tree Star). Live single cells were selected by gating all samples on forward scatter height versus forward scatter width to exclude doublets and then by subsequently gating out any cells staining positively for 7-aminoclonazimycin D or DAPI, respectively (data not shown).

**Culture of bone marrow–derived macrophages**

Rear ankle joints were measured at the time of infection and at 4 wk after infection by using a metric caliper, as described (9). BMDM growth medium consisted of RPMI 1640 medium (Invitrogen) supplemented with 30% L929 conditioned medium and 20% horse serum (HyClone). Strain-specific BMDM were harvested, resuspended in serum-replacement medium at a density of 6 × 10^6/ml, and 500 μl aliquots were replated into 24-well plates and cultured overnight.

**Culture of B. burgdorferi and BMDM coinoculation**

N40 isolate B. burgdorferi cells (provided by Stephen Barthold, University of California Davis, Davis, CA) were cultured in Barbour-Stoenner-Kelly II medium containing 6% rabbit serum (Sigma-Aldrich). Live B. burgdorferi were visualized and counted using a Petroff-Houser counting chamber and a dark-field condenser. An aliquot of B. burgdorferi was centrifuged at 6000 × g on a tabletop centrifuge for 6 min, resuspended in room temperature 1× PBS, centrifuged again, and resuspended in serum-replacement medium at a high concentration of 7.4 × 10^6/ml or a low concentration of 7.4 × 10^5/ml. The 24-well plates of strain-specific BMDM were removed from the incubator, and the serum-replacement culture medium was aspirated and replaced with 500 μl freshly prepared serum-replacement medium, low-concentration B. burgdorferi medium, or high-concentration B. burgdorferi medium and returned to the incubator. The high and low concentrations used produce a multiplicity of infection of ~25 and 2.5, respectively. Twenty-four hours later, supernatants were harvested into individual 1.7-ml microcentrifuge tubes and centrifuged at 6000 × g for 6 min. A total of 400 μl resulting cell-free supernatants was then transferred into a 96-well 500 μl round-bottom assay plate (Axygen). To obtain cell extracts, cells were washed once with 1× PBS and then incubated in extraction buffer (50 mmol NaPO4 [pH 7], 10 mmol BME, 10 mmol EDTA, 0.1% sarcosyl, and 0.1% Triton X-100) on ice for 30 min. Extracts were then homogenized by pipetting up and down, and 400 μl was transferred into a 96-well assay plate.

**β-Galactosidase enzyme assay**

4-Methylumbelliferyl β-D-galactopyranoside (Marker Gene) was used as a fluorogenic substrate to measure β-galactosidase (BGL) enzymatic activity. A total of 10 μl sample (serum, cell extract, or supernatant) was incubated with 1 mmol 4-methylumbelliferyl β-D-galactopyranoside in a total volume of 50 μl assay buffer (100 mmol sodium citrate [pH 4.4]) for 1 or 37°C in a 97-mm well plate (Costar). A total of 150 μl bioluminescence buffer (200 mmol sodium carbonate) was then added, and samples were analyzed on a BioTek Synergy HT microplate reader (BioTek). Fluorescence was measured with an excitation wavelength of 360 nm and emission wave-length of 460 nm. Units were calculated by comparison against a standard curve prepared using free 4-methylumbelliferone (Sigma-Aldrich). Results are expressed as arbitrary units, with the average value of B6 unstimulated control samples within each assay run set at a baseline of 100.

**Gelatin zymography**

A total of 20 μl cell-free BMDM supernatant was added to 20 μl Zymogram Sample Buffer (Bio-Rad) and loaded onto 10% polyacrylamide gels embedded with gelatin (Bio-Rad). Electrophoresis buffer was prepared from a 10× stock solution (13 g Tris base, 72 g glycine, and 5 g NaDodSO4 dissolved in 500 ml Milli-Q water). Zymography gels were electrophoresed at 100 V for 1 h. After electrophoresis, gels were removed from the cassette and incubated in 1× Zymogram Renaturation Buffer (Bio-Rad) at room temperature on a rocker plate for 30 min, followed by overnight incubation at 37°C in Zymography Development Buffer (Bio-Rad). Gels were then stained with 2.5% Coomassie Blue R-250 (Bio-Rad) dissolved in a solution of 50% methanol, 10% glacial acetic acid, and 40% Milli-Q water for 30 min. Gels were destained using 50% methanol, 10% glacial acetic acid, and 40% Milli-Q water and then imaged. Density analysis of the bands was performed using Image J (National Institutes of Health). The appropriate position of the MMP-9 zymography band was verified using recombinant mouse MMP-9 (AnaSpec) (data not shown).

**ELISA**

MMP-9 ELISA was performed according to the manufacturer’s instructions using a Quantikine ELISA Mouse Total MMP-9 kit (R&D Systems). Briefly, washed slides were placed into a 96-well microplate and filled with 50 μl assay diluent. Then, 50 μl cell-free BMDM supernatants, standards, or control solutions were added, mixed, and incubated for 2 h at room temperature. Each well was then aspirated and washed four times with wash buffer. A total of 100 μl Mouse Total MMP-9 Conjugate was added to each well, covered, and incubated for 2 h at room temperature. The plates were again aspirated and washed four times. A total of 100 μl substrate solution was added; the plate was covered and incubated at room temperature for 30 min in the dark. A total of 100 μl stop solution was added to each well, and the plate was read using a BioTek Synergy HT microplate reader (BioTek) at an absorbance of 450 nm and a wavelength correction set to 540 nm.

**Rapamycin treatment of mice**

Rapamycin (LC Laboratories) was dissolved in EtOH to prepare a 100 mg/ml stock solution. This was diluted to 1.2 mg/ml into a vehicle solution of 1× PBS, 5% PEG-400, and 5% Tween-20. B6-Gash mice were given daily i.p. injections of either 8 mg/kg rapamycin or an equivalent volume of vehicle, beginning 2 d prior to the initiation of the experimental arthritis protocols and continuing throughout the course of the experiment with the final injection given the day prior to sacrifice.

**K/B×N serum transfer experiments**

Experiments were carried out as described (6). Briefly, rear ankle joints of mice between 6 and 7 wk of age were measured with a metric caliper prior to infection. A total of 100 μl K/B×N serum was administered in two i.p. injections, with one on day 0 and one on day 2. Ankle swelling was determined by caliper measurements on days 5 and 7. After the final day 7 measurements, serum was collected for enzyme assays, the most swollen rear ankle joint was harvested for histological analysis, and the less swollen joint and the spleen were excised, immersed in RNA Later (Qiagen), and stored at −80°C.

**B. burgdorferi infection of mice**

Mice between 6 and 7 wk of age were infected by intradermal injection with 2 × 10^7 bacteria of the B. burgdorferi N40 isolate. Sonicated B. burgdorferi used for in vitro stimulation was prepared as described (9).

**Lyme arthritis analysis**

Rear ankle joints were measured at the time of infection and at 4 wk postinfection by using a metric caliper, as described (9). Measurements of the thickest anteroposterior portion of the ankle with the joint extended were taken and are reported as the change in ankle swelling over time. For histological evaluation, tissues were fixed in 10% neutral buffered formalin, decalcified, embedded in paraffin, and cut into 3-μm-thick sections; sections were mounted onto glass slides and stained with HE or Alcian blue as described (6).

**Isolation of RNA and quantitative RT-PCR**

Total RNA was recovered from homogenized tissue using TRizol reagent (Invitrogen). 5 μg total RNA was reverse transcribed using random primers and Moloney murine leukemia virus RT (Invitrogen). Transcripts were
quantified using LightCycler Sybr Plus master mix and a LC-480 PCR system (Roche). The primers used were as follows: β-actin forward 5′-GTAAACAATGCCATGTCAAT-3′ and reverse 5′-CTTCATCGTGGC-CGGCTCTAG-3′; and Glb1 forward 5′-GGTGAATAATGGCTGGCATG-3′ and reverse 5′-TACCCAGATGGTACGGGA-3′.

Results

We previously identified an increased extracellular GAG deposition, visualized histochemically with Alcian blue staining, and localized inflammation following *B. burgdorferi* infection in severely Gusb-deficient mice on a B6 genetic background (GusbNull) and several Gusb hypomorphic strains (6, 10). The previously described runted growth and spontaneous rheumatologic abnormalities of GusbNull mice suggest that the presence of excess partially degraded GAGs may be responsible for a shared disease process underlying both pathologies (11). Additionally, the results of radiation chimera studies indicated that the Gusb genotype of joint resident cells determines the severity of arthritis, suggesting that GAG released by these cells could contribute to localized inflammation. To investigate this, we compared the Alcian blue-positive GAG staining patterns of joints from sham-injected and *B. burgdorferi*-infected B6-GusbH, GusbNull, and GusbHet littermate control mice. Following *B. burgdorferi* infection, GusbHet, B6-GusbH, and GusbNull mice develop mild, moderate, and maximal arthritis, respectively (6). The Alcian blue positivity in joints from both sham and infected GusbHet mice is primarily restricted to the articular surfaces of the cartilage and within the bones of the leg, ankle, and foot (Fig. 1). Ankle joints from infected B6-GusbH mice exhibit extracellular GAG deposition in areas associated with histopathology, and this localized deposition is exaggerated further in GusbNull mice. Because the cellularity of the cranial–tibial tendon sheath is so widely variable between sham and infected mice, we attempted to focus high-magnification (×40) images on an adjacent area that is densely cellular in all samples. The extracellular deposition of these GAGs is most apparent at this high magnification. Although GusbNull mice are expected to spontaneously accumulate excess GAGs even in the absence of infection (11), we found that joints from sham-injected GusbNull mice exhibit minimal extracellular GAG staining.

To quantitate intracellular GAG storage, BMDM from GusbHet and GusbNull mice were prepared and visualized by transmission electron microscopy (Fig. 2). As expected, GusbNull cells exhibited many electron sparse storage vacuoles of irregular size and shape.
consistent with a lysosome storage disease phenotype. Together, these data suggest that although \textit{Gusb} deficiency leads to intracellular accumulation of excess GAG, which may have an independent role in arthritogenesis that deserves further study, \textit{Gusb} appears to play a distinct additional role in intracellular vesicular trafficking in response to inflammatory stimuli.

The role of \textit{Gusb} deficiency in lysosomal trafficking was investigated by measuring the amount of the lysosomal integral membrane protein LAMP-1 present on the cell surface following stimulation with the nonspecific agonist thioglycollate. Unelicited peritoneal exudates from naive B6 control mice, as well as \textit{Gusb} \textsuperscript{Null} and \textit{Gusb} \textsuperscript{Het} littermate mice, were collected, and the cells were stimulated ex vivo for 2 h and analyzed by flow cytometry. We found that unstimulated peritoneal exudate cells from these strains exhibited a uniformly low LAMP-1 baseline condition (Fig. 3A, 3B, Control). Thioglycollate treatment of naive peritoneal cells markedly induced LAMP-1 cell surface staining in all groups, as reflected in an increased percentage of LAMP-1–positive cells and in the elevated mean fluorescence intensity of all single cells (Fig. 3A, 3B, +3% Thio). \textit{Gusb} \textsuperscript{Null} cells exhibited significantly greater LAMP-1 cell surface staining than either B6 or \textit{Gusb} \textsuperscript{Het} cells by each of these metrics \((p < 0.001)\).

To determine which exudate cell types were responsible for this elevated LAMP-1 positivity, the experiments were repeated using a mixture of additional cell type–specific Abs. CD19\textsuperscript{+} B cells (60–80%) and F4/80\textsuperscript{+} macrophages (10–25%) comprise the largest percentage of cells in the unelicited peritoneal exudate of naive

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Severe GUSB deficiency is associated with intracellular storage vacuolation under basal conditions. Representative transmission electron micrographs of BMDM produced from \textit{Gusb} \textsuperscript{Het} or \textit{Gusb} \textsuperscript{Null} littermates. \textit{Gusb} \textsuperscript{Null} cells exhibit many electron sparse storage vacuoles of irregular size and shape, consistent with a lysosome storage disease phenotype.

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Severe GUSB deficiency is associated with exuberant lysosomal exocytosis following stimulation in multiple cell types. Naive peritoneal exudate cells from B6, \textit{Gusb} \textsuperscript{Het}, or \textit{Gusb} \textsuperscript{Null} mice were maintained ex vivo in serum-replacement medium (Control) or in medium with 3% thioglycollate (+ 3% Thio) for 2 h and then washed and analyzed by flow cytometry. Cell surface LAMP-1 positivity was measured by the percentage of LAMP-1–positive cells (A) and by the mean fluorescence intensity of all single cells (B). Significance assessed by one-way ANOVA followed by Bonferroni multiple comparison test (pooled data from three independent experiments, \(n = 3\) to 9 samples/group; overall \(p\) value < 0.0001). (C) Overlay of representative flow cytometry plots of stimulated (3% Thio, blue) and unstimulated (Medium, red) peritoneal exudate obtained from \textit{Gusb} \textsuperscript{Het} (left panel) or \textit{Gusb} \textsuperscript{Null} (right panel) mice. Numbers at each corner of the flow cytometry plots represent the percentage of stimulated cells located in each quadrant. The LAMP-1 horizontal axes for all four plots are on an equivalent scale. Quadrants for F4/80-positive peritoneal macrophages (top panel) are drawn to separate LAMP-1 Low from LAMP-1 High cells. Quadrants for CD19-positive peritoneal B-bells (bottom panel) are drawn to separate LAMP-1–negative from LAMP-1–positive cells. \(*p < 0.001, \,**p < 0.0001.\)
mice (data not shown). Under basal conditions, naive peritoneal macrophages from both GusbHet and GusbNull mice are LAMP-1 positive, whereas peritoneal B cells from both strains are predominantly LAMP-1 negative (Fig. 3C). Following thioglycollate stimulation, a subset of GusbHet peritoneal macrophages further mobilized LAMP-1 onto the cell surface to adopt a LAMP-1 high phenotype. In contrast, GusbNull peritoneal macrophages almost exclusively adopted a LAMP-1 high phenotype following stimu-

FIGURE 4. GUSB hypomorphism is associated with exuberant lysosomal exocytosis following stimulation in multiple cell types. BMDM from B6, B6-Gusb\(^h\), and C3H strains were cultured for 24 h in the absence or presence of either a low (7.4 \(\times\) 10\(^5\)/ml) or high (7.4 \(\times\) 10\(^6\)/ml) concentration of live B. burgdorferi. Lysosomal exocytosis was evaluated by testing cell-free supernatants (A) and cell extracts (B) for BGAL activity (pooled triplicate samples from three independent experiments, \(n = 9\) samples/group; overall \(p\) value < 0.001). Notably, exocytosis is significantly induced by the low concentration in C3H BMDM (\(p < 0.0001\), Student \(t\) test), but not in the two strains on a B6 genetic background. Cell-free supernatants (C) and cell extracts (D) from MEFs were evaluated in a similar manner using high B. burgdorferi concentration (pooled triplicate samples from two independent experiments, \(n = 6\) samples/group; overall \(p\) value < 0.0001). Significance assessed by one-way ANOVA followed by Bonferroni multiple comparison test of all B6 and B6-Gusb\(^h\) samples. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), ****\(p < 0.0001\). CTL, control.

FIGURE 5. GUSB hypomorphism is associated with elevated MMP-9 release following stimulation. (A) Gelatin zymography for MMP-9 enzyme activity, using cell-free supernatants from B6, B6-Gusb\(^h\), and C3H BMDM, with or without coincubation with live B. burgdorferi (+ B.b.). Top panel shows two adjacent zymography gels representative of three independent experiments; bottom panel shows the Image J (National Institutes of Health) band densitometry traces used for quantitation. (B) Statistical comparison of gelatin zymography data (pooled triplicate samples from three independent experiments, \(n = 9\) samples/group; overall \(p\) value < 0.0001). (C) MMP-9 protein levels of cell-free BMDM supernatants, evaluated by ELISA (pooled triplicate samples from two independent experiments, \(n = 6\) samples/group; overall \(p\) value < 0.0001). Significance assessed by one-way ANOVA followed by Bonferroni multiple comparison test of all B6 and B6-Gusb\(^h\) samples. *\(p < 0.05\), ***\(p < 0.001\), ****\(p < 0.0001\).
Gusb<sup>het</sup> peritoneal B cells exhibited minimal LAMP-1 positivity, but strikingly, a majority of stimulated Gusb<sup>Null</sup> peritoneal B cells became LAMP-1 positive. These data indicate that severe GUSB deficiency impacts lysosomal trafficking and fusion with the plasma membrane in response to cellular activation in multiple cell types.

Based on previous reports suggesting that B. burgdorferi coincubation induces exocytosis in a variety of myeloid cell types (12), a second experimental approach was pursued to evaluate the impact of Gusb hypomorphism on lysosomal exocytosis. BMDM were generated from B6, Gusb hypomorphic (B6-Gusb<sup>b</sup>), and C3H mice. Cells were plated overnight, washed, and stimulated by coincubation with live B. burgdorferi in serum-replacement medium. After 24 h of coincubation, cell-free supernatants were harvested and analyzed for release of the lysosomal enzyme BGAL using a fluorogenic enzymatic activity assay. We observed that coincubation with a high concentration of B. burgdorferi was associated with a marked induction of BGAL release into the supernatant by macrophages from all three strains (Fig. 4A, HIGH). Interestingly, despite an otherwise shared B6 genetic background, BGAL release by B6-Gusb<sup>b</sup> macrophages following coincubation was significantly elevated above that of B6 control macrophages. In addition, BGAL activity in corresponding cell extracts was constant across the two strains under basal or stimulated conditions (Fig. 4B), suggesting that the observed increase in supernatant activity is not a consequence of bulk changes in cellular BGAL production. Coincubation with a 10-fold lower concentration of live B. burgdorferi (Fig. 4A, LOW) triggered considerable BGAL release by BMDM from C3H mice, but not by BMDM from either B6 strain. This confirms the dose dependency of the effect and indicates that other unidentified genetic differences between B6 and C3H strains also influence this cellular response.

Due to the intriguing effect of severe Gusb deficiency on lysosomal trafficking in nonmyeloid cells (Fig. 3C), we next investigated whether Gusb hypomorphism would have a similar impact on fibroblasts, a nonmyeloid cell type that is potentially relevant to disease pathogenesis in Lyme and rheumatoid arthritis. We cultured mouse embryonic fibroblasts (MEFs) derived from B6, B6-Gusb<sup>b</sup>, and C3H strains and measured their levels of BGAL release following 24 h of coincubation with live B. burgdorferi. B6 control MEFs displayed no significant change in BGAL release following stimulation, whereas B6-Gusb<sup>b</sup> MEFs exhibited a significantly elevated BGAL signal (p < 0.001) (Fig. 4C, 4D). These data suggest that Gusb<sup>b</sup> allele alone can contribute to elevated lysosomal exocytosis following cell activation in multiple distinct cell types. Interestingly, the increase observed in B6-Gusb<sup>b</sup> was not seen in C3H control MEFs, suggesting that the C3H strain may harbor additional unknown balancing genetic polymorphisms expressed in fibroblasts that stabilize their response to cellular activation.

Although release of BGAL is a reasonable surrogate marker for lysosomal trafficking, it and other related lysosomal enzymes are catalytically inactive at the near neutral pH of the extracellular space and are therefore unlikely to contribute to pathogenesis in any meaningful way. To investigate a plausible downstream effector, we considered matrix metalloproteinase 9 (MMP-9). MMP-9 is essential for murine arthritogenesis following B. burgdorferi infection (13), and intracellular MMP-9 strongly colocalizes with LAMP-2–positive vesicles by confocal microscopy (14). To determine whether the Gusb<sup>b</sup> allele leads to elevated MMP-9 release, BMDM supernatants obtained after 24 h of coincubation with a high dose of 7.4 × 10<sup>9</sup>/ml B. burgdorferi were tested for MMP-9 activity by gelatin zymography (Fig. 5A). Supernatants from unstimulated B6 and B6-Gusb<sup>b</sup> BMDM exhibited low basal MMP-9 enzymatic activities, which were significantly elevated in samples coincubated with live B. burgdorferi. The MMP-9 activities of coincubated B6-Gusb<sup>b</sup> supernatants were significantly elevated above those of B6 (Fig. 5B, p < 0.001). In this assay, C3H-derived BMDM supernatants displayed an elevated basal level of MMP-9 activity, which was further induced by B. burgdorferi coincubation, suggesting that other unknown genetic differences in this strain influence MMP-9 levels. To validate this result with another experimental approach, supernatant MMP-9 protein levels were also evaluated by ELISA (Fig. 5C). Both the elevated basal MMP-9 levels in C3H-derived cells and the exuberant release of MMP-9 associated with the GUSB<sup>b</sup> allele were corroborated by this second approach.

Based on a previous report that rapamycin treatment suppresses lysosome exocytosis (15), we examined the impact of rapamycin treatment on BGAL release in vitro. MEFs derived from B6-Gusb<sup>b</sup> mice were stimulated with 5 µg/ml sonicated B. burgdorferi for 24 h in serum-replacement medium containing either vehicle or 100 nmol rapamycin. We found that rapamycin significantly reduced release of BGAL into supernatants (Fig. 6). To determine if transcriptional downregulation could be responsible, transcripts from the BGAL gene (Glb1) were assessed by quantitative RT-PCR (qRT-PCR). We found that rapamycin caused no significant change in the abundance of BGAL transcripts relative to vehicle-treated control samples in MEFs (data not shown).

Next, we evaluated the impact of daily rapamycin treatment on arthritis severity following K/B×N treatment. We observed that K/B×N-treated B6-Gusb<sup>b</sup> congenic mice receiving rapamycin developed significantly less severe ankle swelling than those treated with vehicle (Fig. 7A, p < 0.01). Rapamycin treatment was associated with reduced serum BGAL levels, consistent with inhibition of bulk cellular release (Fig. 7B). qRT-PCR analyses of K/B×N-treated mice were conducted at day 7 on spleens as a tissue distant from the inflammatory response and on ankle joints as the primary site of inflammation, and we found that our daily systemic delivery of rapamycin had no significant impact on the abundance of BGAL transcripts in either case (Fig. 7C, 7D). These findings are consistent with our results in MEFs, in which rapamycin mitigated cellular release of BGAL without altering its transcription. Alcian blue staining was also used to visualize the marked differences in ankle joint histology between mice treated with rapamycin or vehicle, as indicated (Fig. 7E, 7F).

Then we evaluated rapamycin on Lyme arthritis severity following B. burgdorferi infection. We observed that B6 control mice developed mild ankle swelling and that vehicle-treated B6-Gusb<sup>b</sup>
congenic mice exhibited significantly more severe arthritis (Fig. 8A, p < 0.05). However, this elevated ankle swelling was significantly reduced in rapamycin-treated B6-Gusb<sup>h</sup> congenic mice (p < 0.01), and this treated group was statistically indistinguishable from our B6 negative controls. Serum BGAL levels of B. burgdorferi–infected B6-Gusb<sup>h</sup> congenic mice were also significantly suppressed by rapamycin treatment (Fig. 8B). To determine whether rapamycin treatment influenced the bacterial burden of infected mice, qRT-PCR analysis was conducted on samples prepared from heart tissue. We found that the bacterial burden was significantly higher in rapamycin-treated mice (Fig. 8C, p < 0.0001), despite their markedly lower arthritis severity (Fig. 8A). These data suggest that rapamycin treatment has important and distinct impacts on host defense and arthritogenesis, although the effective suppression of arthritogenesis conferred by Gusb hypomorphism despite elevated bacterial burden is remarkable.

Taken together, these data are consistent with a cellular model by which GUSB plays distinct but synergistic roles in GAG homeostasis and vesicular trafficking (Fig. 9). Under resting conditions, GUSB hypomorphism leads to mild intracellular accumulation of GAGs. In the absence of a second pathological trigger, this accumulation remains asymptomatic, but may progress with advancing age (16). Severe GUSB deficiency leads to spontaneous intracellular accumulation of large amounts of GAG in storage vacuoles and induces a spontaneous disease process. Even among individuals carrying identical deficiency mutations, the severity of disease and age of onset may be variable (7). Challenge with an inflammatory trigger leads to lysosomal fusion,
cascade. This leads to less severe arthritis in mouse models of both K/B×N-induced and Lyme-associated arthritis, despite the presence of equivalent or even elevated levels of inflammatory stimuli.

**Discussion**

Our forward genetic approach identified *Gusb* as an unexpected regulator of murine arthrogenesis. The novelty of this discovery has refocused the importance of lysosomes and associated intracellular vesicles in the inflammatory response. The homeostatic degradation of GAGs by *Gusb* and other closely related lysosomal enzymes is an essential biological process that has primarily been studied in the context of rare, severe congenital enzymatic deficiencies that induce a spontaneous disease process. Although the pathological mechanism by which lysosomal storage diseases induce musculoskeletal symptoms is not fully understood, one suggestion is that partially digested substrate fragments may directly activate proinflammatory signaling pathways. This idea is bolstered by numerous studies that have demonstrated the therapeutic benefits of anti-inflammatory drugs in animal models of various lysosomal storage diseases (17, 18). These substrates can be detected in the serum and urine of severely affected individuals, and the presence of large excesses or altered relative proportions of distinct GAGs are used for clinical diagnosis (19). This necessitates a pathway for transport out of cellular lysosomes and storage vacuoles, into the extracellular milieu, and eventually into the systemic circulation. However, we observed that intracellular deposition of GAGs in the joint tissue is minimal under basal conditions, even in *Gusb* null mice, despite evidence that intracellular GAG accumulation is proceeding as expected (Figs. 1, 2). Instead, the extracellular deposition of GAGs appears to be linked to a localized inflammatory response and is greatly exacerbated in *Gusb* null or B6-*Gusb* mice. This dichotomy between accumulating excess GAGs intracellularly in the absence of a coincident inflammatory response and depositing GAGs extracellularly during localized pathogenesis strongly infers a role for *Gusb* in vesicular trafficking.

A role for lysosomal trafficking in the pathogenesis of Lyme and rheumatoid arthritis is implied by the elevated levels of lysosomal exoglycosidases, including GUSB and BGAL, in the serum and synovial fluid of patients (20). These levels are reported to be especially high in the synovial fluid of Lyme arthritis patients (21), consistent with a localized response. Alterations in plasma GAG profiles have also been identified in patients with rheumatoid arthritis and correlated with the level of ongoing disease activity (22).

Vesicular trafficking is vital to the regulation of cellular inflammatory responses, influencing the expression of adhesion molecules and the release of cytokines, chemokines, ATP, and other effector molecules from a variety of specialized granules, secretory lysosomes, and related vesicles (23, 24). We observed that inflammatory stimuli induce lysosomal exocytosis in peritoneal B cells, macrophages, and fibroblasts (Figs. 3, 4), and this cellular process may be uniformly conserved (25). Lysosomes are a vital source of lipids during plasma membrane repair (26). This repair process is hijacked by the protozoan *Trypanosoma cruzi* to gain entry to the cell (27) and is essential for cellular retention of the parasite (28). Remarkably, C3H inbred mice are highly susceptible to *T. cruzi* infection, whereas B6 mice are resistant, and this C3H susceptibility correlates with exacerbated macrophage activation and cytokine release in vitro (29).

In addition to *Gusb*, severe deficiencies in 10 other GAG-degrading lysosomal enzymes (*Idua*, *Idxs*, *Gns*, *Hgsnat*, *Naglu*, *Sgsh*, *Galns*, *Glb1*, *Arsb*, and *Hyal1*) also result in mucopoly saccharidosis (MPS I–IX, collectively), and many exhibit musculoskeletal manifestations (30). Severe deficiencies in an additional seven genes (*Asah1*, *Ctsa*, *Gba*, *Gnptab*, *Gnptg*, *Man2b1*, and *Neu1*) linked to lysosomal storage disease of other substrates also produce
It is noteworthy that among this latter group, neuraminidase (Neu1) has previously been shown to regulate lysosomal exocytosis (32), suggesting that perhaps other lysosomal enzymes may have a similar effect.

The potentiated release of MMP-9 we observed in Gusb hypomorphic cells following stimulation with live B. burgdorferi provides a direct link to a known effector of Lyme arthritogenesis (Fig. 5), but also highlights the wide impact that alterations in lysosome exocytosis could have on a variety of proinflammatory effectors. In particular, the extracellular release of intracellular GAGs may trigger localized TLR activation (18), modulate cytokine signaling (33, 34), or contribute to the establishment of chemokine gradients (35). These effects are not mutually exclusive and could all act in concert upon arrival of infectious Borrelia in the joint to establish a proarthritogenic state (Fig. 9).

The mammalian target of rapamycin (mTOR) inhibitor rapamycin has been previously described as a pharmacological inhibitor of lysosomal exocytosis (15). We corroborated these findings in vitro and in vivo, observing marked reductions of BGAL abundance in cell-free supernatants and serum following rapamycin treatment. mTOR has been proposed as a potential therapeutic target in clinical osteoarthritis, and this is supported by a correlation between mTOR expression and disease in patients (36) and by preclinical mouse models (37). Introduction of rapamycin was also reported to induce long-term disease remission in a patient with juvenile rheumatoid arthritis (38). An earlier study found that rapamycin blunted the development of adjuvant arthritis in rats (39). We found that rapamycin effectively suppressed the elevated arthritis severity caused by Gusb hypomorphism in our mouse models of K/B×N and Lyme arthritis (Figs. 7, 8). Because K/B×N arthritis represents the effector phase of disease progression, and because Lyme arthritis severity was reduced despite a marked increase in the bacterial burden of rapamycin treated animals, this infers that mTOR activation plays an im-

**FIGURE 9.** Model of alterations in lysosomal composition and trafficking related to Gusb genotype. Top panels depict homeostatic changes in lysosomal composition determined by lysosomal GUSB levels. Bottom panels depict alterations in cellular response following inflammatory stimulation. In resting cells, LAMP-1 is predominantly localized to lysosomal membranes, with GUSB, BGAL, and MMP-9 proteins sequestered within intracellular vesicles. Resting Gusb hypomorphic cells develop mild lysosomal GAG storage, with spontaneous formation of enlarged GAG storage vesicles in Gusb−/−deficient cells. Inflammatory stimuli induce lysosomal fusion and release of lysosomal contents in all groups. Gusb hypomorphism or deficiency each increase the amount of lysosomal fusion following stimulation, as detected by measuring BGAL and MMP-9 release or LAMP-1 cell surface staining, respectively. These alterations in lysosomal trafficking and composition contribute to downstream inflammatory responses, culminating in altered Lyme and rheumatoid arthritis severity (lightning bolts). WT, wild-type.
portant role in the pathogenic cascade induced by the host re-
response, downstream of the initial inflammatory triggers.

This research has clear implications for human populations, in
whom GUSB levels are widely variable (40, 41). Even more in-
triguing, the reported elevation of circulating lysosomal enzymes
and alterations in GAG profiles within general populations of
patients with arthritis suggest that lysosomal exocytosis may be
a conserved process during inflammatory arthropathogenesis, raising
its potential diagnostic and therapeutic value. The large impact
that GUSB hypomorphism has on experimental arthritis severity
and its ubiquitous expression in all cell types infer that lysosomal
function and trafficking are important determinants of the patho-
logical trajectory of inflammation following a primary insult.

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