An NLRP3 Mutation Causes Arthropathy and Osteoporosis in Humanized Mice

Graphical Abstract

Highlights
- Syntenic replacement humanizes the NLRP3 inflammasome in mice
- The common NLRP3 gene replaces the function of the excised mouse gene
- An NLRP3 disease allele causes arthritis, osteoporosis, and endotoxin hypersensitivity
- NLRP3-humanized mice provide a platform for evaluating anti-NLRP3 therapies

Authors
John N. Snouwaert, MyTrang Nguyen, Peter W. Repenning, ..., Ted A. Bateman, Jenny P.-Y. Ting, Beverly H. Koller

Correspondence
bkoller@correspondence.unc.edu

In Brief
In this study, Snouwaert et al. use syntenic replacement to generate the first mouse model in which human rather than mouse NLRP3 triggers inflammasome assembly. Introduction of a gain-of-function mutation into the humanized locus results in development of progressive arthritic disease in adult animals.
SUMMARY

The NLRP3 inflammasome plays a critical role in host defense by facilitating caspase I activation and maturation of IL-1β and IL-18, whereas dysregulation of inflammasome activity results in autoinflammatory disease. Factors regulating human NLRP3 activity that contribute to the phenotypic heterogeneity of NLRP3-related diseases have largely been inferred from the study of Nlrp3 mutant mice. By generating a mouse line in which the NLRP3 locus is humanized by syntenic replacement, we show the functioning of the human NLRP3 proteins in vivo, demonstrating the ability of the human inflammasome to orchestrate immune reactions in response to innate stimuli. Humanized mice expressing disease-associated mutations develop normally but display acute sensitivity to endotoxin and develop progressive and debilitating arthritis characterized by granulocytic infiltrates, elevated cytokines, erosion of bones, and osteoporosis. This NLRP3-dependent arthritis model provides a platform for testing therapeutic reagents targeting the human inflammasome.

INTRODUCTION

Cryopyrin (NLRP3) is a large protein with distinct domains to which unique functions have been assigned. These include the N-terminal effector domain, the Pyrin domain for binding downstream signaling molecules, a nucleotide binding/oligomerization domain, a winged helix domain, a superhelical domain, and a C-terminal leucine-rich repeat receptor domain (Ting et al., 2008). Perturbation of cell homeostasis alters the secondary/tertiary structure of the protein, resulting in oligomerization of the cytoplasmic monomers (Martinon et al., 2002). The structural changes allow the NLRP3 pyrin domains of the complex to associate with the bipartite protein PYCARD. The CARD domain of PYCARD becomes available to interact with pro-caspase 1 and 11, leading to proximity-dependent self-cleavage of the pro-enzymes into their active forms (Agostini et al., 2004). A primary function of these caspases is proteolytic processing of pro-interleukin 1β (IL-1β) and pro-IL-18 to their biologically active forms, IL-1β and IL-18, a prerequisite for their release from intact cells (Dinarello, 2009). The majority of our knowledge concerning the function of this important innate immune pathway in human disease has been extrapolated from mouse studies in which the immune response of wild-type (WT) mice is compared with that in mice lacking these proteins (Elliott and Sutterwala, 2015). Direct studies of the response of the human protein have largely been limited to the study of transformed myeloid cell lines and primary immune cell populations. Studies using macrophages from WT and NLRP3-deficient mice have identified a diverse and extensive list of agents that lead to NLRP3-dependent release of IL-1β from cells, including not only microbial agents such as bacteria, toxins, and fungi but also particulate matter such as urate crystals, asbestos, alum, silica, amyloid β, and ATP (Leemans et al., 2011). It is largely presumed that these same agents can trigger assembly of the human inflammasome and thus initiate and/or amplify inflammatory responses in humans.

Mutations in NLRP3 have been identified in most patients with autoinflammatory disorders, including familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), chronic infantile neurological cutaneous and articular syndrome (CINCA), and neonatal onset multisystem inflammatory disease (NOMID) (Kastner et al., 2010). Identification of mutations in the same gene suggests that these syndromes represent a disease spectrum, with the severity of the disease likely related to the degree to which a particular mutation alters the structure and various functions of the protein (Aksentijevich et al., 2007). Collectively termed cryopyrin-associated periodic syndromes (CAPS), these diseases share many symptoms, including rash, episodic unprovoked fever, abdominal pain, elevated acute-phase proteins, fatigue, redness of the eyes, and arthralgia. Both the intensity and the frequency of the symptoms can vary, with the most severe disease resulting in bone deformities, hearing loss, mental impairments, and organ damage (Stojanov and Kastner, 2005). The majority of CAPS-associated mutations map to an inner surface of the hexameric ring in the cryopyrin model. It is hypothesized that the mutations disrupt the closed
form of the monomeric protein (Aksentijevich et al., 2007), leading to inappropriate assembly of the NLRP3 complex in the absence of a second signal and subsequent release of IL-1β and IL-18 (Agostini et al., 2004).

The primary structure of NLRP3 is well conserved between mouse and human, allowing animal models of CAPS to be generated by introducing the disease-associated mutations into the mouse gene (Bonar et al., 2012; Brydges et al., 2009; Meng et al., 2009). Important characteristics of the human disease are reiterated in these models, including autoinflammatory disease accompanied by elevated production of IL-1β. However, the inflammatory disease reported is more severe than that observed in patients with comparable mutations. Most of the mice inheriting Nlrp3 genes harboring CAPS mutations die before adulthood. In addition, the severity of the inflammatory disease associated with particular mutations does not correlate well with the effects of the corresponding mutations in humans. For example, a FCAS-associated mutation, which represents the mildest CAPS in humans, results in early lethality in mice, whereas a higher percentage of the mice carrying MWS-associated mutations survive past weaning.

Differential impact of a given amino acid substitution in proteins of two different species is perhaps not surprising. Epistatic interactions can occur both within and between molecules, and the effect of individual mutations may depend on the genetic context in which they occur (Lehner, 2011; Camps et al., 2007). The position of the amino acid substitution in NLRP3 is certainly a key determinant of the impact on its function. But this impact is likely to be further defined by primary interactions between neighboring amino acids and by secondary and tertiary interactions with more distant peptide motifs. Thus, the effects of introducing a mutation corresponding to a human disease-associated polymorphism into the murine homolog of the affected protein might be expected, in some cases at least, to differ substantially from those responsible for the human disease. An interesting example of this is the impact of the loss of residue 508 on CFTR. This deletion mutation dramatically alters the stability and function of the human protein, whereas both the swine and mouse Δ508 proteins show some activity (Ostedgaard et al., 2007). Perhaps the best evidence supporting the importance of epistasis in determining the physiological consequence of non-synonymous mutations comes from the observation that, in some cases, these disease-associated residues occur as the wild-type residues in functionally orthologous proteins (Baresić et al., 2010; Kondrashov et al., 2002). Intramolecular epistatic interactions will limit the ability to directly compare the impact of different CAPS-associated mutations and common variants on the functions of the NLRP3 protein by generation of mutant mouse Nlrp3 alleles.

To develop a platform for the study of common as well as CAPS-associated variants of human NLRP3 in vivo, we have generated a mouse line in which the mouse Nlrp3 locus is excised and replaced with either a common allele or a disease-associated human NLRP3 allele. We report here the functioning of the human NLRP3 human gene in the place of the mouse gene and the development of disease in juvenile and adult mice carrying a CAPS-associated, non-synonymous SNP.

RESULTS

Generation of Mice Carrying the Human NLRP3 Locus
An embryonic stem cell (ESC) line in which the 35-kb mouse Nlrp3 locus is deleted and the establishment of co-isogenic 129S6 mice carrying this deletion have been described previously (Kovarova et al., 2012). We refer to this mutation as ΔNlp3 to distinguish this line from other NLRP3-deficient mouse lines that still contain Nlrp3 introns and exons. ΔNlp3 ES cells were used to generate an ESC line in which the deleted DNA was replaced with a single copy of the corresponding syntenic NLRP3 DNA (Figure 1A). This 48,199-base pair (bp) DNA segment includes ~12 kb of DNA 5′ to the ATG site, all NLRP3 introns and exons, and 3′ intragenic DNA. The human gene is present as a single copy, the orientation and relative distance of NLRP3 to the endogenous murine upstream and downstream genes is preserved, and, with the exception of a mutant loxP and frt site, no vector or marker sequences remain in the humanized locus. These ESCs were used to generate co-isogenic 129S6-NLRP3 mice. Using standard nomenclature, the human gene is capitalized (NLRP3), whereas Nlrp3 refers to the mouse gene.

Evaluation of the Expression of NLRP3 in the Mouse
Tissues and cells were collected from mice heterozygous for a WT mouse and human gene, referred to as NLRP3/Nlrp3 mice, as well as from animals homozygous for either the mouse or human gene. Human NLRP3 was robustly expressed in a pattern that generally followed that of the mouse gene (Figure 1B). The only exception was in neutrophils, in which NLRP3 expression was significantly lower than that of Nlrp3, suggesting that regulation of the human and mouse genes may differ in these cells. To further examine NLRP3/Nlrp3 expression, we measured changes in mRNA levels in peritoneal macrophages after lipopolysaccharide (LPS) exposure. Both mouse and human NLRP3 mRNA levels showed a rapid but transient increase after stimulation (Figure 1C). In contrast, evaluation of parallel LPS-stimulated cultures with an NLRP3-specific antibody reported to interact with both human and mouse protein suggests that not only are the levels of NLRP3 in the humanized mice lower in resting cells, but the levels achieved on stimulation of the cells with LPS are lower and may decrease more rapidly after stimulation, suggestive of differences in the stability and or regulation of the human and mouse proteins (Figure 1D).

Functioning of the Human NLRP3 Inflammasome in Mouse Macrophages
To determine whether the human gene could restore NLRP3 function in mice, we examined whether mice in which the Δ locus had been restored with the human gene could produce IL-1β in response to stimuli that have been shown previously to be NLRP3-dependent. Peritoneal macrophages from WT, ΔNlrp3/ΔNlrp3, and 129 NLRP3/NLRP3 mice were exposed to LPS to induce IL-1β production and then to alum, silica, uric acid crystals, imidazoquinoline compounds, or ATP. In addition, cells were exposed to nigericin, a pore-forming agent that stimulates the NLRP3 inflammasome by depleting intracellular K+ (Solie et al., 2001). The human gene restored the response of the
mouse macrophages to these various agents (Figure 2). As reported previously, a robust release of IL-1β was observed in response to silica (Cassel et al., 2008); monosodium urate (MSU), an etiological agent in gout; or calcium pyrophosphate dihydrate (CPPD) crystals (Martinon et al., 2006). The humanized cells released IL-1β in response to all agents that triggered IL-1β production by mouse NLRP3, although the response was significantly lower, except in the case of MSU treatment. The response to MSU did not differ between humanized and WT macrophages. As expected, an NLRP3-dependent release of IL-1β was observed in response to ATP in the humanized cells, although, while the response was robust, it was significantly lower than that observed with mouse macrophages. We did not detect release of IL-1β in response to the imidazoquinoline compounds R837 and R848. This is not surprising because the level of IL-1β released on exposure to these agents is very low (Kanneganti et al., 2006).

in that the autoinflammatory disease-associated D305N SNP was introduced into the DNA used to restore the DNLrp3 locus (numbering is based on uniprot.org, and this SNP is often referred to as D303N) (Figure S1A). We expect the missense mutation to be the only difference between this mouse line and that described above. Heterozygous pups were present in litters at the expected frequency and displayed no apparent phenotype. No difference was seen in the expression of the two genes in various tissues or in LPS-treated macrophages (Figures S1B and S1C). This is not surprising given that the strategy used to generate the two lines ensures that the only difference between the two mouse ES cell lines is the SNP encoding D305N. For simplicity, we refer to the 129S6-NLRP3D305N mouse as D305N.

The pro-inflammatory characteristics of this disease-associated NLRP3 variant were examined by exposure of macrophages to LPS (Figure 3). As expected, only low levels of IL-1β were detected in supernatants from macrophages carrying the
common human NLRP3 gene. In contrast, robust release of IL-1β was observed in the supernatant from macrophages carrying the D305N mutation at all LPS concentrations (Figure 3A). Addition of ATP resulted in the expected increase in IL-1β release from LPS-primed macrophages expressing the common NLRP3 allele, and, after exposure to this agent, the levels of IL-1β in the supernatant of the control and mutant cultures were not significantly different (Figure 3B). To determine whether activation of the D305N inflammasome by a priming signal alone was unique to LPS, this experiment was repeated using tumor necrosis factor α (TNF-α) as the priming signal (Figure 3C). Similar to LPS, TNF-α alone was sufficient to induce IL-1β release by macrophages carrying the CAPS mutation. However, the magnitude of the release was smaller than that observed on exposure to LPS, and the release of IL-1β from the mutant cells could be further enhanced by exposure to a second stimulus, in this case ATP.

Response of D305N and NLRP3 Mice to LPS

The in vivo response of mice expressing common and mutant NLRP3 to LPS was examined first in congenic 129B6F1 animals of three different genotypes. Each mouse carried the ΔNlrp3 locus on the C57BL/6-derived chromosome and one of three different alleles on the 129-derived chromosome: WT (Nlrp3/ΔNlrp3), the common NLRP3 allele (NLRP3/ΔNlrp3), or the disease-associated variant (D305N/ΔNlrp3). Mice of each of the three genotypes were present in litters at expected frequencies and could not be distinguished by observation alone. Mice were exposed intraperitoneally (i.p.) to a low dose (2.5 mg/kg) of LPS, and the ensuing hypothermic shock was monitored over 8 hr. A small but significant drop in temperature was measured in the Nlrp3/ΔNlrp3 and NLRP3/ΔNlrp3 mice.
Levels continued to increase, peaking 2 hr after treatment, and levels of IL-1β cytokine was observed. In contrast, a dramatic increase in the temperature of mice expressing the NLRP3 allele, a small but significant increase in the level of this NLRP3 D305N variant, was dramatically higher in the D305N mice. Gr1+ myeloid cells, which is composed of activated monocytes and granulocytes, was dramatically higher in the D305N mice. Fraction of CD69, an early marker of activation was also not increased in this population. However, the fraction of CD4+/CD8+ ratio were altered in the mutant mice. Expression of CD4, CD8, CD11b, CD69, and Gr-1 indicated that neither the lymphocyte numbers nor the CD4+/CD8+ ratio were altered in the mutant mice. Expression of CD69, an early marker of activation was also not increased in this population. However, the fraction of CD11b+ Gr1+ myeloid cells, which is composed of activated monocytes and granulocytes, was dramatically higher in the D305N mice.

Characterization of Mice Homozygous for the D305N Mutation

Mice homozygous for the D305N mutation were present in litters at the expected frequency and did not differ in size from WT littermates. Analysis of a cohort of 6-month-old co-housed 129S6 and D305N/D305N mice showed a slight decrease in weight that did not achieve statistical significance, and the D305 mice occasionally developed blepharitis. No hepatomegaly was apparent; however, significant splenomegaly was observed in the mutant animals (Figure S2). Analysis of splenocytes with CD4, CD8, CD11b, CD69, and Gr-1 indicated that neither the lymphocyte numbers nor the CD4+/CD8+ ratio were altered in the mutant mice. Expression of CD69, an early marker of activation was also not increased in this population. However, the fraction of CD11b+ Gr1+ myeloid cells, which is composed of activated monocytes and granulocytes, was dramatically higher in the D305N mice.

Figure 4. Response of Mice Expressing Nlrp3, NLRP3, or D305N to LPS

(A) Mice of the indicated genotype received a low dose (2.5 mg/kg) of LPS i.p., and the core body temperature was monitored for 8 hr. (B–F) Cytokine levels in the peritoneal fluid were determined in an identically treated cohort at the indicated time points. Mice carrying a single copy of the D305N mutation showed a dramatic hypothermic response accompanied by an early increase in (B) IL-1β and (C) IL-18 production and later increases in (D) IL-1α, (E) TNF-α, and (F) IL-6. (G) The rapid drop in temperature observed in the D305N mice was attenuated by pretreatment with the small-molecule NLRP inhibitor CRID3/MCC. Results are expressed as mean ± SEM, n = 4.

(Figure 4A). The magnitude and the time course were similar in mice carrying a single copy of the human or mouse gene. In contrast, a dramatic hypothermic response was apparent 1 hr after LPS exposure of mice expressing the NLRP3 D305N variant, and these animals remained hypothermic through the duration of the experiment. In a parallel experiment, peritoneal exudate was collected and evaluated for the production of cytokines (Figures 4D–4F). In animals expressing the WT mouse and the common NLRP3 allele, a small but significant increase in the level of this cytokine was observed. In contrast, a dramatic increase in the levels of IL1β and IL-18 (Figures 4B and 4C) was observed in exudates collected from D305N animals 1 hr after LPS exposure. Levels continued to increase, peaking 2 hr after treatment, and achieved concentrations 20-fold higher than those observed in mice expressing either normal human or mouse NLRP3. Other inflammatory mediators associated with LPS-mediated sepsis, but which are not dependent on the inflammasome for processing and release, including IL-1α (Figure 4D), TNF-α (Figure 4E), and IL-6 (Figure 4F), were present at significantly higher levels in the exudate collected from D305N animals. The dramatic hypothermic response to LPS of the D305N mice provides a sensitive and rapid means of evaluation of the in vivo efficacy of small-molecule inhibitors of NLRP3. To demonstrate this, we evaluated the recently described NLRP3 inhibitor CRID3. A dose-dependent attenuation of hypothermia was observed in D305N mice treated with the compound prior to LPS exposure, with complete protection observed at 50 mg/kg (Figure 4G).
This population represented approximately 1% of splenocytes from WT animals, whereas approximately 10% of cells in the D305N mice were CD11b+ Gr-1+ (Figure 5A). Consistent with the presence of an ongoing inflammatory response in the D305N mice, levels of the major acute-phase protein, serum amyloid P, were elevated in the D305N animals (Figure 5B), as were serum levels of the natural IL-1β antagonist, IL1RA (Figure 5C). Hematological parameters were assessed in co-housed mice with the following genotypes: Nlrp3/Nlrp3, NLRP3/NLRP3, D305N/D305N, and D305N/NLRP3 (Table S1). White cell counts (WBC) were higher in mice heterozygous and homozygous for the mutant allele, although the increase was modest in the Nlrp3/D305N mice and did not achieve significance with this group size (n = 3). The WBC of the D305N mice was more than 6-fold higher than that of control groups. This reflected a modest but significant increase in lymphocytes and monocytes and a large but variable increase in circulating granulocytes. Histological evaluation of the sagittal section of the brain showed inflammation of the meninges in the D305N mice (Figure S3), although no deficit in hearing could be documented (Figure S4). Staining of organs with Congo red revealed no deposits of amyloid in the kidney or brain. To determine whether the inflammatory changes observed in the D305N mice were mediated by IL1β, we generated D305N/D305N:Il1b−/− mice. Not only was the spleen size normal in these animals (Figure 2S), but serum amyloid P (SAP) and IL1RA levels were no longer elevated (Figures 5B and 5C).

**Development of Arthropathy**

Although not observed at weaning, arthritic changes in the limbs and paws of the D305N/D305N mice became apparent within the first 3 months of life and progressed as the mice aged (Figure 6A). To examine this further, myeloperoxidase (MPO) activity, which is high in mature neutrophils and relatively specific for this cell type, was assessed non-invasively using a chemiluminescent reagent. No signal was observed at the joints and paws of control animals, whereas luminescence was easily detected at the joints of the mutant mice (Figure 6B). To examine further the inflammation present at the joints, a cohort of mice was euthanized, and cytokines present in tissue homogenates prepared from the paws were analyzed. IL-1β, which could not be detected in the lysates prepared from paws of normal mice, was elevated in the samples from the mutant animals. IL-18 and IL-6 levels were significantly higher, as were the levels of the IL-1β receptor antagonist IL-1RA (Figure 6C). Levels of IL-17 and IL-23 in homogenates from both mutant and WT animals were below the sensitivity of the assay. To determine whether the swelling of the paws corresponded with edema formation, serum protein...
extravasation into the paws and knee joints was quantified using Evans blue. Significant increases in serum protein levels were observed in both the front paws and knee joints (Figure 6D). Histological evaluation of the knee showed the presence of cellular infiltrate in soft tissues, including entheses—sites where tendons, ligaments, joint capsules, and muscles connect to bone. These infiltrates contained large numbers of granulocytes, identified based on their segmented nuclei (Figures 6E–6G). Although hyperplasia of the synovium was observed, inflammatory cells were not present in the joint space, and no loss of articulating cartilage was apparent on this analysis. Infiltrating granulocytes and mononuclear cells were also abundant in the entheses, ligaments, and synovial membrane, whereas the joint space is largely spared. Higher magnification of the indicated regions reveals an increased number of granulocytes in these areas, including the junction between muscle and tendons and tibial tuberosity. A total of seven D305N and seven control mice were examined.

Development of Osteoporosis in D305N Mice

Kyphosis was observed in approximately 10% of the D305N mice with advancing age. In addition, X-rays of the hindlimbs showed increased radiolucency and thinner cortices in all visualized bones of mice carrying the D305N allele relative to those of control mice (Figure 7A). This is consistent with osteoporosis, which may be either a primary condition or secondary to inflammation, decreased use, or both. Microcomputed tomography (microCT) followed by three-dimensional reconstruction was used to further characterize changes in the bone microarchitecture of the NLRP3 mutant animals (Figure 7B). Although D305N mice showed no differences relative to mice humanized with the common NLRP3 allele in the amount or density of trabecular bone at the proximal tibia, they showed a significant decrease in connectivity density and a significant increase in trabecular thickness (Table S2; Figures S1–S6), suggesting a compensatory response to the loss of trabecular connections. Compared with control mice, D305N mice had less cortical bone, and it was more porous. The medullary/marrow cavity and “footprint” of cortical bone in these animals was also smaller, contributing to a 33%
lower polar moment of inertia, meaning that the femur has a lower ability to resist torsional and bending loads. A small but significant decline of tissue mineral density was observed, suggesting that material properties may also be negatively affected in D305N mice. Lower mineral density is also indicative of greater bone turnover, consistent with a greater activation of osteoclasts and a general state of systemic inflammation (Table S2).

Articular subchondral erosion was prominent in both the ankles and knees of the D305N mice (Figure 7B). To rule out possible developmental differences in bone density, X-ray and microCT analysis of D305N mice were carried out at 6 weeks of age. The D305N mice could not be distinguished at this age (Figures S6A and S6B) from the WT.

**DISCUSSION**

We have generated mice in which the mouse Nlrp3 locus has been excised and replaced with its human counterpart. The extent to which we chose to extend the replacement of mouse sequences with corresponding human sequences upstream and downstream of the introduced human NLRP3 gene was based on striking a balance between two goals. The first was to maximize the likelihood that the expression pattern of the introduced gene would mimic that of the human gene rather than the host species, whereas the second was to avoid interfering with the normal expression of neighboring endogenous genes. The length of the flanking human sequences was therefore chosen based on available knowledge of transcriptional regulatory elements associated with NLRP3 as well as the mouse genes flanking the replaced locus. For both human and mouse NLRP3, expression is highest in cells of myeloid lineage, with only low levels of expression detected in lymphoid cells and most organs (Anderson et al., 2004; Manji et al., 2002). This expression pattern was largely preserved in our humanized mice, indicating that the regulatory elements in the human NLRP3 gene are recognized by the mouse trans-acting transcription factors that confer this pattern of expression. Of the tissues examined, only neutrophils displayed a significant difference in expression between the human and the mouse genes, with the human NLRP3 gene being expressed at a lower level.

The humanized NLRP3 locus provides the opportunity to directly compare the function of human and mouse NLRP3 both in primary immune cells and in vivo. Comparison of macrophages expressing the human protein with macrophages from WT littermates showed that, although LPS exposure alone was insufficient to trigger inflammasome assembly in macrophages expressing either the mouse or human protein, both the mouse and the humanized inflammasome released IL-1β in response to a similar spectrum of danger-associated molecular patterns (DAMPs). Overall, the relative ability of specific DAMPs to mediate assembly of the mouse or human NLRP3 inflammasome was well conserved. This is consistent with the hypothesis that the NLRP3 inflammasome responds to perturbations in intracellular K+ and, thus, that the magnitude of the response reflects the ability of the particulate matter or toxin to alter cell permeability to this ion. Contrary to this model, some studies have noted differences in the contribution of NLRP3 to IL1β production by both human and mouse macrophages in response to specific stimuli (Atianand et al., 2011). However, these studies are hard to interpret because identification of equivalent cell populations for this type of study is difficult. Differences observed may not reflect differences in the cell’s ability to form an inflammasome but, rather, upstream differences in the response of the cell to the pathogen-associated molecular pattern (PAMP); for example, the binding of the toxin to the
cell. The mice described here should provide a model for direct comparison of the assembly of the human and mouse NLRP3 inflammasome under different in vitro and in vivo experimental conditions in different cell populations. Although the macrophages expressing human NLRP3 responded to all DAMPs that elicited a response by WT macrophages, the magnitude of the response was generally smaller. This could be the result of lower expression of the human protein compared with the mouse protein. It is also possible that the decreased release of IL-1β reflects inefficiencies in the assembly of the human protein with other components of the inflammasome, such as caspase I. Alternatively, the reduced release may reflect an inherent difference between species revealed by direct comparison of responses mediated by WT and humanized inflammasomes. For example, it could reflect differential sensitivity of the mouse and human proteins to the changes in intracellular K+ that trigger inflammasome assembly.

To test the suitability of our humanized NLRP3 mice as a model for examining the effects on NLRP3 function of CAPS-associated mutations or SNPs associated with common diseases such as arthritides, we generated a second mouse line from ESCs in which the DNLpr3 locus was reconstituted with an NLRP3-D305N locus. These mice developed a disease that modeled aspects of CAPS in humans, although the disease we observed in our D305N mice was milder than that reported for patients with the analogous mutation, which has been identified in both MWS and NOMID patients (Aksentijevich et al., 2002; Feldmann et al., 2002). This might reflect an important and interesting species difference and raises the possibility that the severe disease previously observed in mice carrying Nlrp3 genes harboring CAPS mutations reflects subtle differences in the expression pattern of the gene in the two species and that the milder disease observed in the line described here could result from the lower expression in neutrophils. Alternatively, the observed protein levels in the humanized mice may result from species differences in NLRP3 stability. This raises the possibility that the severe disease observed upon introduction of mutations into the mouse protein results from increased duration of inflammasome activity.

Similar to all CAPS mutations, the D305N-associated autoinflammatory disease we observed in our mice was inherited in an autosomal dominant manner. Consistent with this, the response of mice carrying a single copy of the humanized locus to LPS was significantly greater than that of the WT animals. However, this heightened LPS sensitivity of the heterozygous animals did not translate into a dramatic change in their overall health under normal housing conditions. In this setting, the mice presented mild inflammation of their joints that was discernable only by evaluation of cytokines in lysates from these tissues, and, although peripheral WBC was higher, the increase was slight and did not achieve statistical significance. The phenotype of animals homozygous for the D305N locus was more severe. Similar to MWS and FCAS patients, the mice developed normally but displayed a number of features, including elevated WBC, splenomegaly, elevated serum IL-1β, and elevated acute-phase proteins characteristic of the human disease. The D305N/D305N mice responded to elevated IL-1β by increasing circulating and tissue levels of the natural receptor antagonist IL-1RA, an adaptive response also observed in CAPS (Aksentijevich et al., 2002). Interestingly, unlike CAPS patients, the D305N mice generally failed to develop skin inflammation, with skin lesions limited to a small area surrounding the eye in a very small percentage of animals later in life. The lack of inflammation in the skin might reflect differences in the regulation of the immune composition of human and mouse skin (Pasparakis et al., 2014). It is also possible that the danger signals that lead to the activation of the inflammasome in human skin are not present in our specific pathogen-free animals.

Macrophages from the D305N mice, similar to monocytes from CAPS patients, released IL-1β without the need for a second signal. We also found that this sensitizing signal was not unique to the LPS/TLR4 pathway. Exposure of D305N macrophages to TNF-α was sufficient to cause IL-1β release, consistent with a model in which assembly of the mutant inflammasome does not require a second signal rather than one in which the LPS can provide a second signal to D305N. It also indicates that inflammatory events that induce production of TNF-α may be able to trigger activation of the inflammasome in CAPS patients carrying this mutation. Elevated levels of serum TNF-α have been measured in CAPS patients, and our experiments indicate that they may contribute to an autostimulatory amplification of IL-1β production. However, the fact that this is only one of the many pathways that can provide the primary signal is shown by the limited benefit observed when treating patients with TNF-α inhibitors such as etanercept (Church et al., 2008).

We also observed arthropathy in our D305N mice that displayed both similarities and differences to that seen in CAPS patients. The acute inflammatory changes we observed, including redness, swelling, and accumulation of granulocytes, model the joint involvement observed in MWS, and the progressive bone loss we observed is consistent with the osteoporosis seen in the majority of NOMID patients. In addition, similarly to both the DIRA and NOMID patients, the articulating surface and synovial space in our D305N mice were largely spared despite extensive inflammatory infiltrate. The importance of IL-1β in osteoclast activation and inhibition of osteoblastogenesis is well documented (Redlich and Smolen, 2012). Therefore, it is not surprising that painful and swollen joints are a common symptom of MWS, whereas osteopenia and osteoporosis are observed in the majority of NOMID patients. However, although lytic bone lesions do not develop in patients with NOMID despite the increase in IL-1β production, erosion of bone at sites of inflammation is evident in both the microCT scans and histological evaluation of the D305N mice. On the other hand, deficiency of the interleukin-1 receptor antagonist (DIRA) patients present with periostitis and lytic bone lesions, and BALB/c mice lacking the orthologous mouse gene develop progressive spontaneous polyarthritis with bone erosion (Aksentijevich et al., 2009; Horai et al., 2000; Reddy et al., 2009). Interestingly, in the IL-1RA-deficient mice, similar to the D305N mice, the synovial space was relatively free of granulocytes, and the articulation cartilage was intact (Horai et al., 2000). The lack of disease in mice on other genetic backgrounds carrying the same mutation indicates that, at least in mice, genetic modifiers can alter the pathophysiology of unregulated IL-1β activity (Nicklin et al., 2000). Similar factors may also be partially responsible for
phenotypic differences observed between humans and mice carrying various CAPS-associated mutations. Our D305N mice also differ in several respects from previously published descriptions of mice in which CAPS-associated mutations were introduced into conserved residues in the mouse protein (Brydges et al., 2009; Meng et al., 2009). For instance, the relative severity of the disease phenotype associated with hemizygous inheritance of knockin mutations corresponding to the CAPS-associated A352V and L353P mutations was the opposite of what would have been predicted based on human studies. Although the human L353P mutation is considered milder, mice hemizygous for this mutation were either stillborn or died soon after birth (Brydges et al., 2009). In contrast, mice carrying the A352V mutation, associated with the more severe forms of disease, often survived to adulthood (Brydges et al., 2009; Meng et al., 2009). The milder form of disease observed in our D305N mice is unlikely to be specific for this mutation because introduction of the analogous mutation into the orthologous mouse codon has recently been reported to lead to death in most hemizygous animals by 2–3 weeks of age (Bonar et al., 2012). A possible explanation for the severe disease in the knockin models is that disease-associated substitutions have a greater effect on the stability and structure of the mouse protein. Subtle differences in the regulation of the expression of the human and mouse genes, such as higher expression in neutrophils, could further alter disease presentation.

In conclusion, we have used syntenic replacement to generate a mouse line that allows study of the human NLRP3 gene in diverse primary cells and in vivo after challenge with damage-associated molecular pattern molecules. The mechanism used in generation of the mice allows introduction of mutations into both conserved and non-conserved residues of the human gene as well as into non-coding regions of the gene. Mice carrying various NLRP3 alleles will allow direct genotypic/phenotype evaluation of NLRP SNPs, including evaluation of the relative sensitivity of mutant NLRP3 proteins to environmental factors. The ease with which these mice can be bred should make it possible to generate sufficient numbers of animals to detect relatively subtle effects on inflammasome function. The mice carrying the normal and mutant proteins also provide a platform for testing drugs designed to inhibit the activity of NLRP3 because species differences in the efficacy of inhibitors are avoided.

**EXPERIMENTAL PROCEDURES**

**Generation of the Mouse Lines**

Generation of mouse ESCs and mouse lines lacking the entire 35-kb mouse Nlrp3 locus, extending from base pair 59,534,790 to base pair 59,569,921 on chromosome 11, has been reported previously (Kovarova et al., 2012). The ΔNlrp3 locus contains a neomycin resistance gene and the 3′ half of a human HPRT minigene preceded by a mutant loxP site. We corrected the ΔNlrp3 locus using the 48.2-kb syntenic segment of human DNA (base pair 247,570,114 to 247,618,312 human chromosome 1 derived from BAC RP11-243D8). This DNA segment carries the common NLRP3 allele in which codon 305 (numbering based on uniprot.org) corresponds to aspartic acid. The DNA was incorporated into a vector, referred to as a Replacer, that carries the 5′ half of the HPRT minigene followed by a mutant loxP site complementary to that in the deleted locus. Cre-mediated recombination of the Replacer vector with the deleted locus resulted in reconstitution of a selectable HPRT minigene. The same segment of DNA used in the Replacer vector was mutagenized by introducing a transition mutation at the first position of codon 305 to create the D305N mutant. A second ESC line and a mouse line were generated by correction of the ΔNlrp3 locus with the D305N NLRP3-containing DNA fragment. Mice were housed within a specific pathogen-free animal facility. Control mice were either littermates or mice matched and co-housed at weaning with control animals bred in the same room. Studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee guidelines for the University of North Carolina at Chapel Hill.

**Expression Analysis**

RNA was isolated using RNA-Beet (Tel-Test), and cDNA was generated with a high-capacity cDNA archive kit (Applied Biosystems). cDNA was amplified with Taqman PCR Universal Master Mix using commercially available probes/primers (Applied Biosystems). Samples were run in duplicate, and relative expression was determined by normalizing samples to 18S RNA (ΔCt). Data were analyzed using the comparative Ct method as described by Applied Biosystems. In some experiments, samples from mutant animals were normalized to values obtained from co-housed control animals (ΔΔCt).

**Activation of Macrophages**

Macrophages were cultured in RPMI medium containing 10% fetal bovine serum (FBS), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), L-glutamine, penicillin/streptomycin (pen/strep), and 2-mercaptopethanol with LPS alone (E. coli serotype 055:BS, 1 μg/mL unless otherwise indicated) or LPS plus one of the following agents: silica (250 μg/mL), MSU (100–500 μg/mL), CPPD (100–500 μg/mL), R837 (5 μg/mL), or R848 (5 μg/mL). ATP (5 mM) or nigericin (20 μM) was added to LPS-treated cultures 30 min before harvest. In some experiments, macrophages were collected 72 hr after treatment of mice with 2.5 mL of 4% thioglycollate broth.

**LPS-Induced Hypothermia**

Mice received the indicated LPS dose i.p. in saline. The core body temperature was measured using a rectal probe (Physitemp Instruments). Peritoneal exudate was collected from the peritoneal cavity after injection of 3 mL of PBS. In some experiments, mice were treated with CRID3/MCC950, catalog no. 210826-40-7, dissolved in saline, i.p. 30 min before LPS exposure.

**Quantification of Cytokines and Inflammatory Mediators**

ELISA was used to quantify IL-1α, IL-1β, TNF-α, and IL-6 (eBioscience); IL-18 and IL-1ra (R&D Systems); and serum amyloid (Immunology Consultants Laboratory). Homogenates were prepared from hindpaws, including the tarsus, as described previously (Lu et al., 2010) using Fast Prep24 126-48 and Roche protease inhibitor cocktail tablets.

**Edema Formation**

Evans’ blue (0.05% in PBS) was delivered intravenously (i.v.) (0.01 mL/g) 30 min later, mice were euthanized, tissue was collected, and extravasated dye was extracted in formamide at 55°C and quantitated by spectrophotometry at 610 nm as described.

**Flow Cytometry**

Flow cytometry was performed using a Beckman Coulter CyAn ADP analyzer, and data were analyzed using FloJo (TreeStar) software after staining with CD4-FITC, CD8-PE, CD69-APC, or isotype-matched control antibodies (BD Biosciences).

**Non-invasive Monitoring of Inflammation and Bone Loss**

Animals received 200 mg/kg of chemiluminescent reagent (XenoLight Redi-Active Probe, PerkinElmer) by i.v. injection. Images were captured 5 min later using an indocyanine green (ICG) filter set for IVIS Lumina. Radiographic images were obtained using a Faxitron X-ray MX-20 specimen radiograph system (10 at 18 kV) and analyzed with Agfa CR-35X digital X-ray. For microCT imaging and bone analysis, the left hindlimbs of 6-month-old female mice were imaged in 70% ethanol with the knee joint intact using microCT at a resolution of 10 μm (µCT80, Scanco Medical). The trabecular bone microarchitecture was
analyzed in the proximal tibia metaphysis in a region beginning just distal to the epiphyseal growth plate and extending 1 mm in length away from the knee joint.

**Isolation of Peritoneal Mast Cells**

Peritoneal cells were collected by lavage, and mast cells were purified by Percoll density centrifugation as described previously (Hachisuka et al., 1988). The purity of the cell populations was determined to be greater than 99% based on staining of a sample of cells with toluidine.

**Western Blot Analysis**

Macrophages were stimulated with 1 μg/mL LPS and lysed at the indicated time. 20 μg of total protein was separated by SDS-PAGE (Hybond-P, Amersham, GE Healthcare) under reducing conditions, transferred onto a polyvinylidene difluoride membrane, and incubated with an antibody that can bind both human and mouse NLRP3 (Cryo-2 Adipogen) and anti-mouse-IgG HRP (#7076) from Cell Signaling Technology.

**Statistical Analysis**

Unless otherwise indicated in the figure legends, groups were compared by two-tailed Student’s t test.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at [http://dx.doi.org/10.1016/j.celrep.2016.11.052](http://dx.doi.org/10.1016/j.celrep.2016.11.052).

**AUTHOR CONTRIBUTIONS**


**ACKNOWLEDGMENTS**

We thank Anne Latour for assistance with tissue culture and Joseph Snouwaert for artwork. This work was supported by NIH grants AR061491 (to J.P.-Y.T.), DK094779, and AI067798 (to J.P.-Y.T.).

Received: November 22, 2015

Revised: August 29, 2016

Accepted: November 16, 2016

**REFERENCES**


Supplemental Information

An NLRP3 Mutation Causes Arthropathy and Osteoporosis in Humanized Mice

John N. Snouwaert, MyTrang Nguyen, Peter W. Repenning, Rebecca Dye, Eric W. Livingston, Martina Kovarova, Sheryl S. Moy, Brian E. Brigman, Ted A. Bateman, Jenny P.-Y. Ting, and Beverly H. Koller
Figure S1 related to Figure 3: Generation and characterization of expression of an NLRP3 allele encoding the D305N mutation. A. Sequence at codon 305 of the mutant human NLRP3 gene. A transition mutation at the first position of this codon switches the encoded amino acid from an aspartate to an asparagine. The segment containing the D305N mutation was amplified using primers 5'-GACTGGGCGTCGGGGACACTCTAC-3' and 5'-GCAGTTTCTCCAGGGCACAGGTC-3' and sequenced to verify the presence of the mutation. B. Expression of the mutant NLRP3 gene carrying the D305N SNP was compared to that of the wild type human allele in various tissues and cell types by quantitative PCR normalizing to 18S RNA. The levels of the mutant transcript did not differ significantly from that of the human wild type mRNA. C. To address this further we determined whether differences would become apparent after activation of macrophages with LPS. Peritoneal macrophages were obtained from wild type animals carrying a single copy of either wild type or mutant NLRP3. The expression of NLRP3 and II1b present in cells from control mice expressing the common NLRP3 gene mice was assigned a value of 1. No significant difference was observed in the levels of the mutant and wild type human NLRP3 transcripts. n = 3 for all samples.
Figure S2 related to Figure 6: Growth of the D305N/D305N mice. The weights of wild type male 129 and D305N/D305N mice co-housed at weaning were examined at 6 months of age. Although the mean weight of the mutant mice was approximately 3 g less than that of the wild type, the difference did not achieve significance. No difference in the weight of the livers was observed; however the spleens of the mutant animals were significantly larger, with weights approximately twice that of the normal 129 animals. Results are expressed as mean ± SEM. *p < 0.05. D305N mice were bred to congenic 129-Il1b mice (N8) to generate NLRP3 mutant animals unable to produce IL1-β. Spleen weights in these animals did not differ significantly from wild type mice, indicating that this cytokine is predominately responsible for the increase in elevated numbers of splenic neutrophils in the D305N mice.
Figure S3 related to Figure 6. Inflammation in the subarachnoid space of the D305N mice. Comparison of similar sagittal sections showing the cerebellum and associated meninges from NLRP3/NLRP3(A) and D305N/D305N mice (B) shows extensive accumulation of inflammatory cells in the leptomeninges of mutant animals. C. Higher magnification of the area indicated with an asterisk (*) shows both granulocytes (arrow) and monocytes (arrow head) in the infiltrates. D. In some cases granulocytes could also be detected in the molecular layer of the cerebellum. P; Pukinjie cell. Slides represent one of four D305N mice examined at 6 months of age.
Figure S4 related to Figure 6. Magnitude of startle response in the acoustic startle test of D305N mice compared to mice expressing the wild type gene. Trials included no stimulus (No S) trials and acoustic startle stimulus (AS; 120 dB) alone trials. No significant difference was observed between the animals homozygous for the mutant allele and wildtype animals. Data shown are means (± SEM) for each group. See Supplemental Methods for description of statistical analysis.
Figure S5 related to figure 6: Longitudinal section through the hind digit of a D305N/D305N mouse stained with hematoxylin and eosin at increasing magnification. Left to right. Cellular infiltrate was observed in the dorsal connective tissue and ligaments (asterisk) of one of seven mutant mice examined. High magnification shows presence of mononuclear cells as well as cells with segmented nuclei, the latter consistent with the recruitment of granulocytes (arrows). Bone loss was apparent (arrow head), while the skin/hair follicles were relatively free of inflammatory cells. B, cuneiform bones; Sk, skin.
Figure S6 related to Figure 7: Impact of the D305N mutation on the radiological features of the bones of wild type and D305N mice at 6 weeks of age. A. X-ray of hind legs from *Nlrrp3/Nlrrp3* (WT) and D305N/D305N mice. B. Three-dimensional reconstructed bones of ankle joints of *Nlrrp3/Nlrrp3* and D305N/D305N mice. At this age mutant mice were difficult to distinguish from wild type animals, indicating that the presence of the arthropathy in the adult animals was progressive and not the result of developmental abnormalities.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10^3/\mu l)</td>
<td>0.85 ± 0.25</td>
<td>1.73 ± 0.20</td>
<td>1.23 ± 0.23</td>
<td>2.33 ± 0.92</td>
<td>8.33 ± 1.35*</td>
</tr>
<tr>
<td>LYMF (10^3/\mu l)</td>
<td>0.40 ± 0.30</td>
<td>1.33 ± 0.34</td>
<td>0.88 ± 0.24</td>
<td>1.5 ± 0.70</td>
<td>3.43 ± 0.64*</td>
</tr>
<tr>
<td>GRAN (10^3/\mu l)</td>
<td>0.25 ± 0.05</td>
<td>0.20 ± 0.06</td>
<td>0.18 ± 0.03</td>
<td>0.47 ± 0.13</td>
<td>4.1 ± 1.2b</td>
</tr>
<tr>
<td>MONO (10^3/\mu l)</td>
<td>0.20 ± 0.00</td>
<td>0.30 ± 0.10</td>
<td>0.18 ± 0.03</td>
<td>0.38 ± 0.09</td>
<td>0.8 ± 0.17a</td>
</tr>
<tr>
<td>LYMF - %</td>
<td>42.25 ± 17.65</td>
<td>76.3 ± 10.33</td>
<td>64.83 ± 10.19</td>
<td>54.7 ± 13.35</td>
<td>42.28 ± 7.11</td>
</tr>
<tr>
<td>GRAN - %</td>
<td>35.75 ± 11.15</td>
<td>15.20 ± 6.27</td>
<td>20.25 ± 4.92</td>
<td>27.33 ± 6.74</td>
<td>48.16 ± 8.93</td>
</tr>
<tr>
<td>MONO - %</td>
<td>22 ± 6.50</td>
<td>12.75 ± 0.35</td>
<td>14.93 ± 5.27</td>
<td>17.97 ± 6.62</td>
<td>9.57 ± 1.88</td>
</tr>
<tr>
<td>HCT - %</td>
<td>45.75 ± 1.55</td>
<td>46.83 ± 0.78</td>
<td>48.1 ± 0.83</td>
<td>46.03 ± 1.72</td>
<td>44.96 ± 2.40</td>
</tr>
<tr>
<td>MCV - fl</td>
<td>56.50 ± 0.20</td>
<td>57.5 ± 0.21</td>
<td>54.93 ± 0.52</td>
<td>57.37 ± 0.24</td>
<td>52.96 ± 1.92</td>
</tr>
<tr>
<td>RBC (-10^6/\mu l)</td>
<td>8.09 ± 0.31</td>
<td>8.14 ± 0.16</td>
<td>8.75 ± 0.15</td>
<td>8.01 ± 0.31</td>
<td>8.48 ± 0.21</td>
</tr>
<tr>
<td>HGB - g/dl</td>
<td>12.70 ± 0.50</td>
<td>12.97 ± 0.18</td>
<td>13.58 ± 0.19</td>
<td>12.7 ± 0.40</td>
<td>12.17 ± 0.62</td>
</tr>
<tr>
<td>MCH - pg</td>
<td>15.75 ± 0.05</td>
<td>15.97 ± 0.15</td>
<td>15.55 ± 0.21</td>
<td>15.83 ± 0.15</td>
<td>14.37 ± 0.55</td>
</tr>
<tr>
<td>MCHC - g/dl</td>
<td>27.85 ± 0.15</td>
<td>27.73 ± 0.22</td>
<td>28.33 ± 0.31</td>
<td>27.53 ± 0.13</td>
<td>27.13 ± 0.33</td>
</tr>
<tr>
<td>RDW%</td>
<td>11.95 ± 0.15</td>
<td>12.97 ± 0.07</td>
<td>12.78 ± 0.31</td>
<td>12.2 ± 0.21</td>
<td>13.93 ± 0.78</td>
</tr>
<tr>
<td>MPV - fl</td>
<td>5.70 ± 0.00</td>
<td>5.77 ± 0.07</td>
<td>5.8 ± 0.14</td>
<td>5.8 ± 0.06</td>
<td>5.73 ± 0.07</td>
</tr>
<tr>
<td>PLT (10^9/\mu l)</td>
<td>350.50 ± 73.50</td>
<td>346.33 ± 24.46</td>
<td>398.25 ± 17.59</td>
<td>375 ± 47.09</td>
<td>594 ± 101.86</td>
</tr>
</tbody>
</table>

Table S1 related to Figure 5: Hematological parameters in Nlrp3/Nlrp3, NLRP3/NLRP3, ΔNlrp3/ΔNlrp3, Nlrp3/D305N, and D305N/D305N mice. Blood samples were collected in EDTA (5 mM) from deeply anesthetized mice of the indicated genotypes by cardiac puncture and analyzed using Heska's Animal Blood Counter. Values obtained for the D305N mice are expressed as mean ± SEM.

*, p < 0.05

a, p < 0.05, when compared to combined values from the two wild type groups, Nlrp3/Nlrp3 and NLRP3/NLRP3.

b, Although the mean number of granulocytes in the blood of the mutant animals was more than 10 times higher than that of normal mice, the difference did not achieve significance because of the high animal to animal variation.

### Table S2 related to Figure 7. Evaluation of bone structure of NLRP3 and D305N mice by mCT.
Seven mice of each genotype were included in the study. Significance is determined using a t-test with significance set at p<0.05.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NLRP3/NLRP3</th>
<th>D305N/D305N</th>
<th>%diff</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td>7.74±0.63</td>
<td>7.68±0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conn-Dens. (1/mm^3)</td>
<td>34.6±2.9</td>
<td>24.3±3.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DT-Tb.N (1/mm)</td>
<td>3.03±0.09</td>
<td>2.78±0.1</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>DT-Tb.Th (mm)</td>
<td>0.0479±0.00</td>
<td>0.0568±0.00</td>
<td>-29.8</td>
<td>0.0496</td>
</tr>
<tr>
<td>DT-Tb.Sp (mm)</td>
<td>0.332±0.010</td>
<td>0.367±0.020</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vBMD (mg HA/ccm)</td>
<td>93.9±7.2</td>
<td>108.9±6.4</td>
<td>18.5</td>
<td>0.016</td>
</tr>
<tr>
<td>Ct.Po (%)</td>
<td>3.97±0.05</td>
<td>4.69±0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DT-Ct.Th (mm)</td>
<td>0.276±0.00</td>
<td>0.242±0.00</td>
<td>18.2</td>
<td>0.004</td>
</tr>
<tr>
<td>pMOI (mm^4)</td>
<td>0.359±0.03</td>
<td>0.239±0.01</td>
<td>-12.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T.Ar (mm^2)</td>
<td>1.63±0.06</td>
<td>1.33±0.02</td>
<td>-33.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B.Ar (mm^2)</td>
<td>1.001±0.030</td>
<td>0.814±0.014</td>
<td>-18.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M.Ar (mm^2)</td>
<td>0.626±0.035</td>
<td>0.520±0.009</td>
<td>-18.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BA/TA (%)</td>
<td>61.7±0.7</td>
<td>61.0±0.2</td>
<td>-17.0</td>
<td>0.01</td>
</tr>
<tr>
<td>TMD (mg HA/ccm)</td>
<td>1195±0</td>
<td>1191±1</td>
<td>-0.3</td>
<td>0.003</td>
</tr>
</tbody>
</table>

BV/TV: bone volume fraction,
Conn.Dens: connectivity density
Tb.N: trabecular number
Tb.Th: trabecular thickness
Tb.Sp: trabecular separation
vBMD: volumetric bone mineral density
Ct.Po: cortical bone porosity
Ct.Th: cortical bone thickness
T.Ar: total cross-sectional area
B.Ar: total bone area
M.Ar: marrow cavity area
pMOI: polar moment of inertia
vBMD: volumetric bone mineral density
TMD: tissue mineral density
Supplemental Experimental Procedures

Evaluation of Hearing in mice. Acoustic startle procedure. The acoustic startle test was used to measure hearing ability, reactivity to environmental stimuli, and sensorimotor gating. Measures were taken of startle magnitude. The test was conducted using a San Diego Instruments SR-Lab system. Each mouse was placed in a small Plexiglas cylinder within a larger, sound-attenuating chamber. The cylinder was seated upon a piezoelectric transducer, which allowed vibrations to be quantified. The chamber included a ceiling light, fan, and loudspeaker for the acoustic stimuli (bursts of white noise). Background sound levels (70 dB) and calibration of the acoustic stimuli were confirmed with a digital sound level meter. Each test session consisted of 42 trials, presented following a 5-min habituation period. There were 7 types of trials: no-stimulus trials, trials with the acoustic startle stimulus (40 ms; 120 dB) alone, and trials in which a prepulse stimulus (20 ms; 74, 78, 82, 86, or 90 dB) had onset 100 ms before the onset of the startle stimulus. The different trial types were presented in blocks of 7, in randomized order within each block, with an average intertrial interval of 15 sec (range: 10 to 20 sec). Measures were taken of the startle amplitude for each trial, defined as the peak response during a 65-ms sampling window that began with the onset of the startle stimulus. Data were analyzed using repeated measures ANOVA, with the factors genotype and decibel level. Fisher's protected least-significant difference (PLSD) tests were used for comparing group means only when a significant F value was determined in the ANOVA. For all comparisons, significance was set at p < 0.05.

MicroCT Imaging and Bone Analysis. Measured parameters included trabecular bone volume fraction (BV/TV), volumetric bone mineral density (vBMD), connectivity density (Conn.Dens), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). Cortical bone parameters were obtained from the mid-diaphysis of the femur in a region spanning 0.5 mm beginning immediately distal to the third trochanter and extending towards the knee joint. Measurements included cortical bone porosity (Ct.Po), cortical bone thickness (Ct.Th), total cross-sectional area (T.Ar), total bone area (B.Ar), marrow cavity area (M.Ar), polar moment of inertia (pMOI), volumetric bone mineral density (vBMD), and tissue mineral density (TMD). T.Ar measures the cross-sectional area encompassed by both the cortical bone and the medullary/marrow cavity, the cortical bone ‘footprint’. pMOI is a direct measurement of the ability of a structure to resist torsional loads, and is independent of material properties. It is also mathematically related to a structure’s ability to resist bending loads.