Growth Hormone Receptor Deficiency Protects against Age-Related NLRP3 Inflammasome Activation and Immune Senescence

Graphical Abstract

Highlights

- The long-lived GH-R-deficient mice are protected from inflamming

- Loss of GH-R deactivates NLRP3 inflammasome and increases naive T cells in aging

- Ablation of macrophage IGF1-IGF1R axis inhibits the inflammasome

- Macrophage somatotrophic axis regulates NLRP3 inflammasome in aging

Authors

Olga Spadaro, Emily L. Goldberg, Christina D. Camell, ..., Andrzej Bartke, Liou Y. Sun, Vishwa Deep Dixit

Correspondence

vishwa.dixit@yale.edu

In Brief

Reduction in endocrine GH and IGF1 axis extends lifespan in model organisms. In this work, Spadaro et al. show that the ablation of the somatotropic axis within macrophages dampens the NLRP3 inflammasome-mediated inflammation seen with aging and prevents against age-related loss of naive T cells.

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Growth Hormone Receptor Deficiency Protects against Age-Related NLRP3 Inflammasome Activation and Immune Senescence

Olga Spadaro,1 Emily L. Goldberg,1 Christina D. Camell,1 Yun-Hee Youm,1 John J. Kopchick,2 Kim Y. Nguyen,1 Andrzej Bartke,1 Liou Y. Sun,4 and Vishwa Deep Dixit1,5,*

1Section of Comparative Medicine and Program on Integrative Cell Signaling and Neurobiology of Metabolism, Yale School of Medicine, New Haven, CT 06520, USA
2Edison Biotechnology Institute, Ohio University, Athens, OH 45701, USA
3Department of Internal Medicine, Southern Illinois University School of Medicine, Springfield, IL 62701, USA
4Department of Biology, University of Alabama at Birmingham, Birmingham, AL 35203, USA
5Department of Immunobiology, Yale School of Medicine, New Haven, CT 06520, USA
*Correspondence: vishwa.dixit@yale.edu
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SUMMARY

The hallmarks of age-related immune senescence are chronic inflammation, aberrant expansion of effector memory, and loss of naive T lymphocytes due in part to systemic activation of innate immune sensor NLRP3 inflammasome in myeloid lineage cells. The endogenous mechanisms that regulate inflammasome activation during aging are unknown. Here, we present evidence that growth hormone receptor (GH-R)-dependent downregulation of NLRP3 inflammasome in macrophages is linked to pro-longevity effects that maintain immune system homeostasis in aging. Deletion of GH-R prevented the macrophage-driven age-related activation of inflammasome in response to NLRP3 ligands and also increased the preservation of naive T cells, even in advanced age and with higher IFNγ secretion from effector cells. The mechanism of inflammasome inhibition is linked to autocrine somatotropic axis as ablation of IGF1R in macrophages lowered the NLRP3 inflammasome activation. Together, our findings show that functional somatotropic axis in macrophages controls inflammation, thus linking NLRP3-mediated innate immune signaling to health span and longevity.

INTRODUCTION

Systemic low-grade inflammation contributes to the development of chronic diseases and degenerative changes during aging (Ferrucci et al., 2005; Goldberg and Dixit, 2015). The increase in inflammatory cytokines in the elderly often referred to as “inflammaging” is a proposed driver of chronic diseases and shortened health span (Franceschi and Campisi, 2014). Although the cellular origin of age-related inflammation and molecular mechanism is not fully understood, emerging evidence suggests that accumulation of cellular damage, due to exhaustion of endogenous mechanisms in tissue macrophages that clear the damage-associated molecular patterns (DAMPs), may play an important role in this process (Goldberg and Dixit, 2015). These new data support Metchnikoff’s original prediction that phagocytes or macrophages drive aging-associated degenerative diseases (Metchnikoff, 1908).

It is now understood that accumulation of DAMPs, such as byproducts of necrotic cells, extracellular ATP, ceramides, saturated fatty acids, uric acid, amyloid fibrils, or free cholesterol crystals, are sensed by pattern recognition receptors (PRRs) in macrophages (Medzhitov, 2008; Schroder and Tschopp, 2010) to trigger chronic low-grade inflammation seen during aging (Youm et al., 2013). The NLRP3 inflammasome is the primary sensor of structurally diverse DAMPs and initiator of a sterile inflammatory cascade (Lamkanfi and Dixit, 2014). Assembly of the NLRP3 inflammasome in response to DAMPs requires pyrin-pyrin interaction of NLRP3 with adaptor protein ASC. The active inflammasome complex is formed through CARD-CARD (caspase activation recruitment domain) interaction of ASC with procaspase-1. Once assembled, the NLRP3 inflammasome activates caspase-1, which in turn controls the secretion of IL-1β and IL-18 (Martinon et al., 2002). The ablation of NLRP3 inflammasome prevents age-related inflammation and functional decline (Youm et al., 2012, 2013). This raises the question, what are the endogenous pathways that regulate NLRP3 inflammasome during aging, and are these mechanisms relevant to longevity and maintenance of immune homeostasis?

Downregulation of somatotropic axis (GH, IGF-1, and hypothalamic factors that control GH release) is a well-established endocrine feature of the aging process that is associated with deficits in physiological processes (Bartke et al., 2013). The question whether this decline causes age-related disorders or represents a compensatory homeostatic mechanism to promote longevity has been addressed using several model organisms (Bartke et al., 2013; Kenyon, 2010). For example, the mutant mice lacking GH (growth hormone), GH-R (GH receptor), and
Figure 1. GH-R-Deficient Mice Are Protected from Nlrp3 Inflammasome Activation in the Visceral Adipose and from T Cell Diversity Restriction in Aging

(A–D) VAT from young (3 month) and old (30 month) WT and Ghr−/− mice was analyzed for Nlrp3, Casp-1, Asc, and IL1β gene expression by real-time PCR (data are expressed as mean ± SEM; n = 3/age group/genotype).

E

WT (3m)WT (30m) Ghr−/− (30m) Ghr−/− (3m)

IL1β Asc Actin

F

3 month 30 month

Procas1 p37 22 kDa p20 Actin

G

WT (3m) Ghr−/− (3m) WT (30m) Ghr−/− (30m)

CD4+CD62L+ Naive 71% ERM 10%

CD8+CD62L+ Naive 71% ERM 5%

H

CD4+ CD44+

CD8+ CD44+

I

CD62L+ CD44+

CD62L− CD44+

J

CD62L+ CD44+

CD62L− CD44+

K

IFN-γ (pg/mL) LOD

(legend continued on next page)
GHRH (GH-releasing hormone) outlive their normal siblings by ~40% with better metabolic function, low risk of cancer, and severely reduced IGF-1-circulating levels (Brown-Borg et al., 1996; Coschigano et al., 2003; Sun et al., 2013). Pro-longevity intervention caloric restriction is also associated with reduction in IGF-1 in rodents. Interestingly, unlike in rodents, the recent human CALERIE-II trial, which evaluated the effect of 15% reduction in energy intake over 2 years, shows no decrease in serum-circulating IGF-1 concentration but rather increased serum IGFBP-1 and decreased IGF-1:IGFBP-1 ratio levels; this suggests an overall reduction in IGF-1 bioactivity in humans post-calorie restriction (Fontana et al., 2016). Consistent with these data, fasting and caloric-restriction-induced reduction in IGF-1 protects against age-related defects in hematopoietic stem cells and T cell progenitors (Cheng et al., 2014; Longo and Finch, 2003). Interestingly, GH-R expression increases with age in hematopoietic stem cells, but GH signaling is dispensable for normal hematopoiesis and bone marrow reconstitution post-cytoablation (Stewart et al., 2014). GH-R in macrophages is required for inflammation and glucose homeostasis in the context of diet-induced obesity (Lu et al., 2013). Given a central role of macrophage-expressed NLRP3 inflammasome in age-related inflammation, we investigated the contribution of endogenous pro-aging GH-IGF-1 pathway in controlling the innate immune-sensing machinery and immune senescence. Our findings show that downregulation of GH-R-mediated signals in macrophages represents an endogenous regulatory “break” mechanism that limits age-related inflammation by downregulation of NLRP3 inflammasome activation. These data also suggest that longevity in GH-R-deficient mice is associated with reduction in NLRP3 inflammasome.

RESULTS

Deletion of GH-R Protects against Age-Related Inflammasome Activation and T Cell Senescence

GH controls lipolysis and can likely impact the generation of metabolic DAMPs that are sensed by the NLRP3 inflammasome to induce inflammation. Given the important role of visceral adipose tissue (VAT) in age-related inflammation and insulin sensitivity (Huffman and Barzilai, 2009; Youm et al., 2013), our initial studies investigated whether GH-R-deficient long-lived mice exhibit altered regulation of inflammasome machinery during aging. Compared to young WT mice, 36-month-old mice displayed significant upregulation of IL-1β, caspase-1, Nlrp3, and the Asc mRNA in VAT that was significantly reduced in age-matched GH-R-deficient (Ghr−/−) mice (Figures 1A–1D). Ablation of GH-R prevented the age-related increase in pro-IL-1β, whereas the secreted p17 isoform could not be detected in VAT lysates (Figure 1E). Consistent with this, aged VAT shows an increased expression of inflammasome adaptor protein ASC, which was significantly reduced in age-matched Ghr−/− mice (Figure 1E). To further define the role of GH-R in downregulation of inflammasome activation in aging, we then quantified the caspase-1 cleavage into enzymatically active p20 heterodimer. Consistent with our prior data (Youm et al., 2013), aging is associated with an increase in caspase-1 activation (Figure 1F). Compared to age-matched control animals, 36-month Ghr−/− mice have reduced p20 subunit of caspase-1 (Figure 1F). These data suggest that GH-R-dependent signaling promotes age-related inflammasome activation in VAT.

We previously demonstrated that ablation of NLRP3 inflammasome in aged mice prevents the homeostatic expansion of effector memory (E/M) T cells and increases naive T cell output due to delayed thymic involution (Youm et al., 2012, 2013). Therefore, we determined whether GH-R-dependent reduction in age-related inflammasome activation impacts T cell senescence. As expected, WT mice in advanced age displayed expansion of E/M cells and loss of naive T cells in both CD4 and CD8 subsets (Figures 1G–1J) with no difference in the total CD4 and CD8 T cells subsets (Figures S1A and S1B). Strikingly, the 36-month-old age-matched Ghr−/− mice had increase in naïve CD4 and CD8 cells and were protected from E/M T cell expansions (Figures 1G–1J). Furthermore, ablation of GH-R in old mice increased the central memory CD8 cells (Figure 1H). To determine the functional capacity of E/M T cells, we sorted CD4 E/M T cells and stimulated them in a TCR-dependent manner to evaluate the effector cytokine production. Interestingly, compared to WT mice, the CD4 E/M T cells of middle-aged Ghr−/− mice secreted significantly higher levels of IFNγ (Figure 1K), whereas no differences in TNFα, IL-4, and IL-5 production were observed (Figures S1C–S1E). These data suggest that long-lived GH-R-deficient mice are protected from age-related inflammasome activation and associated defects in T cell immune compartment.

Age-Dependent Increase in Macrophage Activation Is Mediated in Part by GH-R

The inflammasome activation in macrophages requires an initial priming “signal 1” via TLRs-NF-κB pathway, which leads to transcriptional upregulation of cytokines and components of the inflammasome machinery (Gross et al., 2011). To determine the mechanism of GH-R-dependent inhibition of inflammasome during aging, we investigated the potential regulation of signal 1 in macrophages. We first investigated the expression of GH-R in BMDMs of young and old mice either primed with LPS or..
Figure 2. GH Signals in BMDMs Sustaining the M1 Signature

(A) Real-time PCR analysis of Ghr either in WT BMDMs untreated, LPS treated, and differentiated to the M1 phenotype (LPS + IFNγ) or in liver and VAT.

(B) pERK 1/2 protein expression was checked after rGH treatment in cultured WT 3-month-old BMDMs at different time points.

(C) pNF-kB and pERK signaling were evaluated in 30-month-old WT and Ghr−/−/− mice BMDMs either untreated or LPS primed in in vitro cell culture.

(D) Real-time gene expression analysis of the inflammasome components Nlrp3, Asc, and Casp-1 in cultured BMDMs under LPS and LPS + IFNγ (M1 phenotype) treatment. BMDMs were derived from 3-month- and 30-month-old WT and Ghr−/−/− mice as indicated.

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polarized to classical M1 activation. We found that GH-R expression is upregulated with aging in activated macrophages, but not in liver and VAT (Figure 2A), suggesting a role for GH in inducing signal 1 and in the pro-inflammatory macrophage phenotype upon aging. Next, to determine whether the GH-R in macrophages is functional, BMDMs were stimulated by GH and MAPK signaling was evaluated. Consistent with a functional response, the GH-R ligation increased ERK phosphorylation (Figure 2B). To determine whether signal 1 induction in old macrophages is dependent on GH-R, the BMDMs from WT and 30-month-old long-lived GH-R-deficient mice were stimulated with LPS to investigate the NFKB and MAPK signaling. These experiments revealed that LPS-induced NFKB and ERK phosphorylation was diminished in GH-R-deficient macrophages of old mice (Figure 2C), confirming the involvement of GH signaling in the NF-kB-dependent signal 1.

We next investigated the GH-R-dependent changes in inflammasome signal 1 and inflammatory mediators in BMDMs of young and old mice that were treated with LPS or polarized to classical M1 or alternative M2 activation. In LPS-stimulated BMDMs, ablation of GH-R prevented the age-dependent increase in inflammasome components ASC and caspase-1, whereas NLRP3 was not affected (Figures 2D and S2A). M1 macrophages demonstrated age-dependent increase in Nlrp3 (Figure 2D) and I1β (Figure 2E) mRNA expression that was attenuated in GH-R-deficient cells with no change in ASC or caspase-1 (Figure S2A). Interestingly, no age-related increase upon LPS and M1 stimulation in BMDMs was found for NLRC4 inflammasome (Figure S2B), suggesting the NLRP3 inflammasome specifically regulates age-related inflammation in mice.

Compared to young cells, the aged BMDMs displayed significantly higher expression of pro-inflammatory cytokines I12b, Tnf, and I6 that is GH-R dependent (Figures 2F–2H, S2C, and S2E). Furthermore, ablation of GH-R enhanced the expression of Cc22, Cc24, Arg1, and Il4r in M2 BMDMs, suggesting enhanced M2 polarization (Figures 2I–2L, S2D, and S2E). Together, these data suggest that GH-R controls transcriptional regulation of inflammasome components as well as age-related macrophage activation and inflammation.

**GH-R and Macrophage-Derived IGF1 Controls the Inflammasome Assembly in Response to Diverse “Danger Signals”**

After signal-1-dependent increase in transcription, the functional inflammasome is assembled when NLRP3 senses “signal 2,” which can be provided by a wide range of DAMPs (Henao-Mejia et al., 2014; Martinon et al., 2006; Wen et al., 2012). Therefore, we investigated whether GH-R expression controls NLRP3 inflammasome assembly in aged macrophages. Interestingly, compared to macrophages derived from 36-month-old WT mice, the age-matched GH-R-deficient BMDMs showed reduced caspase-1 activation and active p17 subunit response to canonical NLRP3 ligands ATP, urate crystals, and ROS damage induced by sodium arsenite (Figures 3A and 3B). Notably, ablation of GH-R attenuated the caspase-1 cleavage and IL-1β activation when NLRP3 inflammasome was primed by substituting LPS with a different TLR4 agonist lipid A (LA) or TLR2 ligands, peptidoglycan (PGN) and lipoteichoic acid (LTA), and signal-2-dependent inflammasome assembly was induced by ATP and urate (Figures 3C and 3D). Thus, the ablation of GH-R in old macrophages controls the NLRP3 inflammasome activation. Interestingly, aged GH-R-deficient macrophages expressed low levels of ASC and NLRP3, suggesting that the mechanism of reduced NLRP3 inflammasome activation is related to decrease in ASC-NLRP3 assembly availability upon activation by ATP, urate, and sodium arsenite (Figure 3E).

Given IGF-1 is a downstream effector of GH-R-dependent anabolic responses (Junnilla et al., 2013), we next investigated the mechanism of inflammasome regulation by the somatotropic axis. Surprisingly, compared to liver, which is the predominant endocrine source of circulating IGF-1, we identified that macrophages expressed IGF-1 similar to levels measured in liver (Figure 3F). Furthermore, macrophages also highly expressed the IGF-1 receptor (Igfr), suggesting an autocrine/paracrine role of somatotropic axis in innate immune signaling (Figure 3G). Therefore, we next specifically deleted Igfr1 from macrophages by myeloid-lineage-specific LysM-Cre (Figure S3). Interestingly, deletion of Igfr in macrophages led to significant reduction in NLRP3 inflammasome-dependent caspase-1 and IL-1β activation induced by extracellular ATP, urate crystals, sodium arsenite, and ceramides (Figures 3H and 3I). Together, these data suggest that downregulation of somatotropic axis in macrophages protects against NLRP3 inflammasome activation.

**Deletion of GH-R Protects against NLRP3-Dependent Urate-Crystal-Induced Inflammation**

The long-lived Ghr−/− mice show reduced expression of inflammatory markers and display significant increase in insulin sensitivity, despite an increase in adipose tissue mass (Masternak and Bartke, 2012). Given our prior data that VAT is a significant source of inflammation and displays increased NLRP3 inflammasome activation (Youn et al., 2013), we challenged the middle-aged WT and Ghr−/− mice with urate crystals and quantified myeloid cell infiltration into the VAT. The Ghr−/− displayed significant reduction in F4/80+CD11b+ adipose tissue macrophages (ATM) (Figure 4A). Importantly, compared to sham-treated mice, urate crystals induced significant increase in ATM infiltration in VAT that was significantly reduced in 12-month-old Ghr−/− animals (Figure 4B). Total number of B lymphocytes (B220+ MHCII+) shows an increase in VAT of urate-treated old WT mice, which was reduced in animals with GH-R deletion (Figure S4). Collectively, these data suggest that lack of GH-R-dependent signaling protects against NLRP3-driven inflammation in vivo.

(E–H) Real-time PCR analysis of I1β, I12b, TNFα, and I6 in in vitro M1-differentiated BMDMs.

(I–L) Real-time PCR analysis of Cc22, Cc24, Arg1, and Il4r in in vitro M2-differentiated BMDMs.

All real-time data are presented as mean ± SEM (n = 3–10/age group/ genotype); statistical differences were calculated by two-way ANOVA with Tukey’s test (*p < 0.05).
Figure 3. GH-R Ablation Protects against NLRP3 Inflammasome Activation, Suggesting an IGF1-IGF1R Autocrine-Paracrine Pathway in BMDMs

(A and B) LPS-primed 36-month-old WT and 36-month-old Ghr−/− BMDMs were stimulated with multiple aging-relevant DAMPs (ATP, MSU, and NaArs), and supernatants were analyzed for the active caspase-1 p20 subunit (A) and active IL-1β p17 subunit (B) by immunoblotting. n = 3–10/group; each blot is representative of one mouse/genotype.

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DISCUSSION

Chronic diseases such as kidney disease, atherosclerosis, gout, cancer, diabetes, Alzheimer’s disease, and immunopathology resulting from aberrant immune responses are the major contributors to death and disability in the elderly (Goldberg and Dixit, 2015; Pawelec et al., 2014). Epidemiological evidence suggests that aging is the single biggest risk factor for these chronic diseases, and mechanistically, inflammation is thought to be a common link between aging and disease. Despite increasing awareness of the health and economic impact of the growing elderly population, limited progress has been made in understanding the immunological mechanisms that control age-related inflammation and whether harnessing the endogenous regulatory pathways can enhance health span and reduce disease burden. Here, we show that downregulation of GH-R-IGF1 axis in macrophages is one such signal that links longevity to innate immune sensor NLRP3 and control of age-related inflammation.

Given a vital role of innate immune system in maintaining tissue homeostasis and regenerative responses through regulated production of growth factors and pro-inflammatory cytokines, tissue macrophages located in every organ are the prime cellular candidates for potential role in age-related inflammation and degenerative changes (Medzhitov, 2008). Thus, sustained activation of tissue macrophages by endogenous metabolic danger signals or exposure to exogenous environmental contaminants during the lifespan can propagate inflammatory damage and tissue dysfunction. Interestingly, macrophages can sense diverse DAMPs via nucleotide-binding domain leucine-rich repeat (NLR) protein such as NLRP1, NLRP3, NLRC4, NLRC5, NLRP6, or NLRP7 (Martinon et al., 2009). Among these NLRs, the NLRP3 has been demonstrated to sense the environmental danger signals such as silica and asbestos and endogenous DAMPs such as urate (Martinon et al., 2006), extracellular ATP (Mariathasan et al., 2006), cytosolic DNA (Muruve et al., 2008), mitochondrial damage (Zhou et al., 2011), ceramides, and fatty acids (Youm et al., 2012). Importantly, downregulation of the NLRP3 inflammasome complex in macrophages protects against age-related inflammation (Youm et al., 2013). Thus, the endogenous-tissue-derived mechanisms that negatively control the NLRP3-dependent inflammatory responses may be especially relevant to aging, longevity, and health span extension. Our data show that downregulation of GH-R signaling, which extends lifespan, controls inflammation in aging by inhibiting both the transcriptional signal1 as well as signal-2-dependent post-translational processing of NLRP3 inflammasome.

Importantly, T cells are not the predominant sources of inflammasome-dependent cytokines IL-1 or IL-18 and NLRP3 masome-dependent cytokines IL-1 or IL-18 (Guarda et al., 2011). Thus, GH-R-dependent increase in naive T cells in advanced aging could result from indirect effect of overall reduction in systemic inflammation or reduced inflammasome activation in thymic macrophages that could result in protection from thymic involution and higher T cell export. Importantly, data from long-lived GH-R-deficient mice showing higher naive T cells are intriguing as prior studies show that treatment of mice and humans with GH increases thymic naive T cell production (Dixit, 2010; Murphy et al., 1992; Napolitano et al., 2008). Future studies using aged cell-specific gene-targeted mice would be required to gain definitive mechanistic insights of potential cell-intrinsic thymic effects of GH and inflammasome interactions. The GH-R deficiency enhanced the naive T cell repertoire and reduced the compensatory effector/memory T cell expansions typically seen in aging. Functionally, the E/M T cells of aged GHRKO mice displayed enhanced IFNγ secretion upon TCR-mediated activation. These findings are consistent with recent reports on the importance of the increased fibroblast responsiveness to IFNγ in long-lived primates (Pickering et al., 2015), which imply a role for the interferon response in the biology of aging.

The importance of the immune endocrine cross-talk mediated by GH-GH-R interactions in aging adipose tissue suggests that changes in secretion of adipocytes-derived hormones adiponectin and leptin may impact the adipose inflammation (Masternak et al., 2012). GH-IGF1 axis is thought to primarily operate at the endocrine level (Junnila et al., 2013). Surprisingly, we found macrophages are a substantial source of IGF-1 and have significant IGF-1R expression. Existing transcriptional profiling data sets from Immunological Genome Project (http://www.immgen.org) further confirmed that macrophages and myeloid origin cells highly express IGF1 axis, suggesting an auto/paracrine function. Consistent with this hypothesis, ablation of IGF1R in macrophages and downregulation of somatotrophic axis demonstrated reduced inflammasome activation in response to diverse NLRP3 activators. In addition, downregulation of GHRH-GH-IGF1 axis exerts regulatory effects on the immune system by lowering experimental ocular inflammation (Qin et al., 2014). Furthermore, upon challenge with urate-crystal induced NLRP3 inflammasome activation in vivo, the GH-R-deficient middle-aged mice are protected from VAT leukocytosis. Collectively, our data show that inhibition of NLRP3 inflammasome by ablation of GH-R and IGF1R is one of the mechanisms that links reduced inflammation to pro-longevity effects.

EXPERIMENTAL PROCEDURES

Experimental Animals

Young and long-lived Ghr−/− mice and WT have been described previouly (Zhou et al., 1997). Mice were sacrificed 10 days after the quarantine period (C and D) Caspase-1 p20 active subunit immunoblot analysis of supernatants in LA-, PGN-, and LTA-primed BMDMs stimulated with ATP (C) or urate (D). (E) Immunoblot analysis of ASC and NLRP3 within the protein fraction of BMDMs, LPS-primed, and stimulated ATP, MT, or Naars as indicated in 36-month-old WT and 36-month-old Ghr−/−. (F and G) Real-time PCR analysis of Ifg1 (F) and Ifg1r (G) gene expression in liver and BMDMs from 3-month WT, LysM-Cre−, and LysM-Cre+ IGF1Rfl/fl(n = 5–7/group). (H and I) LPS-primed 3-month-old LysMCre− and LysMCre+ IGF1Rfl/fl BMDMs were stimulated with multiple aging-relevant DAMPs, and cells (H) and supernatants (I) were analyzed for active caspase-1 (p20) and active IL-1β (p17) by immunoblotting.
Subcutaneous Adipose Tissue

WT (12m)  Ghr−/− (12m)

CD11b+  F4/80+

5%  2.1%

Visceral Adipose Tissue

WT Sham (12m)  WT Urate (12m)  Ghr−/− Urate (12m)

CD11b+  F4/80+

7%  24%  18%

F480+ CD11b+

# cells/g

SHAM  MSU

(legend on next page)
period; during this time mice were multi-housed and fed ad libitum with normal chow diet (5002; LabDiet). Igf1R-flox strain can be purchased from Jackson Laboratories (B6;129-igf1r^floxed^). The colony was multi-housed, fed ad libitum with normal chow diet (5002; LabDiet), and kept following guidelines issued by Yale University’s Institutional Animal Care and Use Committee (IACUC).

**Cell Culture**
The murine bone marrow was collected from femurs in RPMI (Life Technologies) + 10% FBS (R10 Omega Scientific) + 5% antibacterial/antimycotic (Life Technologies), and red blood cells were lysed using ACK lysis buffer. Bone-marrow-derived macrophages (BMDMs) were differentiated using M-CSF (10 ng/ml; R&D Systems) and L929-conditioned media for 7 days. Non-adherent BMDMs were re-plated and treated the following day (see Supplemental Experimental Procedures).

For FACS analysis, CD4^+^ CD62L^-^ CD44^-^ E/M T cells were sorted from splenocytes and cultured on anti-CD3-coated plates (BD Pharmigen) + CD28 soluble antibody 2 µg/ml (BD Pharmigen) added to the media (RPMI + 10% FBS + 5% antibacterial/antimycotic). After 96 hr, the supernatants were measured for IFN-γ, TNFα, IL-4, and IL-5 by Bio-Plex Pro Mouse Cytokine Th1/Th2 Assay (Bio-Rad no. M60-00003J7) on Luminex xPONENT system.

**Western Blot**
BMDM cell lysates were prepared in RIPA buffer by vortexing samples every 10 min for 30'. Samples were then centrifuged at 14,000 xPONENT system. Supernatants were measured for IFN-γ, TNFα, IL-4, and IL-5 by Bio-Plex Pro Mouse Cytokine Th1/Th2 Assay (Bio-Rad no. M60-00003J7) on Luminex xPONENT system.

**Gene Expression Analysis**
Total RNA was extracted using the Trizol method and transferred to the QIAGEN RNeasy mini kit and purified according to the manufacturer’s instructions. Synthesis of cDNA and qPCR was performed as described previously (Nolan et al., 2006). The primer pairs used for real-time PCR are listed in the Supplemental Experimental Procedures.

**Flow Cytometry**
Antibodies used were CD4, CD8, CD44, and CD62L (eBioscience Affymetrix). Cells were acquired on a BD FACSCalibur and data were analyzed in FlowJo (TreeStar), SVF was stained for Fixable Viability Dye Aqua (Life Technologies), B220, MHCII, F4/80, and CD11b. Data were acquired on a BD LSR II and analyzed in FlowJo.

**Statistical Analyses**
Statistical significance of the differences between groups was calculated either by two-tailed paired Student’s t test or by two-way ANOVA using Tukey’s test, which protects the significance of all pair combinations (GraphPad Prism 6 software).

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.01.044.

**AUTHOR CONTRIBUTIONS**
O.S. designed and conducted the majority of experiments, analyzed/interpreted the data, and participated in writing the manuscript. E.L.G. performed FACS analysis and interpreted the data, and C.D.C. performed the sorting analyses and interpreted the data. Y.-H.Y. conducted the analyses of T cell senescence, and K.Y.N. assisted in generation and validation of macrophage Igf1R mice and assisted in data analysis. J.J.K. generated the GH-R-null mice and assisted in data interpretation. A.B. and L.Y.S. aged the GH-R-deficient and control mice and participated in study design and data analysis. V.D.D. conceived and supervised the project, interpreted the data, and wrote the manuscript.

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**Figure 4.** GH-R-Deficient Mice Are Protected from MSU-Induced Macrophage Infiltration in Adipose Tissue  
(A) The SVF separated from subcutaneous adipose tissue was analyzed for F480^+^ CD11b^+^ cells by FACS staining in 12-month WT and 12-month Ghr^-/-^ mice. (B) Twelve-month WT and twelve-month Ghr^-/-^ mice were challenged with MSU, and 4 hr later, the SVF from the VAT was analyzed for F480^+^ CD11b^+^ cells by FACS staining.

All data are presented as mean ± SEM (n = 4 or 5/genotype/treatment); statistical differences were calculated by two-tailed paired Student’s t test (p < 0.05).
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Supplemental Information

Growth Hormone Receptor Deficiency Protects against Age-Related NLRP3 Inflammasome Activation and Immune Senescence

Olga Spadaro, Emily L. Goldberg, Christina D. Camell, Yun-Hee Youm, John J. Kopchick, Kim Y. Nguyen, Andrzej Bartke, Liou Y. Sun, and Vishwa Deep Dixit
Figure S1 Related to Figure 1, Spadaro et al.
Figure S2 Related to Figure 2, Spadaro et al
Figure S3 Related to Figure 3, Spadaro et al
Subcutaneous Adipose Tissue

WT (12m)  

B220+ MHCII+

Visceral Adipose Tissue

WT-Sham (12m)  WT Urate (12m)  Ghr-/- Urate (12m)

B220+ MHCII+

Figure S4 Related to Figure 4, Spadaro et al
Figure S1. Related to Figure 1

CD4 and CD8 T cells subsets in the splenocytes of 3Mo-36Mo WT and Ghr −/− mice represented as FACS staining (A) and as percentages of total gated cells (B).

(C-E) Luminex analysis of cytokine secretion from CD4⁺ CD44⁺ effector-memory sorted and activated T cells from splenocytes in 12 month WT and Ghr −/− mice siblings. (one dot represents one mouse; n=3/group; error bars are representative as mean ± SEM; *p<0.05).

Statistical differences were calculated by two tailed paired Student’s t-test (*p<0.05) (C-E).

Figure S2. Related to Figure 2

A. 3Mo-30Mo Ghr −/− and WT siblings real-time PCR analysis in in vitro differentiated BMDMs of Nlrp3 (LPS treated), Asc and Casp-1 (LPS +IFNγ treated -M1).

B. Real time PCR analysis of the NLRC4 Inflammasome in in vitro differentiated BMDMs, untreated (M0), LPS treated (LPS) or LPS +IFNγ (M1) treated in 3Mo-30Mo WT mice.

C. 3Mo-30Mo Ghr −/− and WT siblings real-time PCR analysis of Il1β, Il12β, TNFa, Il6 and (D) Ccl22, Ccl24, Arg-1, Il4R in in vitro differentiated BMDMs, untreated (M0) or LPS treated (LPS).

E. 3Mo-30Mo Ghr −/− and WT siblings real-time PCR analysis of iNOS, IRF-5, Retnla, Chi3l3, in in vitro M1 (LPS+IFNy) - M2 (IL-4) differentiated BMDMs (n=3-10/age group).
Figure S3. Related to Figure 3

PCR products in the lysozyme 2 gene locus for the Cre allele mutant band (750bp; lane 2) and wild type band (350 bp; lane 1) and in the Igf1r gene locus for Igf1r floxed mutant allele (220 bp; lane 3) and Igf1r wild type allele (124 bp; lane 4).

Figure S4. Related to Figure 4

A. The SVF separated from the subcutaneous adipose tissue of WT and Ghr<sup>-/-</sup> 12-month-old mice was stained for B220<sup>+</sup> MHCII<sup>+</sup>.

B. Upon 4 hour challenge with MSU, the SVF was separated from VAT of WT and Ghr<sup>-/-</sup> 12 month-old-mice and stained for B220<sup>+</sup> MHCII<sup>+</sup> cells. (n=7-8/group; each dot represents one mouse; error bars are representative as mean ± SEM; *p<0.05).
Supplemental Methods

Cell culture treatment

BMDMs cells were treated with ultrapure LPS (L6529-1mg, Sigma-Aldrich) alone or in combination with 5 mM ATP (1h) (1A7699-1G; Sigma-Aldrich) or 250 μg/ml MSU (5 hr) (tlrl-msu, InvivoGen), 0.2mM Sodium Arsenite (1h) (Fluka Analytical), or Ceramide C6, 80 μg/ml (6 hr) (62525-10, Cayman). The BMDMs were also primed with ultrapure lipid A (10 μg/ml; tlrl-mpla; InvivoGen), lipoteichoic acid (10 μg/ml; tlrl-pslta; InvivoGen), or peptidoglycan (10 μg/ml; tlrl-pgnsa; InvivoGen).

BMDMs were also differentiated into M1-M2 subtypes by treatment with LPS (1μg/ml) + IFNγ (10 ng/ml; eBioscience), or IL-4 alone (20 ng/ml; eBioscience) respectively for 24 hours. CD4+ CD62L- CD44+ effector-memory T cells were sorted from splenocytes and cultured for 96 hr on anti-CD3 coated plates (BD Pharmingen) in the presence of soluble anti-CD28 2 μg/ml (BD Pharmingen).

As for GH recombinant protein, BMDMs were starved for 12 hours in RPMI w/o supplements and rGH (Origene, TP720039) was added at the concentration of 0.5 μg/ml.

Breeding strategies

Igf1Rfl/fl were crossed to LysMCre/Cre (B6.129P2-Lyz2tm1(cre)Ifo, Jackson Laboratory) to generate the Het littermate Igf1Rfl/fl LysMcre/-. Het mice were either intercrossed or backcrossed to Igf1Rfl/fl to generate experimental LysMcre/-Igf1Rfl/fl and controls LysMcre/- and LysM+/Igf1Rfl/fl. Both controls were used in the experiments as no phenotypical differences were observed.
**Intraperitoneal injection**

For IP MSU injection mice were given a single dose of 2.5 mg MSU (tlrl-msu, InvivoGen) diluted in PBS and were sacrificed 4 hours after injection. Control mice were injected with the same amount of vehicle (PBS).

**Adipose tissue digestion**

Visceral and subcutaneous adipose tissues were harvested at sacrifice and weighed. Tissue was digested in 0.1% collagenase I enzyme (Worthington Biochemicals) in Hanks Buffered Salt Solution (Life Technologies, Inc.) for 45 min at 37°. Stromal vascular fraction (SVF) was pelleted by centrifugation at 1500 rpm for 10’, washed twice in PBS and filtered. ACK lysing buffer was used to remove red blood cells. The SVF was then incubated with Fc Block and stained.
Table S1. Related to gene expression analysis in Methods.

| Gene name | Sequence                  |
|-----------|---------------------------|
| Il1b Fw   | AAGAGCTTCAGGCAGGCAGTATCA  |
| Il1b Rev  | ATGAGTCACAGAGGATGGGCTCTT |
| Il12b Fw  | AGCAGTAGCAGTTCCCCCTGA    |
| Il12b Rev | AGTCCCTTGGTCCAGTGTC      |
| Tnfα Fw   | CCACCACGCTCTTCTGTCTAC    |
| Tnfα Rev  | AGGGTCTGGGCCCATAGAACT    |
| Il6 Fw    | AGCAAAAGCCAGAGTCCCTTCAGAG|
| Il6 Rev   | TTTGTCCTTACGCACTCTTCTGT  |
| Nlrp3 Fw  | GCTAAGAAGGACCAGCCAGA     |
| Nlrp3 Rev | CAGCAAAACCATCCACTCTT     |
| Asc Fw    | TACAGCCAGAAACAGGCACCTTT  |
| Asc Rev   | AAAGCATCCAGCACTCCGTCA    |
| Casp-1 Fw | GGACCTCAGAATTGGCCCTCT    |
| Casp-1 Rev| AGACGTGTACGAGTGTTGTT     |
| Arg1 Fw   | ATTAACGAGCAGCTTTCTC      |
| Arg1 Rev  | TTTTTCAGCAGACGAGCTT      |
| Il4r Fw   | TGTGGGCCTGTAGTCTTCTT     |
| Il4R Rev  | GGGAAAGTGCGGATGTAGTCA    |
| Ccl22 Fw  | ACCTCTGATGCGTCCTCCTAT    |
| Ccl22 Rev | AGGGTGACGGATGTAGTCTCT    |
| Ccl24 Fw  | GCGTGTTGCATCTCCTCCCAT    |
| Ccl24 Rev | GATGAAGATGACCCCTGCCTTG   |
| Gapdh Fw  | TCAACAGCAAATCCTCCACTCCTC |
| Gapdh Rev | ACCCTGTTGCTGTAGCCGTATCA  |