



# HHS Public Access

Author manuscript

*Nat Med.* Author manuscript; available in PMC 2011 August 01.

Published in final edited form as:

*Nat Med.* 2011 February ; 17(2): 179–188. doi:10.1038/nm.2279.

## The NALP3/NLRP3 Inflammasome Instigates Obesity-Induced Autoinflammation and Insulin Resistance

**Bolormaa Vandanmagsar<sup>1,5</sup>, Yun-Hee Youm<sup>1,5</sup>, Anthony Ravussin<sup>1,5</sup>, Jose E. Galgani<sup>2,5</sup>, Krisztian Stadler<sup>3,5</sup>, Randall L. Mynatt<sup>5</sup>, Eric Ravussin<sup>2,5</sup>, Jacqueline M. Stephens<sup>4</sup>, and Vishwa Deep Dixit<sup>1,5,6</sup>**

<sup>1</sup>Laboratory of Neuroendocrine-Immunology, Louisiana State University System, Baton Rouge, LA 70808

<sup>2</sup>Human Physiology Laboratory, Louisiana State University System, Baton Rouge, LA 70808

<sup>3</sup>Oxidative stress and Disease Laboratory, Louisiana State University System, Baton Rouge, LA 70808

<sup>4</sup>Department of Biological Sciences, Louisiana State University System, Baton Rouge, LA 70808

<sup>5</sup>Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808

### Abstract

Emergence of chronic ‘sterile’ inflammation during obesity in absence of overt infection or autoimmune process is a puzzling phenomenon. The Nod Like Receptor (NLR) family of innate immune cell sensors like the Nlrp3 inflammasome are implicated in recognizing certain non-microbial origin ‘danger–signals’ leading to caspase-1 activation and subsequent IL-1 $\beta$  and IL-18 secretion. We show that reduction in adipose tissue expression of Nlrp3 is coupled with decreased inflammation and improved insulin–sensitivity in obese type-2 diabetic patients. The Nlrp3 inflammasome senses the lipotoxicity–associated ceramide to induce caspase-1 cleavage in macrophages and adipose tissue. Ablation of Nlrp3 prevented the obesity–induced inflammasome activation in fat depots and liver together with enhanced insulin–signalling. Furthermore, elimination of Nlrp3 in obesity reduced IL-18 and adipose tissue IFN $\gamma$  along with an increase in naïve and reduction in effector adipose tissue T cells. Collectively, these data establish that Nlrp3 inflammasome senses obesity–associated ‘danger–signals’ and contributes to obesity–induced inflammation and insulin–resistance.

### Keywords

Inflammation; obesity; type 2 diabetes; IL-18; T cells; adipocytes; adipose tissue; macrophages; cryopyrin; Pycard; IL-1 $\beta$ ; caloric restriction; caspase-1; Nlrp3; Nalp3; ASC; LPS; Ceramide; Lipotoxicity; MAPK; Akt

---

Users may view, print, copy, download and text and data- mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: [http://www.nature.com/authors/editorial\\_policies/license.html#terms](http://www.nature.com/authors/editorial_policies/license.html#terms)

<sup>6</sup>Address and Correspondence to: Vishwa Deep Dixit, Ph.D, Laboratory of Neuroendocrine-Immunology, Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808, Vishwa.Dixit@pbrc.edu, Phone: 225-763-2719, Fax: 225-763-0261.

## Introduction

Despite no overt infection or auto-antigen mediated immune cell activation, the chronic inflammation during obesity is an established instigator of several diseases like the type 2 diabetes, defective immunity, atherosclerosis, certain cancers, CNS dysfunction and dementia<sup>1–4</sup>. It is recognized that activation of adipose tissue macrophages and T cells within fat depots is coupled with the development of obesity-induced pro-inflammatory state and insulin resistance<sup>5, 6</sup>. The activation of classically activated M1 macrophages at the expense of anti-inflammatory M2 macrophages has been causally linked to the development of adipose-tissue inflammation and metabolic syndrome<sup>7, 8</sup>, a pathophysiological state aptly termed as “metainflammation”<sup>9</sup>. It is recognized that several pro-inflammatory cytokines<sup>10</sup>, including IL-1 $\beta$ , are implicated in disrupting insulin-signaling<sup>11</sup>. Consistent with these data, randomized clinical trials have demonstrated that blockade of Interleukin1 $\beta$  (IL-1 $\beta$ ) signalling by Anakinra, a recombinant human IL-1 receptor antagonist leads to sustained reduction in systemic inflammation and improvement of type-2 diabetes<sup>12, 13</sup>. However, the origin of inflammation during obesity and the underlying molecular mechanisms are not fully understood. Furthermore, although the physiological rationale of release of pro-inflammatory cytokines by macrophages during infection is established<sup>14, 15</sup>, the mechanism underlying the maladaptive chronic inflammation observed during adipose-tissue expansion and obesity is still speculative.

Innate immune cells such as macrophages discriminate infectious agents from self proteins by detecting ‘pathogen-associated molecular patterns’ (PAMPs) through expression of pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs)<sup>15–17</sup>. Emerging evidence suggests that macrophages can also recognize the injured or damaged-cell derived ‘danger-associated molecular patterns’ (DAMPs) and release pro-inflammatory cytokines such as IL-1 $\beta$ <sup>18–20</sup>. The release of bioactive IL-1 $\beta$  and IL-18 from macrophages is in turn dependent on autocatalytic activation of procaspase 1 zymogen into enzymatically active 10 and 20 kDa caspase-1 heterodimers<sup>21–23</sup>. The presence of a large N-terminal homotypic protein-protein interaction motif called caspase activation recruitment domain (CARD) is critical for formation of multiprotein scaffolds called inflammasome wherein caspase-1 undergoes conformational change required for its cleavage and full activation<sup>18–20, 24</sup>.

Structurally, the NLR proteins contain N-terminal CARD or pyrin domains needed for homotypic protein-protein interaction, an intermediate nucleotide binding self-oligomerization NACHT domain and a C-terminal domain containing leucine-rich repeats (LRRs)<sup>18–20, 24</sup>. Among the NLR family members, the Nlrp3 inflammasome (for NACHT, LRR and pyrin domain containing protein) has been implicated in sensing the non-microbial origin DAMPs such as extracellular ATP, urate crystals, asbestos, silica and  $\beta$ -amyloid<sup>25–28</sup>. The assembly of the Nlrp3 inflammasome requires interaction of pyrin domain (PYD) of ASC (for apoptosis-associated speck like protein containing carboxy-terminal CARD) with PYD of Nlrp3, and a functional inflammasome complex is formed through CARD-CARD interaction of ASC with procaspase 1<sup>24, 29</sup>.

It has been shown that obesity-induced elevation in specific saturated free-fatty acids may activate the TLR4 mediated signalling in macrophages and participate in inducing insulin resistance<sup>30, 31</sup>. However, it remains unclear whether cytosolic PRRs such as Nlrp3 play a role in sensing obesity related 'danger signals' or if Nlrp3 inflammasome participate in immune dysfunction leading to chronic inflammation and insulin-resistance in diet-induced obesity. We show that Nlrp3 inflammasome activation in obesity promotes macrophage-T cell activation in adipose tissue and impairs insulin-sensitivity.

## Results

### Nlrp3 is associated with obesity-induced insulin resistance

Our initial studies evaluated the expression of IL-1 $\beta$  and Nlrp3 in the adipose tissue. These data show that in mice the mRNA expression of IL-1 $\beta$  and Nlrp3 in the visceral adipose tissue (VAT) was correlated with body weight and adiposity (Fig. 1a,b). The anti-thesis of obesity, calorie restriction (CR) extends lifespan, reduces inflammation and enhances insulin sensitivity<sup>32</sup>, therefore we examined whether CR impacts the inflammasome transcriptional machinery in fat depots. Compared to *ad libitum* (AL) chow fed control mice, chronic CR (40% reduction in food intake) in age-matched middle aged animals (12 month old) resulted in a significant reduction ( $P < 0.01$ ) in *Nlrp3*, *Asc* and *Il-1 $\beta$*  mRNA in both visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) (Fig. 1c-e) in parallel with reduction in fat cell size (Fig. 1f,g).

To test the clinical relevance of data generated from mouse models, we investigated the obese-type 2 diabetic mellitus (T2DM) patients before and after weight loss achieved by intensive behaviour modifications such as CR and exercise (Supplementary Table 1). The intervention was designed to achieve and maintain weight loss through decreased caloric intake and increased physical activity with an expected 1-year weight loss of 7% of the initial value<sup>33</sup>. The weight loss in obese-T2DM subjects resulted in significant reduction in fat cell size and improvement of insulin-sensitivity (Supplementary Table 1). We collected the abdominal SAT biopsies from obese-T2DM male Caucasian subjects ( $n = 10$ ) before and after 1 year weight-loss intervention. To prevent observer bias, the real time PCR analysis to quantify the mRNA levels of *NLRP3*, *ASC* and *IL-1 $\beta$*  was performed in blinded fashion. The weight loss enhanced the insulin sensitivity in obese-T2DM which was associated with significant reduction in *IL-1 $\beta$*  and *NLRP3* mRNA expression in SAT with no change in *ASC* (Fig. 1h). Of note, the reduction in *IL-1 $\beta$*  and *NLRP3* expression in SAT was coupled with lower glycemia and the improvement in HOMA-IR in these subjects (Fig. 1i), (Supplementary Table 1). Together, these experiments in mouse models and humans indicate that increased adipose tissue Nlrp3 expression is correlated with obesity associated insulin-resistance. Conversely, chronic caloric restriction and weight-loss induced improvements in insulin-sensitivity is associated with reduction in adipose tissue expression of Nlrp3 inflammasome and IL-1 $\beta$ .

### Nlrp3 inflammasome activation in obesity regulates IL-1 $\beta$ and IL-18

Nlrp3 is known to be present in several tissues and cell types<sup>34</sup> but it is not known which cellular compartments in adipose tissue express the inflammasome components. The

immunostaining of adipose tissue sections of obese mice revealed strong co-localization of Nlrp3 with macrophage marker F4/80 (Fig. 2a) in crown-like structures (CLS). Consistent with immunofluorescence data, the examination of purified F4/80<sup>+</sup> adipose tissue macrophages (ATMs), stromal vascular fraction (SVF) and mature 3T3L1 adipocytes revealed that both *Nlrp3* and *Asc* are highly expressed in ATMs and SVF cells with low expression in adipocytes (Fig. 2b,c). We also analyzed the *Nlrp3* expression in enriched primary adipocytes from adipose tissue. Normalization of *Nlrp3* mRNA with differentiated macrophage marker *Cd11c* in adipocytes fraction revealed that almost all *Nlrp3* expression in enriched primary adipocytes maybe be attributed to contaminating lipid engorged macrophages (data not shown).

Notably, the development of progressive adiposity in high-fat diet (HFD) fed mice led to strong caspase-1 auto activation in adipose tissue (Fig. 2d), (Supplementary Fig. 1a,b). Consistent with progressive caspase-1 activation in obesity, we found an increase in IL-1 $\beta$  processing in adipose tissue with highest expression in 9 month old diet-induced obese (DIO) mice (Fig. 2d). Since the highest caspase-1 activation was detected in 9 month old DIO mice (Fig. 2d), (Supplementary Fig. 2a) and high prevalence of obesity 'epidemic' and related co-morbidities in middle-age population, we next studied the role of Nlrp3 inflammasome using chronic DIO model. This rationale is based on the hypothesis that 7 months of HFD feeding is likely to induce a more severe disease than the 6–8 weeks of HFD feeding in mice. Furthermore, although it is established that Nlrp3 inflammasome regulates post-translational processing of caspase-1 *in vitro* in the bone marrow derived macrophages, specificity of this pathway *in vivo* in HFD induced obesity is not established. Of note, compared to healthy-lean mice, obesity led to marked increase in caspase-1 autoactivation in VAT, SAT and liver (Fig. 2e), (Supplementary Fig. 1). Ablation of Nlrp3 partially blocked the obesity induced caspase-1 autoactivation in VAT, SAT and liver (Fig. 2e), (Supplementary Fig. 1), but did not affect caspase-1 cleavage in kidney (Fig. 2f). These findings demonstrate that in obesity, caspase-1 is specifically activated in adipose tissue and liver through Nlrp3 inflammasome dependent mechanism but in kidney this process is independent of Nlrp3 and is tissue specific.

The post-translational processing of IL-1 $\beta$  is complex and can be regulated through several inflammasomes including Ipaf inflammasome, and AIM2 inflammasome<sup>19–20</sup>. Furthermore, pro-IL-1 $\beta$  can be processed through neutrophil-derived serine proteases in a caspase-1 and inflammasome independent fashion<sup>21, 35</sup>. Our data show that compared to wild type (WT) mice the ablation of Nlrp3 inflammasome reduced the expression of active IL-1 $\beta$  in adipose tissue of diet induced obese (DIO) mice (Fig. 2g), (Supplementary Fig. 2b). The chow fed WT mice and Nlrp3 deficient animals did not show a difference in IL-1 $\beta$  processing in VAT (data not shown). Notably, compared to chow fed mice, obesity was associated with significant increase in serum IL-18 concentrations which was blocked upon ablation of Nlrp3 (Fig. 2h). The serum levels of IL-1 $\beta$  in our WT and *Nlrp3*<sup>-/-</sup> mice were below the detection limit of the ELISA (data not shown). These data demonstrate that Nlrp3 inflammasome is specifically activated in response to high fat diet and controls the production of IL-1 $\beta$  in adipose tissue and IL-18 in obesity.

## Nlrp3 inflammasome impairs insulin–sensitivity in obesity

To further understand the role of Nlrp3 inflammasome in regulating insulin–action, we performed insulin and glucose tolerance test (ITT and GTT) in WT and *Nlrp3*<sup>−/−</sup> mice fed HFD for 6 weeks, 4 months (6 month old) and 7 months (9 month old). The ITT and GTT revealed that in early stages of obesity, the elimination of Nlrp3 inflammasome affords greater protection against HFD–induced insulin–resistance (Fig. 3a). In chow fed controls, ablation of Nlrp3 did not alter insulin action (Supplementary Fig. 3a,b) nor affect glucose homeostasis suggesting specific activation of Nlrp3 inflammasome in diet–induced obesity. Compared to 6 month old WT DIO mice, *Nlrp3*<sup>−/−</sup> mice displayed higher magnitude of reduction in glucose after insulin injection (Fig. 3b,d) as well improved GTT (Fig. 3b). Furthermore, compared to age–matched controls, the 9 month old obese *Nlrp3*<sup>−/−</sup> mice (on HFD for 7 months) showed significant reduction in fasting glucose levels (Fig. 3c) as well as improved glucose homeostasis as revealed by the GTT (Fig. 3c). Due to lower baseline glucose values of *Nlrp3*<sup>−/−</sup> mice, the percent reduction in glucose post insulin injection was not significantly different. Estimation of area under the curve of ITT in 6 and 9 month old DIO mice demonstrated significant reduction in glucose levels in *Nlrp3* deficient mice (Fig. 3d).

Consistent with an association of Nlrp3 with adipocyte size and insulin–sensitivity in obese diabetic humans (Supplementary Table 1), the genetic ablation of Nlrp3 was associated with reduction in the fat cell size in VAT (Fig. 3e) but not in SAT (data not shown). Although the epidymal fat pad weight was lower in 3 month old DIO *Nlrp3*<sup>−/−</sup> mice, the final body weights of male WT and *Nlrp3*<sup>−/−</sup> mice on 60% HFD (or control chow) for 6 weeks, 4 months and 7 months did not show significant differences (Supplementary Fig. 4a,b). We did not detect any change in blood triglycerides and cholesterol levels in middle–aged obese *Nlrp3*<sup>−/−</sup> mice (Supplementary Fig. 4c,d).

Given that metabolic effects of insulin action are dependent on P13K–AKT signalling, we investigated the serine<sup>473</sup> phosphorylation of AKT after 5 and 10 min insulin injection in 8 month old obese WT ( $n = 9$ ) and *Nlrp3*<sup>−/−</sup> mice ( $n = 9$ ). Consistent with higher insulin–sensitivity, the Nlrp3 deficient mice had significantly greater ( $P < 0.05$ ) AKT activity in VAT, SAT, liver and muscle compared to the insulin treated obese WT control animals (Fig. 3f), (Supplementary Fig. 5), while, vehicle injected control mice did not show any AKT phosphorylation (Supplementary Fig. 6a). Consistent with enhanced insulin–sensitivity in the absence of Nlrp3 inflammasome, there was also a reduction in serine phosphorylation of IRS-1 in liver and fat of Nlrp3 deficient obese mice (Fig. 3f), (Supplementary Fig. 5e).

Apart from PI3K–AKT pathways, insulin can also signal by promoting the interaction of adaptor protein, Grb2 with son–of–sevenless to activate the Ras–MAPK signalling<sup>36</sup>. Considering that Ras–MAPK pathway primarily regulates non–metabolic effects of insulin such as cell growth, differentiation and survival<sup>36</sup>, we next tested the specificity of insulin–signalling in Nlrp3 inflammasome deficient obese mice. Interestingly, compared to control animals, we found marked activation of Erks 1 and 2 MAPK specifically in the VAT but not in SAT, liver or muscle (Fig. 3f), (Supplementary Fig. 6b). These data suggest that

improvement of insulin-sensitivity in Nlrp3 deficient mice is related to overall stimulation of PI3K-AKT pathway while the MAPK pathways is selectively activated in visceral fat.

Considering a reduction in Nlrp3 inflammasome dependent caspase-1 activation in liver and improvement in insulin signalling, we also investigated hepatic steatosis in 9 month old DIO *Nlrp3*<sup>-/-</sup> mice. The histological evidence suggest that compared to WT mice, the ablation of Nlrp3 resulted in reduction in hepatic steatosis (Fig. 3g) which is consistent with recent findings that activation of Nlrp3 inflammasome induces hepatic fibrosis and liver injury<sup>37, 38</sup>. Furthermore, the reduction in fatty liver disease in obese *Nlrp3*<sup>-/-</sup> mice was associated with increase in fatty acid oxidation regulators *Ehhad*, *Cpt1a* and *Crat*, with no change in *Acc1* and *Fas* (data not shown), the genes that regulate fatty acid synthesis (Fig. 3h).

### Ceramides activate Nlrp3 inflammasome and ATMs in obesity

Inhibition of obesity-induced caspase-1 activation and insulin-resistance in *Nlrp3*<sup>-/-</sup> mice prompted us to investigate whether the Nlrp3 inflammasome senses specific inducers that activate innate immune cells like macrophages. Recent evidence suggest that during progressive obesity, the development of adipose tissue fibrosis restricts the adipocyte expansion which may ultimately cause lipid spill-over and increases in tissue and circulating levels of free fatty acids and ectopic fat in several organs<sup>39</sup>. Since ATMs can scavenge lipids and generation of ceramide from fatty acids during obesity induces inflammation<sup>41-43</sup>, we tested whether Nlrp3 inflammasome senses ceramide. Bone marrow derived macrophages were primed with LPS to induce transcriptional activation of IL-1 $\beta$ , and secondary inflammasome activation signal was provided by ceramides established previously<sup>27</sup>. The immunoblot analysis revealed that together with the positive control (LPS +ATP) exposure of wild type macrophages to ceramides, causes activation of caspase-1 (Fig. 4a). Notably, ceramide induced caspase-1 activation is blocked in the absence of Nlrp3 (Fig. 4a). Consistent with this data, we observed that ceramide-induced IL-1 $\beta$  secretion from macrophages was reduced in absence of Nlrp3 (Fig. 4b).

Next, we investigated the physiological relevance of these data and tested whether ceramides can induce caspase-1 activation within the adipose tissue. We cultured the epididymal adipose tissue explants from 9 month old WT-DIO and *Nlrp3*<sup>-/-</sup> DIO in presence of LPS and ceramides. The LPS priming and ceramide induced caspase-1 activation was reduced in adipose tissue explants of Nlrp3 deficient mice (Fig. 4c). Considering that within the adipose tissue, the adipose-tissue macrophages (ATMs) highly express Nlrp3 inflammasome (Fig. 2b,c), we investigated whether reduction of Nlrp3-mediated sensing of obesity-associated pro-inflammatory inducers impacts the ATM's pro-inflammatory profiles *in vivo*. The ATMs were enriched from stromal vascular fraction (SVF) through positive selection from VAT and SAT of 9 month old control WT and *Nlrp3*<sup>-/-</sup> DIO mice. We found that loss of Nlrp3 function increased the expression of M2 macrophage associated transcripts, *Il-10* and *Arg1* in SAT-ATMs but not in VAT macrophages (Fig. 4d). These results suggest that the obese *Nlrp3*<sup>-/-</sup> mice specifically retain M2-like macrophage phenotype in the inguinal SAT. In addition, we found that M1-macrophage associated expression of *Tnfa*, *Ccl20* and *Cxcl11* were specifically reduced in the visceral fat derived ATMs but not in SAT macrophages (Fig. 4d). Also, the expression

of *Il-10* was increased in whole SAT while *Tnfa* was reduced in obese *Nlrp3* deficient animals (Supplementary Fig. 7). Compared to WT-obese mice, the ATMs derived from SAT of *Nlrp3*<sup>-/-</sup> obese animals showed lower *Nos2* expression (Fig. 4d). Collectively, these data suggest that ablation of *Nlrp3* protects from obesity-associated pro-inflammatory ATM activation.

### ***Nlrp3* inflammasome regulates adipose tissue T cells in obesity**

Together with macrophages, the expanded adipose tissue during obesity also harbors activated T cell populations which are thought to participate in local adipose tissue inflammation and insulin sensitivity<sup>44-46</sup>. Reduction in adipose inflammation and improved insulin-action due to *Nlrp3* deficiency prompted us to investigate the adipose tissue macrophages and T cell subsets. We found that in VAT of 9 month old DIO mice, the loss of *Nlrp3* function did not affect the frequency of M1 (F4/80<sup>+</sup>CD11c<sup>+</sup>CD206<sup>-</sup>) or M2 (F4/80<sup>+</sup>CD11c<sup>-</sup>CD206<sup>+</sup>) adipose tissue macrophages (ATMs) (Fig. 5a). Notably, we observed reduced numbers of smaller cells (on FSC/SSC, Figure 5A lower panel) in gate 2 of obese *Nlrp3*<sup>-/-</sup> mice. Gating of this population in SVF revealed that these cells are not ATMs as they lacked the expression of F4/80, CD11c and CD206.

Further examination of smaller cells in gate 2 within SVF revealed that in WT mice approximately 60% of these cells are T lymphocytes (Fig. 5b). Compared to WT controls, 9 month old middle-aged *Nlrp3* deficient mice did not show significant differences in the frequency of CD4 and CD8 cells (Fig. 5b). Furthermore, in middle-aged 9 month old obese mice, elimination of *Nlrp3* inflammasome signalling did not affect the overall ratio (percent gated) of CD4 and CD8 effector-memory E/M (CD62L<sup>-</sup>CD44<sup>+</sup>) or CD4/CD8 naïve T cell subsets (CD62L<sup>+</sup>CD44<sup>-</sup>) (Fig. 5b). The ablation of *Nlrp3* in 9 month old DIO mice reduced the overall percentage of lymphocytes (in FSC/SSC - Gate 2) within the SVF of VAT. The estimation of T cell numbers within SVF revealed that loss of *Nlrp3* led to significant reduction in the overall numbers of CD4s, CD4 E/M cells, CD8s and CD8 E/M cells without significant difference in naïve T cells (Fig. 5c). Consistent with these data, *Nlrp3* deficient obese mice had significant reduction in the total number of SVF cells in the visceral fat at both early (3 month old) and chronic stages of obesity (9 month old) (Fig. 5d).

Among various T cell subsets, the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T regulatory (T<sub>reg</sub>) cells constitute an important defence mechanism to dampen pro-inflammatory response in several autoimmune and infectious diseases<sup>44</sup>. Recent studies demonstrate that anti-inflammatory T<sub>reg</sub> cells in adipose tissue are reduced in obesity and increase in the T<sub>reg</sub> cell number improves insulin-action<sup>44</sup>. Analysis of T<sub>reg</sub> cell in visceral fat (epididymal + perirenal), subcutaneous fat (inguinal) in middle-aged DIO mice revealed no significant differences between WT and *Nlrp3* deficient mice (Fig. 5e,f) suggesting specificity of *Nlrp3* dependent effects on adipose tissue effector T cell subsets.

Together with VAT, the SAT also constitutes a significant proportion of adipose mass in obese mice. Considering that obesity-associated caspase-1 autoactivation in SAT is *Nlrp3* dependent, we next investigated whether the reduction in macrophage activation and insulin-sensitivity is associated with changes in distribution of naïve and effector/memory-ART (adipose resident T cells) cell subsets in subcutaneous fat. Notably, elimination of

Nlrp3 inflammasome in 9 month old obese mice increased the number of M2 macrophages (F4/80<sup>+</sup>CD11c<sup>-</sup>CD206<sup>+</sup>) without affecting the M1 macrophage (F4/80<sup>+</sup>CD11c<sup>+</sup>CD206<sup>-</sup>) frequency (Fig. 6a,b). Surprisingly, compared to WT-obese mice, both the CD4/CD8 adipose resident T cell number was significantly higher in *Nlrp3*<sup>-/-</sup> animals (Fig. 6c,d). Furthermore, examination of total CD4 and CD8 cells revealed that there was a preponderance of naïve cells (CD4<sup>+</sup>CD62L<sup>+</sup>CD44<sup>-</sup>, CD8<sup>+</sup>CD62L<sup>+</sup>CD44<sup>-</sup>) within the adipose T cell compartment of Nlrp3 deficient DIO mice (Fig. 6c,d) suggesting a lower inflammatory profile in fat.

Considering that macrophage-derived IL-18 induces a T helper1 (Th1) response for host defence against specific infections, we also investigated the classical Th1 derived cytokine IFN $\gamma$  in context of 'sterile' obesity-induced inflammation in Nlrp3 deficient mice. Consistent with reduction in obesity-induced IL-18 in *Nlrp3*<sup>-/-</sup> mice (Fig. 2h), the ablation of Nlrp3 inflammasome lowered the expression of *Ifn $\gamma$*  mRNA as well as protein in the adipose tissue during obesity (Fig. 6e,f). Furthermore, in absence of Nlrp3 inflammasome, obesity-induced increase in circulating interferon gamma inducible protein IP-10/CXCL10 and MCP-1/CCL2 were significantly reduced (Fig. 6g) without any change in IL-17 levels (data not shown). Collectively, the data suggest that blocking the activation of Nlrp3 inflammasome in response to obesity related 'danger signals' may lower macrophage-T cell interactions that participate in sustaining chronic inflammation (Fig. 6h).

## Discussion

Obesity is associated with self-directed tissue inflammation where local or systemic factors other than infectious agents activate the cells of innate immune system. In addition, evidence that 'sterile' inflammation during obesity is because of underlying autoimmune processes or due to presence of organ specific auto-antibodies has so far not been demonstrated. Therefore, obesity-induced chronic low grade inflammation and the development of metabolic syndrome may qualify the established criterion of "autoinflammatory disease cluster"<sup>47, 48</sup>. The influx of macrophages and T cells in adipose tissue upon chronic caloric excess driven obesity and release of pro-inflammatory mediators by these cells causes insulin-resistance<sup>6-9, 31</sup>. However, an upstream initiating event for obesity-induced immune cell activation in adipose tissue is not established. Here we demonstrate that Nlrp3 inflammasome plays a substantial role in sensing obesity-associated inducers of caspase-1 activation and therefore regulates the magnitude of the inflammation and its downstream effects on insulin signalling.

Our findings that adipose tissue expression of IL-1 $\beta$  and Nlrp3 is correlated with reduction in plasma glucose and insulin sensitivity in obese T2D patients post weight loss and reduction in Nlrp3 expression post CR in mice raised several questions. First, can caspase-1 undergo autoactivation in obesity and whether eliminating the signalling through Nlrp3 reduces adipose inflammation and improves insulin action? We found that induction of high-fat diet induced obesity caused marked caspase-1 activation in adipose tissue and liver. Such a steady state *in vivo* activation of caspase-1 in obesity has so far not been reported in the context of self-directed tissue inflammation. The loss of Nlrp3 function reduced but did not eliminate the caspase-1 activation in visceral fat, subcutaneous fat and liver suggesting



that other inflammasome may contribute in pathophysiology of obesity. In addition, Nlrp3 inflammasome activation was tissue specific as obesity-induced caspase-1 activation in kidney was not affected in Nlrp3 deficient mice. Consistent with these data, obese Nlrp3<sup>-/-</sup> mice were more insulin sensitive.

Another question that emerges from our findings is what is being sensed by Nlrp3 inflammasome to induce caspase-1 activation in obesity? This question is difficult to answer, particularly in a complex disorder such as diet-induced obesity where alterations in several cell-stress associated metabolites could potentially activate Nlrp3 or other inflammasomes (Fig. 6h). It has been shown that saturated fatty acids like oleate and palmitate can induce inflammation by TLR4<sup>30</sup> and loss of TLR4 function can partially protect against obesity induced insulin-resistance<sup>31</sup>. Notably, obesity-related increased levels of lipotoxic ceramides can induce cell death and also trigger inflammation. Our data demonstrates that ceramides induce caspase-1 activation in an Nlrp3 dependent mechanism. However, it is possible that several other inducers may participate in caspase-1 activation. For example Nlrp3 inflammasome has been recently shown to be activated by oxidized LDL and cholesterol crystals in models of high fat diet-induced atherogenesis leading to macrophage activation and IL-1 $\beta$  secretion<sup>49</sup>. Cells undergoing necrosis can also activate Nlrp3 inflammasome in macrophages<sup>50</sup>. Considering that the development of obesity is also associated with hypoxia<sup>51, 52</sup> and adipocyte death<sup>53</sup>, it is likely that ATMs that form 'Crown Like Structures' can be activated via Nlrp3 sensing pathway. Thus, it is possible that reduced sensing of such 'danger associated molecular patterns' (DAMPs) due to Nlrp3 deficiency protects from long-term obesity and hyperglycemia associated insult (Fig. 6h).

Lastly, another question that arises from our data is which mediators downstream of Nlrp3 inflammasome participates in the mechanism of obesity-induced inflammation and insulin-sensitivity? Although, the Nlrp3 inflammasome specifically regulates caspase-1 activation that allows the release of IL-1 $\beta$  and IL-18, both these cytokines in turn can initiate several events that may amplify inflammatory responses<sup>21</sup>. Our prior studies have found that obesity is associated with increase in IFN $\gamma$ <sup>+</sup> T cells in the adipose tissue<sup>54</sup>. It is well known that IFN $\gamma$  alone or together with microbial stimuli (such as LPS) is known to cause induction of classically activated M1 macrophages<sup>17</sup>. In context of obesity-associated inflammation, we provide additional mechanistic insight that reduction in IL-1  $\beta$  and IL-18 processing is regulated by Nlrp3 inflammasome that may deliver secondary signals to adipose tissue T cells and induce an effector Th1 pro-inflammatory profile in adipose tissue. Consistent with our data of reduced IFN $\gamma$  in Nlrp3<sup>-/-</sup> mice and improved insulin-signaling, the reduction in IFN $\gamma$  is associated with improved metabolic outcomes in obesity<sup>55</sup>. Furthermore, increases in M1 macrophage derived cytokines and effector T cells in adipose tissue are causally linked to insulin resistance<sup>8, 45, 46</sup>. Together with our previous studies that show that purified adipose T cells produce several cytokines that further activate macrophages<sup>54</sup>, our present data demonstrates that elimination of Nlrp3 inflammasome reduced M1-like macrophage gene expression and increases M2-like expressed cytokines. These data suggest that Nlrp3 inflammasome sensing pathway participate in the origin of inflammation in obesity by inducing macrophage and subsequent T cell activation (Fig. 6h).

Notably, blocking the obesity-induced gain of Nlrp3 inflammasome function did not affect the T<sub>reg</sub> cell homeostasis in adipose tissue but specifically reduced the E/M cells and increased the naïve CD4 and CD8 T cell populations. Consistent with our data, reduction in E/M cells in VAT may be linked to decrease in CCL20 and CXCL11 chemokine expression<sup>56</sup>. The improvement in overall metabolic profiles upon elimination of Nlrp3 signaling may also be related to lower hepatic steatosis and regulation of genes that control hepatic fatty acid oxidation in obese mice. During the preparation of our manuscript, it was reported that uric acid induced release of reactive oxygen species facilitate the interaction of thioredoxin interacting protein (TXNIP) to Nlrp3, leading to IL-1 $\beta$  release<sup>57</sup>. Consistent with our findings, these studies also support a role of Nlrp3 inflammasome in regulating glucose homeostasis<sup>57</sup>.

The early observations that pro-inflammatory mediator, TNF $\alpha$  induces insulin resistance *in vitro* and in mice, have so far yielded disappointing results in limited randomized clinical trials in obese T2DM patients<sup>58</sup>. On the other hand, inhibiting IL-1 $\beta$  signaling by IL-1 receptor antagonists Anakinra can dampen systemic inflammation including decrease in circulating C-reactive protein (CRP) and IL-6 and reduction in type 2 diabetes in humans<sup>12, 13</sup>. Furthermore, widely used anti-diabetic sulfonyl-urea drugs such as glyburide can also block Nlrp3 inflammasome activation and reduce mortality due to septic shock<sup>59</sup>. Taken together, our data establish that Nlrp3 inflammasome dependent post-translational processing of IL-1 $\beta$  and IL-18 in response to obesity-associated 'danger signals' participates in the development of chronic pro-inflammatory state which impairs insulin-sensitivity. These findings highlight the potential of targeting the molecular pathways regulating caspase-1 activation in obesity for management of insulin-resistance and chronic inflammation induced co-morbidities.

## Material and Methods

### Human Subject population

The participants (10 Caucasian males, obese, with type 2 diabetes) were examined to study the effects of 1-year intensive lifestyle intervention to promote weight-loss<sup>33</sup>. The intervention was designed to achieve and maintain a weight loss of at least 7% through decreased caloric intake and increased physical activity. The inclusion and exclusion criteria have been previously described<sup>33</sup>. We measured the fat mass in all subjects by dual-energy X-ray absorptiometry (Hologic QDR 4500A, Waltham, MA) and the analysis of fasting levels of glucose, FFA, insulin and adiponectin and HOMA – IR calculations were performed as described previously<sup>12,33</sup>. We determined the insulin-stimulated glucose disposal rate using a euglycemic-hyperinsulinemic clamp (90 mg dl<sup>-1</sup>, 80 mU m2 min<sup>-1</sup>). The *NLRP3* and *IL1 $\beta$*  mRNA in the abdominal subcutaneous adipose tissue was determined by quantitative Real Time PCR and *cyclophilin B* as a house keeping gene. All studies were performed under an IRB approved protocol with informed consent from participants.

### Mice and animal care

*Nlrp3*<sup>-/-</sup> mice have been described previously<sup>35</sup>. The mice were fed with ad libitum high-fat diet consisting of 60% calories from fat (D12492i; Research Diets Inc) starting at 8 weeks of

age and control mice with a standard chow diet consisting of 4.5% fat (5002; LabDiet). We purchased the 12 month female calorie restricted mice and *ad libitum* (AL) fed, C57/B6 mice from NIA-aging rodent colony. We performed all experiments in compliance with the NIH Guide for the Care and Use of Laboratory Animals, which were approved by the Institutional Animal Care and Use Committee at Pennington Biomedical Research Center.

### ITT and GTT test

We performed ITT and GTT on 4 hour fasted mice by giving an intraperitoneal injection of insulin ( $0.83 \text{ mU g}^{-1}$  body weight, Sigma, USA) and D-glucose ( $1.8 \text{ mg g}^{-1}$  body weight, Sigma, USA). Glucose levels in tail blood were measured using a glucometer (Breeze, Bayer Health Care, USA).

### Preparation of SVF cells and adipose tissue macrophages isolation

The subcutaneous (inguinal fat pad) and visceral adipose tissue (epididymal fat pad) were digested as described previously<sup>54</sup>. We isolated the the macrophages from SVF cells by positive selection selected using anti-F4/80 antibody (eBiosciences), followed by biotin-conjugated secondary rat IgG and cells were purified using streptavidin labelled magnetic beads (Dynabeads, Invitrogen).

### Flow cytometry

We stained the SVF cells from specific adipose depots with anti-F4/80, CD206, and CD11c (eBiosciences and Biolegend) to indentify macrophage subsets. We labelled the T cell subpopulations in SVF with anti -CD4, CD8, CD25, CD62L, FoxP3 and CD44 (eBiosciences) as described previously<sup>54</sup>. We conducted the FACS analysis using FACS Calibur (BD Pharmingen) and the FACS data was analyzed by post collection compensation using FlowJO (Treestar Inc) software.

### Macrophage culture and cytokine analysis

We prepared the murine bone marrow derived macrophages (BMDM) and cultured them according to previously established protocols<sup>26</sup>. We primed the BMDMs with  $100 \text{ ng ml}^{-1}$  ultrapure LPS (*E coli* serotype 0111:B4; Sigma) and after 12 h, ATP was added in a control well (1 mM) and ceramides in treatment wells with final concentration 0.1 mM. The IL-1 $\beta$  levels in BMDM supernatants were measured by ELISA (eBioscience) and cell lysates were used for western blot analysis.

### Adipose tissue explants culture

We prepared and cultured the adipose tissue explants from epididymal fat pad as described previously<sup>58</sup>. After 24 h of the treatment with LPS and ceramides, we prepared the protein lysates from adipose explants and analysed them for caspase-1 activation by immunoblot analysis.

### Immunohistochemistry

We formalin fixed and paraffin embedded the adipose tissue from mice and stained the tissue sections with haematoxylin and eosin. The immunofluorescence analysis of Nlrp3 and

F4/80 in adipose tissue sections was performed as described previously<sup>54</sup> and images were acquired using a Zeiss 510 Meta multiphoton confocal microscope.

### Cytokine measurement

We measured the levels of IL-1 $\beta$  (eBioscience) and IL-18 by ELISA (MBL, Japan) and the serum levels of IP10 and MCP-1 using milliplex bead assay (Millipore).

### Quantitative Real-time PCR

We isolated the total RNA from human SAT biopsy tissue, mouse fat, liver and from positively selected macrophages using RNeasy Lipid Tissue Mini Kit (Qiagen). All samples were DNase digested to remove potential genomic DNA contamination. We conducted the Quantitative Real-time PCR as described previously (BioRad)<sup>4, 54</sup>.

### Western Blot Analysis

For insulin-signaling experiments, we collected the adipose tissue, liver, kidney and muscle (gastrocnemius) and snap froze them in liquid nitrogen 5 and 10 minutes after the intraperitoneal insulin (0.8 mU g<sup>-1</sup> body weight) injections. We conducted the immunoblot analysis for AKT, IRS1 and MAPK as described previously<sup>54</sup>. For caspase-1 cleavage, we collected the organs of lean and DIO mice from additional cohorts of mice and snap froze them for western blot analysis. The protein immune complexes were detected using specific fluorescent secondary antibodies conjugated with IRDye<sup>TM</sup>800CW (Rockland) and membranes were imaged using Odyssey Infrared Imaging System (LI-COR Odyssey BLOT).

### Statistical Analyses

We used a two-tailed Student's t test to determine significance in differences between genotypes or treatments; \* $P < 0.05$  and  $P < 0.01$ , \*\* $P < 0.005$  and  $P < 0.001$ . We expressed the results as the mean  $\pm$  SEM or the mean  $\pm$  SE. The differences between means and the effects of treatments were also analyzed by one-way ANOVA using Tukey's test (Sigma Stat), which protects the significance ( $P < 0.05$ ) of all pair combinations.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

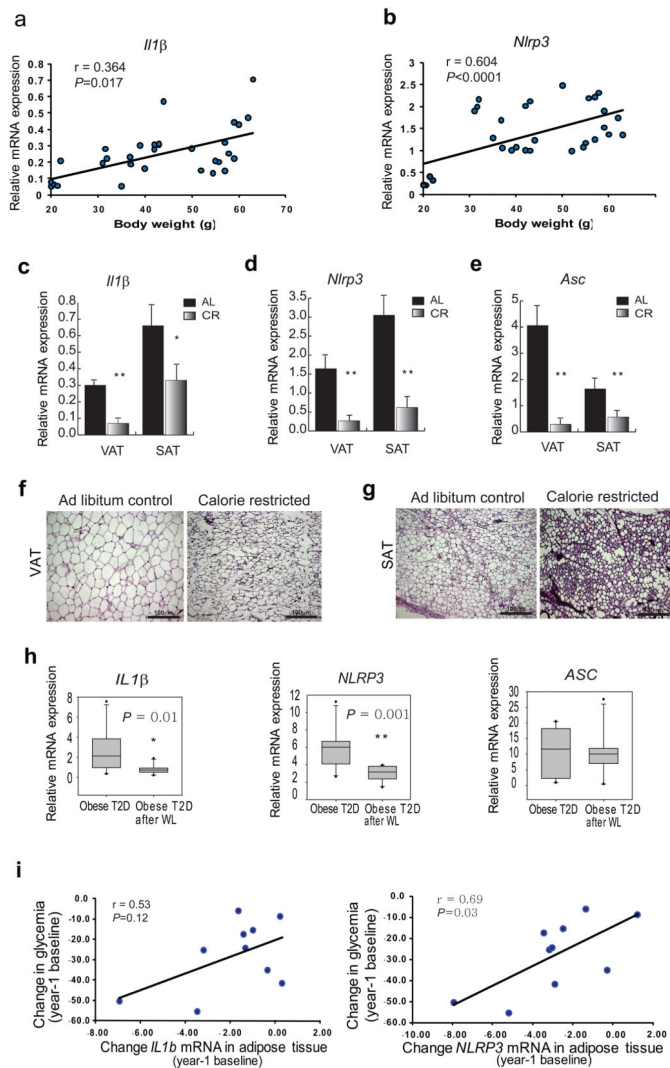
We thank V. Dixit at Genentech Inc for providing anti-Caspase-1 antibody and *Nlrp3*<sup>-/-</sup> mice and J. Suttles from University of Louisville for L929 media. We also thank Steven Bond for expert technical assistance and Drs. Donna H. Ryan, Claude Bouchard and J. Michael Salbaum for helpful discussions. This work was supported in part by pilot grants to BV and VDD from Nutrition and Obesity Research Center (NIH-NIDDK P30 DK072476). The research in Dixit Laboratory is supported in part by the NIH (R01AG31797), Coypu Foundation and Pennington Biomedical Research Foundation. K.S. was partially supported by NIH (DK083615). The present work utilized the facilities of the Genomics and Cell Biology & Bioimaging Core facilities supported by NIH Grant 1 P20 RR02/1945.

## References

1. Dixit VD. Adipose-immune interactions during obesity and caloric restriction: reciprocal mechanisms regulating immunity and health span. *J Leukoc Biol.* 2008; 8:882–92. [PubMed: 18579754]
2. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. *Nat Rev Immunol.* 2008; 8:923–934. [PubMed: 19029988]
3. Luchsinger JA, Gustafson DR. Adiposity, type 2 diabetes, and Alzheimer's disease. *J Alzheimers Dis.* 2009; 16:693–704. [PubMed: 19387106]
4. Yang H, et al. Obesity accelerates thymic aging. *Blood.* 2009; 114:3803–3812. [PubMed: 19721009]
5. Weisberg SP, et al. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest.* 2003; 112:1796–1808. [PubMed: 14679176]
6. Odegaard JI, Chawla A. Mechanisms of macrophage activation in obesity-induced insulin resistance. *Nat Clin Pract Endocrinol Metab.* 2008; 11:619–626. [PubMed: 18838972]
7. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest.* 2007; 117:175–184. [PubMed: 17200717]
8. Patsouris D, Li PP, Thapar D, Chapman J, Olefsky JM, Neels JG. Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. *Cell Metab.* 2008; 8:301–309. [PubMed: 18840360]
9. Hotamisligil GS. Inflammation and metabolic disorders. *Nature.* 2006; 444:860–867. [PubMed: 17167474]
10. Arkan MC, et al. IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med.* 2005; 11:191–198. [PubMed: 15685170]
11. Jager J, Grémeaux T, Cormont M, Le Marchand-Brustel Y, Tanti JF. Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. *Endocrinology.* 2007; 148:241–251. [PubMed: 17038556]
12. Larsen CM, et al. Interleukin-1-receptor antagonist in type 2 diabetes mellitus. *N Engl J Med.* 2007; 356:1517–1526. [PubMed: 17429083]
13. Osborn O, et al. Treatment with an Interleukin 1 beta antibody improves glycemic control in diet-induced obesity. *Cytokine.* 2008; 44:141–148. [PubMed: 18723371]
14. Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature.* 2007; 449:819–826. [PubMed: 17943118]
15. Barton GM, Kagan JC. A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nat Rev Immunol.* 2009; 9:535–542. [PubMed: 19556980]
16. Agostini L, et al. NLRP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity.* 2004; 20:319–325. [PubMed: 15030775]
17. Medzhitov R. Origin and physiological roles of inflammation. *Nature.* 2008; 454:428–435. [PubMed: 18650913]
18. Lamkanfi M, Dixit VM. Inflammasomes: guardians of cytosolic sanctity. *Immunol Rev.* 2009; 227:95–105. [PubMed: 19120479]
19. Franchi L, Eigenbrod T, Muñoz-Planillo R, Nuñez G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol.* 2009; 10:241–247. [PubMed: 19221555]
20. Martinon F, Mayor A, Tschopp J. The inflammasomes: guardians of the body. *Annu Rev Immunol.* 2009; 27:229–265. [PubMed: 19302040]
21. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol.* 2009; 27:519–550. [PubMed: 19302047]
22. Kostura MJ, et al. Identification of a monocyte specific pre-interleukin 1 beta convertase activity. *Proc Natl Acad Sci U S A.* 1989; 86:5227–5231. [PubMed: 2787508]
23. Kuida K, et al. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science.* 1995; 267:2000–2003. [PubMed: 7535475]

24. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell*. 2002; 10:417–26. [PubMed: 12191486]
25. Dostert C, et al. Innate immune activation through Nlrp3 inflammasome sensing of asbestos and silica. *Science*. 2008; 320:674–677. [PubMed: 18403674]
26. Halle A, et al. The NLRP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol*. 2008; 9:857–865. [PubMed: 18604209]
27. Mariathasan S, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature*. 2006; 440:228–232. [PubMed: 16407890]
28. Martinon F, Pétrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NLRP3 inflammasome. *Nature*. 2006; 440:237–241. [PubMed: 16407889]
29. Mariathasan S, et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature*. 2004; 430:213–218. [PubMed: 15190255]
30. Shi H, et al. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest*. 2006; 116:3015–3025. [PubMed: 17053832]
31. Saberi M, et al. Hematopoietic cell-specific deletion of toll-like receptor 4 ameliorates hepatic and adipose tissue insulin resistance in high-fat-fed mice. *Cell Metab*. 2009; 10:419–29. [PubMed: 19883619]
32. Bishop NA, Guarente L. Genetic links between diet and lifespan: shared mechanisms from yeast to humans. *Nat Rev Genet*. 2007; 8:835–44. [PubMed: 17909538]
33. Galgani JE, et al. Metabolic Flexibility in Response to Glucose Is Not Impaired in People With Type 2 Diabetes After Controlling for Glucose Disposal Rate. *Diabetes*. 2008; 57:841–845. [PubMed: 18285553]
34. Kummer JA, et al. Inflammasome components NALP 1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. *J Histochem Cytochem*. 2007; 55:443–452. [PubMed: 17164409]
35. Joosten LA. Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1 beta. *Arthritis Rheum*. 2009; 60:3651–3662. [PubMed: 19950280]
36. Virkamäki A, Ueki K, Kahn CR. Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest*. 1999; 103:931–943. [PubMed: 10194465]
37. Imaeda AB, et al. Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nlrp3 inflammasome. *J Clin Invest*. 2009; 119:305–314. [PubMed: 19164858]
38. Watanabe A, et al. Inflammasome-mediated regulation of hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol*. 2009; 296:1248–1257.
39. Khan T, et al. Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. *Mol Cell Biol*. 2009; 29:1575–1591. [PubMed: 19114551]
40. Håversen L, Danielsson KN, Fogelstrand L, Wiklund O. Induction of proinflammatory cytokines by long-chain saturated fatty acids in human macrophages. *Atherosclerosis*. 2009; 202:382–393. [PubMed: 18599066]
41. Park TS, et al. Ceramide is a cardiotoxin in lipotoxic cardiomyopathy. *J Lipid Res*. 2008; 49:2101–2112. [PubMed: 18515784]
42. Prieur X, R szér T, Ricote M. Lipotoxicity in macrophages: evidence from diseases associated with the metabolic syndrome. *Biochim Biophys Acta*. 2009 Sep 29. (2009). [Epub ahead of print].
43. Shah C, et al. Protection from high fat diet-induced increase in ceramide in mice lacking plasminogen activator inhibitor 1. *J Biol Chem*. 2008; 283:13538–13548. [PubMed: 18359942]
44. Feuerer M, et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med*. 2009; 15:930–939. [PubMed: 19633656]
45. Nishimura S, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat Med*. 2009; 15:914–920. [PubMed: 19633658]
46. Winer S, et al. Normalization of obesity-associated insulin resistance through immunotherapy. *Nat Med*. 2009; 15:921–929. [PubMed: 19633657]

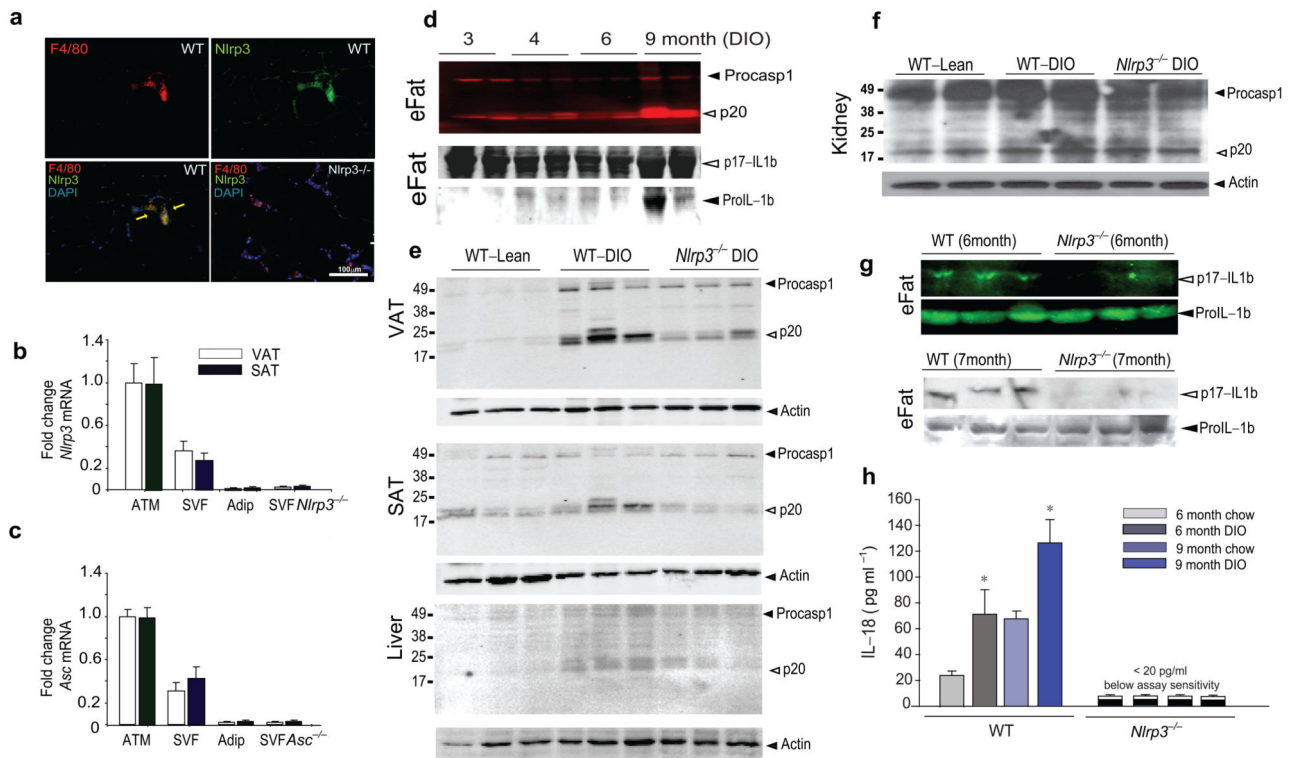
47. Masters SL, Simon A, Aksentijevich I, Kastner DL. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease. *Annu Rev Immunol.* 2009; 27:621–668. [PubMed: 19302049]
48. McGonagle D, McDermott MF. A proposed classification of the immunological diseases. *PLoS Med.* 2006; 3:e297. [PubMed: 16942393]
49. Duewell P, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature.* 2010; 464:1357–1361. [PubMed: 20428172]
50. Li H, Ambade A, Re F. Cutting edge: Necrosis activates the NLRP3 inflammasome. *J Immunol.* 2009; 183:1528–1532. [PubMed: 19596994]
51. Halberg N, et al. Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. *Mol Cell Biol.* 2009; 29:4467–4483. [PubMed: 19546236]
52. Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am J Physiol Endocrinol Metab.* 2007; 293:E1118–28. [PubMed: 17666485]
53. Strissel KJ, et al. Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes.* 2007; 56:2910–2918. [PubMed: 17848624]
54. Yang H, et al. Obesity increases the production of proinflammatory mediators from adipose tissue T cells and compromises TCR repertoire diversity: implications for systemic inflammation and insulin resistance. *J Immunol.* 2010; 185:1836–1845. [PubMed: 20581149]
55. Strissel KJ, et al. T-Cell Recruitment and Th1 Polarization in Adipose Tissue During Diet-Induced Obesity in C57BL/6 Mice. *Obesity (Silver Spring).* 2010 Jan 28. in Press (2010).
56. Duffaut C, et al. Interplay between human adipocytes and T lymphocytes in obesity: CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators. *Arterioscler Thromb Vasc Biol.* 2009; 29:1608–1614. [PubMed: 19644053]
57. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol.* 2010; 11:136–140. [PubMed: 20023662]
58. Dominguez H, et al. Metabolic and vascular effects of tumor necrosis factor-alpha blockade with etanercept in obese patients with type 2 diabetes. *J Vasc Res.* 2005; 42:517–525. [PubMed: 16155368]
59. Lamkanfi M, et al. Glyburide inhibits the Cryopyrin/Nlrp3 inflammasome. *J Cell Biol.* 2009; 187:61–70. [PubMed: 19805629]



### Figure 1. Reduction of *Nlrp3* and *IL-1 $\beta$* expression is associated with improvement of insulin-sensitivity

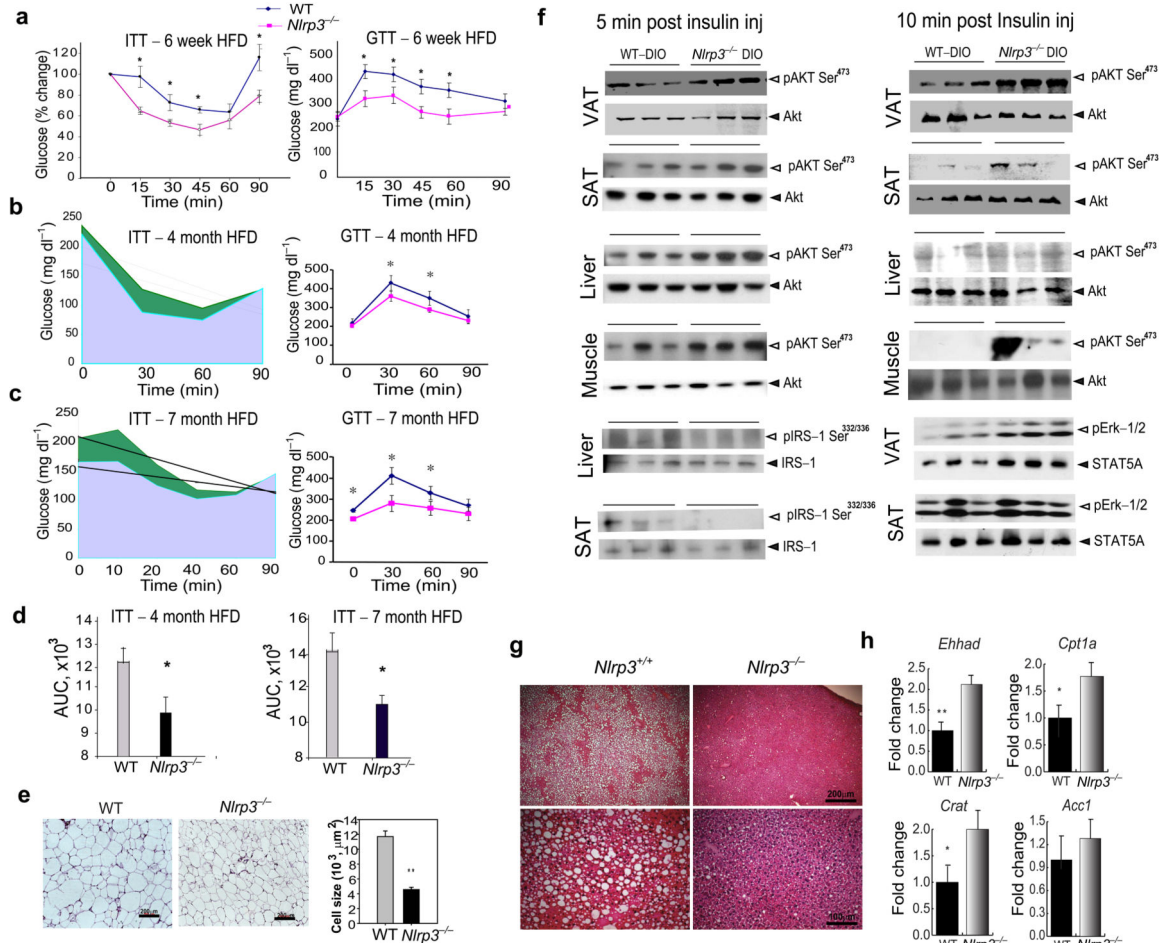
Positive correlation of the visceral fat mRNA expression of (a) *Il-1 $\beta$*  and (b) *Nlrp3* with body weight of C57BL/6 mice ( $n = 32$ ), Pearson's correlations are  $r = 0.364$ ,  $P = 0.0178$  for *Il-1 $\beta$*  and  $r = 0.672$ ,  $P < 0.0001$  for *Nlrp3* respectively. (c) *Il-1 $\beta$* , (d) *Nlrp3* and (e) *Asc* mRNA in visceral and subcutaneous adipose tissue from AL fed control and calorie restricted 12 month old mice,  $n = 6$ ; \* $P < 0.01$ , \*\* $P < 0.005$ . Representative H&E staining showing adipocyte size in (f) visceral and (g) subcutaneous fat tissue from AL fed control and calorie restricted 12 month old C57BL/6 mice. (h) *IL-1 $\beta$*  (left) and *NLRP3* (middle) and *ASC* (right) gene expression, as examined by qRT-PCR in human SAT in obese T2D patients before and after 1-year weight loss. (i) Positive correlation of changes in gene expression of *IL-1 $\beta$*  and *NLRP3* in human abdominal subcutaneous fat with changes in fasting glucose level from baseline to 1-year post intervention; Pearson's correlations are  $r = 0.53$ ,  $P = 0.12$  for *IL-1 $\beta$*  and  $r = 0.69$ ,  $P = 0.03$  for *NLRP3* respectively. Relative gene expression levels are depicted as means  $\pm$  SEM.  $n = 10$ ; \* $P < 0.01$ , \*\* $P < 0.001$ . All samples analyses were performed in a blinded fashion.



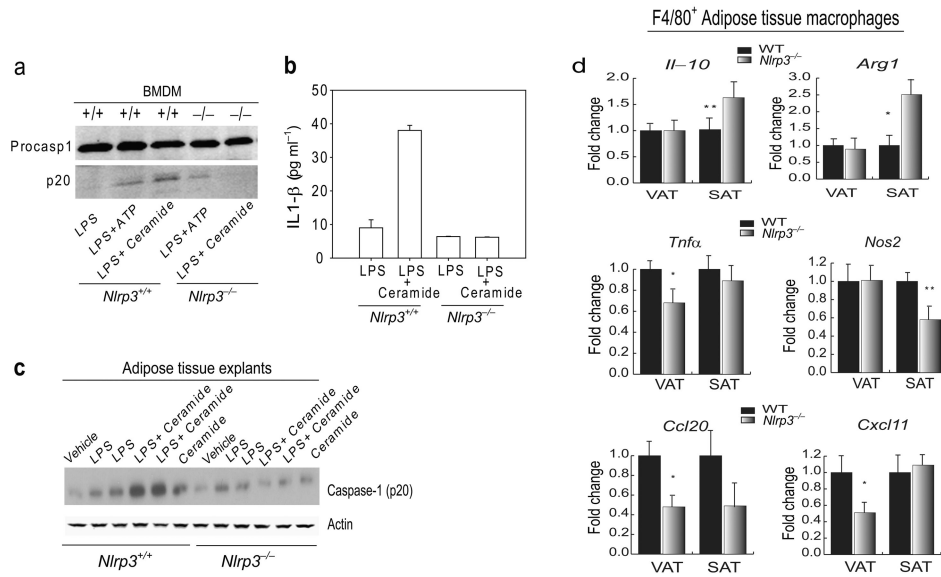


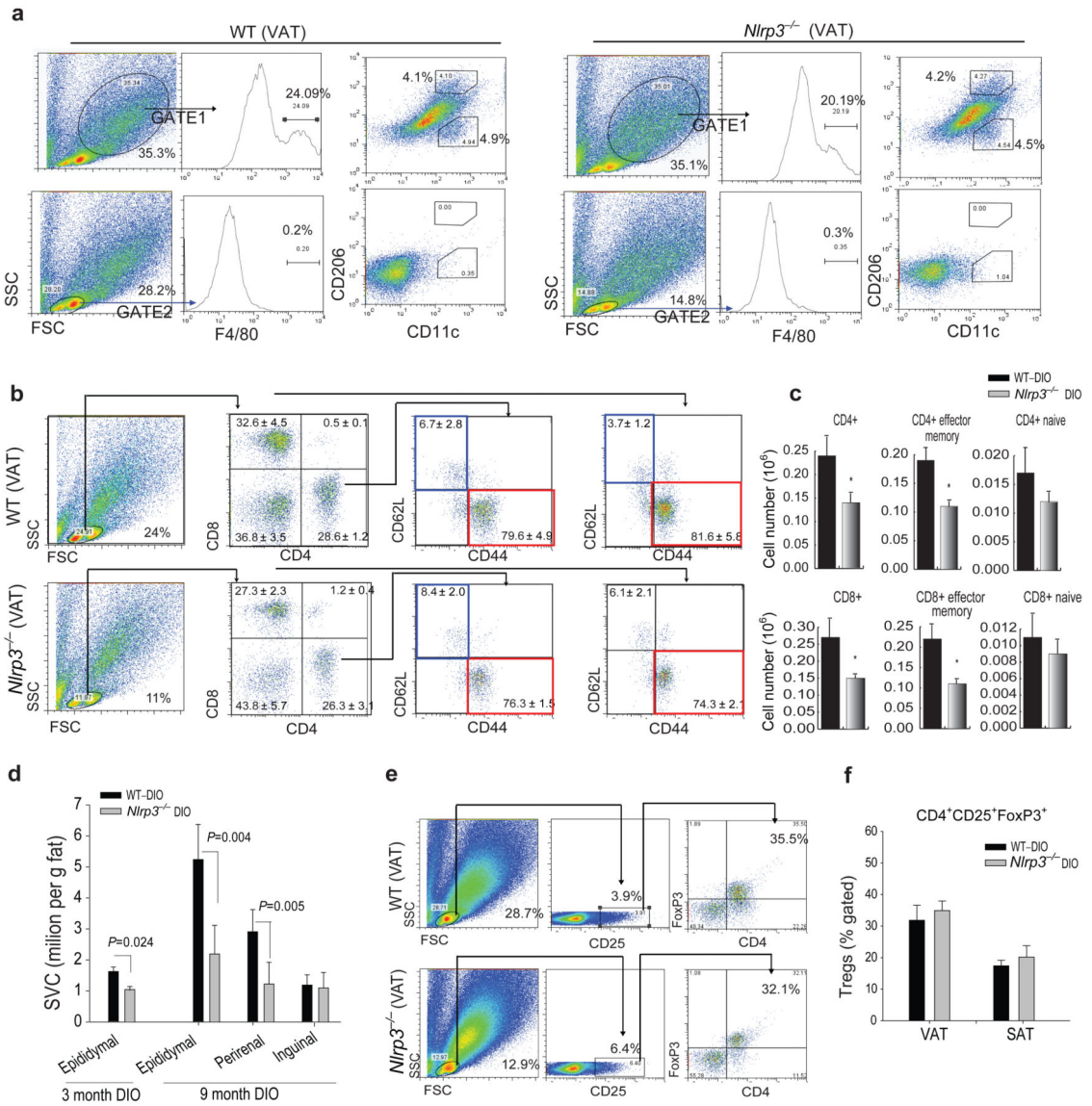
**Figure 2. Elimination of Nlrp3 signaling prevents obesity induced caspase-1 cleavage and IL-1 $\beta$ /IL-18 activation**

(a) Immunofluorescent staining of epididymal fat (eFat) tissue sections stained with antibodies against F4/80 (red), and Nlrp3 (green). Merge of images with nuclear stain DAPI shows co-localization of Nlrp3 with ATM (yellow arrow heads). Negative control staining with addition of antibody to Nlrp3 together with antibody to F4/80 in adipose tissue of *Nlrp3*<sup>-/-</sup> mice displayed reduced Nlrp3 specific immunostaining. (b-c) Quantitative real time-PCR analysis of *Nlrp3* and *Asc* mRNA in purified ATM, SVF derived from SAT and VAT of 6 month old DIO mice and mature 3T3L1 adipocytes (Adip). (d) Immunoblot analysis showing kinetics of caspase-1 (p20) cleavage and active IL-1 $\beta$  (p17) in adipose tissue of mice at different stages of diet-induced obesity. (e) Western blot analysis of activated caspase-1 (p20) in VAT, SAT and liver tissues from 9 month old DIO-WT (C57BL/6) and DIO-*Nlrp3*<sup>-/-</sup> mice. Results shown are representative of three independent experiments. (f) Western blot analysis of activated caspase-1 (p20) in kidney from 9 month old DIO-WT and DIO-*Nlrp3*<sup>-/-</sup> mice. (g) Western blot analysis of IL-1 $\beta$  activation in adipose tissue of 6 and 7 month old DIO mice. (h) Serum IL-18 concentration in age-matched WT and *Nlrp3*<sup>-/-</sup> mice fed chow and 60% HFD for 4 months (6 month old DIO) and 7 months (9 month old DIO). All data are presented as means  $\pm$  SEM,  $n = 6-10$  mice; \* $P < 0.05$ .



**Figure 3. *Nlrp3* inflammasome regulates insulin-sensitivity and steatohepatitis in obesity**  
**(a)** Insulin tolerance test (ITT) and glucose tolerance test (GTT) in male WT and *Nlrp3*<sup>-/-</sup> DIO mice fed 60% HFD for 6 weeks. **(b)** The ITT and GTT in 6 month and **(c)** 9 month old WT and *Nlrp3*<sup>-/-</sup> DIO mice. The ITT graphs display the area under the curve (green for WT and blue for *Nlrp3*<sup>-/-</sup>) with trend lines ( $n = 5-7$  per group). **(d)** The total area under the curve for ITT in 6 and 9 month old WT and *Nlrp3*<sup>-/-</sup> DIO mice. ( $*P < 0.01$ ). **(e)** Adipocyte size in visceral fat tissue from 8 month old DIO-WT (*Nlrp3*<sup>+/+</sup>) and *Nlrp3*<sup>-/-</sup> mice (hematoxylin and eosin staining). The cross-sectional size (area, μM<sup>2</sup>) of adipocytes (100 cells per mouse) were determined in VAT using Image J software. Representative micrographs and the quantified results are shown.  $n = 6$ ;  $*P < 0.001$ . **(f)** Akt, IRS-1 and MAPK signaling *in vivo* as determined by western blotting in VAT, SAT, liver, and muscle from the 8 month old DIO *Nlrp3*<sup>+/+</sup> and DIO *Nlrp3*<sup>-/-</sup> mice. **(g)** Immunohistochemistry (hematoxylin and eosin staining) of section from liver tissues from 9 month old DIO WT and *Nlrp3*<sup>-/-</sup>. **(h)** Fatty acid oxidation and fatty acid synthesis gene expression in liver tissue of *Nlrp3*<sup>+/+</sup> and *Nlrp3*<sup>-/-</sup> mice analyzed by quantitative RT-PCR. Ct of the genes are shown means ± SEM. ( $n = 5$ ),  $*P < 0.05$ ,  $**P < 0.001$ .





**Figure 5. Ablation of *Nlrp3* inflammasome reduces adipose tissue effector T cells without affecting *T<sub>reg</sub>* cells in visceral fat of obese mice**

(a) The FACS plot of SVF cells isolated from VAT of 9 month old WT and *Nlrp3*<sup>-/-</sup> obese mice. First dot plots depict Forward and side scatter (FSC/SSC) and gating strategy for analysis of ATMs and T cells. Gate1 (upper panel) of larger cells shows presence of F4/80<sup>+</sup> cells (histogram) and expression of macrophage markers CD206 and CD11c on ATMs. Gating of smaller cells (gate 2, lower panel) reveals absence of ATMs in this population of SVF. (b) The gate 2 (lymphoid gate) presenting CD4, CD8 T cells in SVF. CD4 and CD8 cells were evaluated for naïve T cells (CD62L<sup>+</sup>CD44<sup>-</sup>, blue box) and effector memory E/M (CD62L<sup>-</sup>CD44<sup>+</sup>, red box) CD4 and CD8 T cells. The FACS analysis was repeated in three independent pooled SVF fractions from a total of 12-14 mice. (c) Gated percentage and absolute numbers (in million cells) of naïve (CD62L<sup>+</sup>CD44<sup>-</sup>) and E/M (CD62L<sup>-</sup>CD44<sup>+</sup>) CD4 and CD8 T cells. (d) Number of stromal vascular cells per gram of fat tissue ( $n = 4-6$ ) in 3 and 9 month old WT and *Nlrp3*<sup>-/-</sup> DIO mice. (e) Representative FACS plots showing

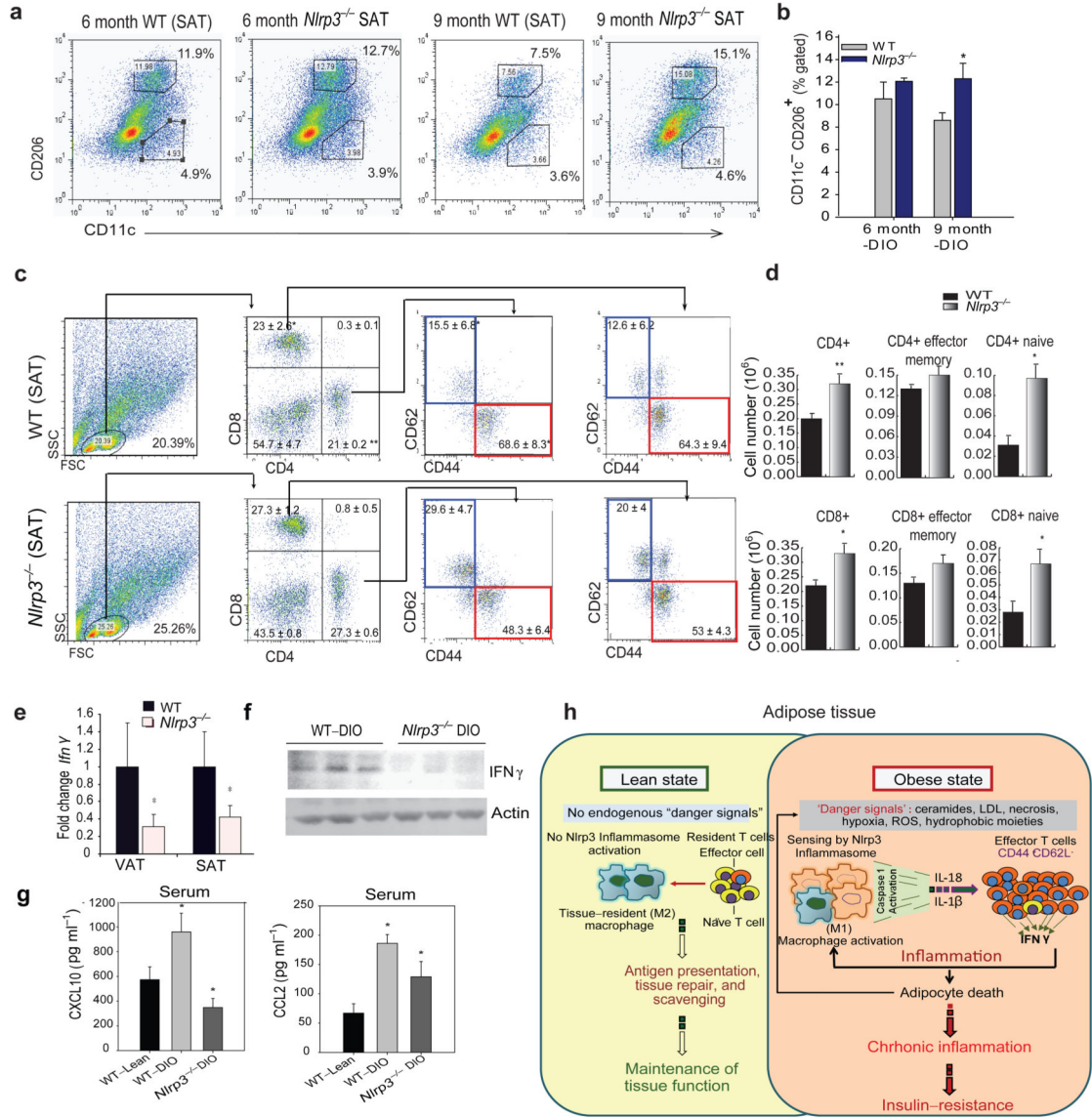
CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T regulatory cells in VAT of 9 month old WT and *Nlrp3*<sup>-/-</sup> DIO mice.  
(f) Gated percentage of T<sub>reg</sub> cells in VAT and SAT of 9 month old WT and *Nlrp3*<sup>-/-</sup> DIO mice (*n* = 6 per group). All data are presented as means ± SEM mean, \**P* < 0.05.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



**Figure 6. Elimination of Nlrp3 inflammasome reduces obesity-induced macrophage-T cell activation in adipose tissue**

(a) The representative FACS plots of SVF cells from SAT of 6 month and 9 month old WT and *Nlrp3*<sup>-/-</sup> DIO mice stained with CD206 and CD11c. (b) The gated percentage of CD11c<sup>-</sup>CD206<sup>+</sup> M2 cells in SAT of 9 month old obese mice. (c) Dot plots showing naïve (CD62L<sup>+</sup>CD44<sup>-</sup>, blue box) and effector memory (CD62L<sup>-</sup>CD44<sup>+</sup>, red box) CD4 and CD8 T cells in SVF from 9 month old obese WT and *Nlrp3*<sup>-/-</sup> mice. (d) Gated percentage and absolute numbers (in million cells) of naïve (CD62L<sup>+</sup>CD44<sup>-</sup>) and effector memory (CD62L<sup>-</sup>CD44<sup>+</sup>) CD4 and CD8 T cells. (e) mRNA level of Th1 cytokine *Ifn*γ in VAT and SAT of 9 month old obese WT and *Nlrp3*<sup>-/-</sup> mice as determined by quantitative RT-PCR. (f) IFNγ (19kD) in VAT of 9 month old WT and *Nlrp3*<sup>-/-</sup> DIO mice as examined by western blotting. (g) IP10/CXCL10 and MCP-1/CCL2 levels in the serum of 9 month old lean WT and obese WT and *Nlrp3*<sup>-/-</sup> mice (*n* = 5). The data shown are mean ± SEM and \*, *P* < 0.05. (h) Hypothetical model of Nlrp3 inflammasome activation in obesity. In absence

of ‘danger signals’ in healthy lean state, the tissue resident macrophages and T cells may participate in the maintenance of adipose tissue function. Nlrp3 inflammasome senses the obesity-associated ‘danger signals’ such as ceramides leading to caspase-1 autoactivation and IL-1 $\beta$  and IL-18 production from ATMs. Secondary signals from activated ATMs to effector adipose T cells sustain the reciprocal proinflammatory feed-forward cascade in obesity leading to insulin-resistance.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript