Retinol binding protein 4 primes the NLRP3 inflammasome by signaling through Toll-like receptors 2 and 4

Pedro M. Moraes-Vieira[a,1,2], Mark M. Yore[a,1,3], Alexandra Sontheimer-Phelps[a,4], Angela Castoldi[a], Julie Norseen[a], Pratik Aryal[a], Kotryna Simonyté Sjödin [a,5], and Barbara B. Kahn[a,6]

[a]Department of Medicine, Division of Endocrinology, Diabetes, and Metabolism, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215

Contributed by Barbara B. Kahn, October 6, 2020 (sent for review July 6, 2020; reviewed by Anthony W. Ferrante and Loredana Quadro)

Adipose tissue (AT) inflammation contributes to systemic insulin resistance. In obesity and type 2 diabetes (T2D), retinol binding protein 4 (RBP4), the major retinol carrier in serum, is elevated in AT and has proinflammatory effects which are mediated partially through Toll-like receptor 4 (TLR4). We now show that RBP4 primes the NLRP3 inflammasome for interleukin-1β (IL1β) release, in a glucose-dependent manner, through the TLR4/MD2 receptor complex and TLR2. This impairs insulin signaling in adipocytes. IL1β is elevated in perigonadal white AT (PGWAT) of chow-fed RBP4-overexpressing mice and in serum and PGWAT of high-fat diet-fed RBP4-overexpressing mice vs. wild-type mice. Holo- or apo-RBP4 injection in wild-type mice causes insulin resistance and elevates PGWAT inflammatory markers, including IL1β. TLR4 inhibition in RBP4-overexpressing mice reduces PGWAT inflammation, including IL1β levels and improves insulin sensitivity. Thus, the proinflammatory effects of RBP4 require NLRP3-inflammasome priming. These studies may provide approaches to reduce AT inflammation and insulin resistance in obesity and diabetes.

macrophage | RBP4 | inflammation | diabetes | obesity

The incidence of type 2 diabetes (T2D) continues to increase worldwide and constitutes a major global health threat (1). Insulin resistance in muscle, adipose tissue (AT), and liver is a hallmark of T2D, which is associated with low-grade chronic inflammation, characterized by increased serum cytokine levels and a proinflammatory immune profile in AT (2–4). Pattern recognition receptors including Toll-like receptors (TLRs) sense pathogenic microbial components (pathogen-associated molecular patterns, PAMPs) and also host-derived damage-associated molecular patterns (DAMPs), which results in nonpathogen-initiated inflammation often referred to as "sterile inflammation" (5). In obesity, TLR2 and TLR4 are implicated in inflammation-induced insulin resistance in AT, and genetic deletion of TLR2 or TLR4 in mice results in improved insulin sensitivity (6, 7). In obese, insulin-resistant humans, TLR2 and TLR4 expression and activation are increased in AT compared to healthy controls (8) and some evidence suggests that people taking an antiinflammatory agent (abatacept, also known as CTLA4-Ig) which blocks antigen presentation, may have improved insulin sensitivity (9, 10). TLR2 and TLR4 signal through the adaptor proteins myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adaptor-inducing IFN (TRIF) to activate proinflammatory signaling cascades including nuclear factor kappa B (NFκB) and c-Jun N-terminal kinase (JNK) (5, 11). This results in AT immune cell migration, activation, and cytokine secretion which augments insulin resistance in adipocytes (12). Thus, TLR2 and TLR4 contribute to AT inflammation and insulin resistance and their targeting may provide treatment strategies for T2D.

Retinol binding protein 4 (RBP4), the major serum retinol transporter, is secreted by the liver and AT (13, 14). Serum RBP4 levels are elevated in obese, insulin-resistant mice and humans (15, 16). Genetic and pharmacological elevation of RBP4 induces insulin resistance in wild-type (WT) mice (16, 17) and reducing RBP4 levels improves insulin sensitivity (16, 18). In humans, RBP4 levels are elevated with prediabetes and correlate positively with many metabolic syndrome-related components, including increased waist/hip ratio, intraabdominal fat mass, dyslipidemia, hypertension, and cardiovascular disease in large epidemiological studies (15, 19–21). A gain-of-function polymorphism in the RBP4 promoter which increases adipose RBP4 expression is associated with an 80% increased risk of T2D in humans (19, 22).

Significance

There is a growing need for insulin-sensitizing therapies to treat type 2 diabetes (T2D). In obese T2D humans, NLRP3 inflammasome activation contributes to insulin resistance. Serum levels of retinol binding protein 4 (RBP4) are elevated in obese, insulin-resistant humans and RBP4 SNPs that increase adipose RBP4 expression confer greater T2D risk. RBP4 levels correlate with many metabolic syndrome-related components, including cardiovascular disease. RBP4 elevation induces insulin resistance in mice by increasing proinflammatory cytokine secretion from macrophages. Reducing RBP4 in mice with dietary obesity decreases adipose inflammation and improves insulin sensitivity. Here we show RBP4 primes the NLRP3 inflammasome which plays a critical role in RBP4-induced insulin resistance. Thus, the NLRP3-RBP4 axis may provide therapeutic strategies for T2D and its comorbidities.

Author contributions: P.M.M.-V., M.M.Y., A.S.-P., A.C., and B.B.K. designed research; P.M.M.-V., M.M.Y., A.S.-P., A.C., J.N., P.A., and K.S.S. performed research; B.B.K. supervised the experimental plan and contributed new reagents/analytic tools; P.M.M.-V., M.M.Y., A.S.-P., A.C., J.N., and P.A. analyzed data; P.M.M.-V., M.M.Y., A.S.-P., and B.B.K. interpreted data; and P.M.M.-V., M.M.Y., and B.B.K. wrote the paper.

Reviewers: A.W.F., Columbia University; and L.Q., Rutgers, the State University of New Jersey.

The authors have no competing interests.

[1]P.M.M.-V. and M.M.Y. contributed equally to this work.

[2]Present address: Obesity and Comorbidities Research Center, Laboratory of Immunometabolism, Department of Genetics, Evolution, Microbiology, and Immunology, Institute of Biology, University of Campinas, SP, Brazil, 13083-862; and Division of Metabolism, Experimental Medicine Research Cluster, University of Campinas, Campinas, SP, Brazil, 13083-862.

[3]Present address: Jounce Therapeutics Inc., Cambridge, MA 02139.

[4]Present address: Wyss Institute for Biologically Inspired Engineering at Harvard University, Boston, MA 02215.

[5]Present address: Department of Clinical Sciences, Pediatrics, Umeå University, 90187, Umeå, Sweden.

[6]To whom correspondence may be addressed. Email: bkahn@bidmc.harvard.edu.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2013877117/-/DCSupplemental.

First published November 19, 2020.

Inflammation is critical for RBP4-induced insulin resistance which is partially mediated through TLR4 (17, 18, 23). RBP4 induces insulin resistance in adipocytes indirectly by increasing proinflammatory cytokine secretion from macrophages (23). Insulin resistance and AT inflammation have been observed in two mouse models of RBP4 overexpression. Mice overexpressing RBP4 driven by a muscle-specific promoter (RBP4-Ox) have elevated serum and perigonadal white adipose tissue (PGWAT) RBP4 protein levels and PGWAT inflammation (17). Elevated AT RBP4 in these mice activates antigen presentation through the JNK pathway which results in proinflammatory CD4 T cell proliferation and Th1 polarization (17). Mice overexpressing RBP4 selectively in adipocytes also have AT inflammation and glucose intolerance even on a chow diet (14). Transfer of RBP4-activated dendritic cells into normal mice is sufficient to cause AT inflammation and insulin resistance (17). Interestingly, overexpression of RBP4 selectively in hepatocytes has been reported not to cause elevated circulating RBP4 levels and is not associated with insulin resistance (24) even though hepatocytes are thought to be the major site for RBP4 secretion (25). Adipocytes can contribute to circulating RBP4 levels especially in obesity (14). Taken together, these data suggest that RBP4 in AT may be an obesity and insulin-resistance-related damage-associated molecule pattern.

Activation of PAMP and DAMP receptors in immune cells leads to the assembly of inflammasomes, which are multiprotein complexes that cleave and activate interleukin-1 beta (IL1β) and interleukin-18 (IL18) (26). The nucleotide-binding domain and leucine-rich repeat containing protein 3 (NLRP3) inflammasome plays a role in the pathogenesis of obesity, type 1 diabetes, type 2 diabetes, and metabolic syndrome (26–28). NLRP3 knockout (KO) protects against insulin resistance in mice (27). NLRP3 activation requires a two-step process. The first "priming" occurs in response to TLR-mediated activation of the NFκB pathway resulting in expression of pro-IL1β (26). The second "activating" step directly induces inflammasome assembly which recruits and cleaves procaspase-1 to its active form caspase-1 (26). Caspase-1 mediates the cleavage of pro-IL1β resulting in IL1β release (26). The NLRP3 inflammasome can be activated by metabolites which are elevated in obesity and insulin resistance, such as palmitate (29). While an ever-expanding list of several metabolites is known to provide the second signal in inflammasome activation, there is a paucity of data on the endogenous proteins and metabolites that provide the first priming signal in obesity and T2D. Here we show that RBP4 is an endogenous NLRP3-inflammasome priming agent and we investigate its upstream signaling pathways.

Our data show that elevating RBP4 levels by RBP4 injection in WT mice or genetically induced RBP4 overexpression markedly elevates adipose IL1β expression, which leads to PGWAT inflammation and insulin resistance. IL1β is elevated in PGWAT of chow-fed RBP4-Ox mice and in serum and PGWAT of HFD-fed RBP4-Ox mice compared to WT mice. The RBP4-mediated proinflammatory effects are mediated specifically through TLR2 and a TLR4/MD2 receptor complex, which does not require the other adaptor proteins LPS-binding protein and CD14. The activation of macrophages by RBP4 through TLR2 and TLR4/MD2 requires signaling through the downstream pathways MyD88 and TRIF. TLR4 inhibition in RBP4-Ox reduces IL1β levels in PGWAT and improves insulin sensitivity. The RBP4-mediated increase in IL1β release from macrophages is glucose dependent. Thus, targeting the NLRP3 inflammasome or the upstream activating receptors or pathways may provide therapeutic avenues to ameliorate RBP4-mediated insulin resistance and T2D.

Materials and Methods

Recombinant RBP4 Preparation. Human RBP4 was expressed in *Escherichia coli* and purified as previously described (16, 17, 23). Holo-RBP4 (RBP4 with retinol bound) was purified by HPLC (HPLC) and dialyzed against 1× phosphate-buffered saline (PBS) for 4 h using a Slide A Lyzer dialysis cassette (Thermo Scientific). The dialysate buffer was used as control in all studies. Endotoxin was removed using sequential affinity absorption with Endotrap matrix (Hyglos GmbH) and Detoxigel (Pierce). Endotoxin levels were quantified using the limulus amebocyte lysate (LAL) QCL-1000TM (Lonza) and found to be <0.001 endotoxin unit per microgram (17). Protein function was measured by RBP4-TTR coimmunoprecipitation and Western blotting and retinol binding was assessed by ultraviolet-visible (UV-Vis) spectrometry (16, 23). To generate retinol-free RBP4 (apo-RBP4), holo-RBP4 was first incubated with 40% butanol–60% diisopropyl ether at 30 °C overnight to remove retinol and centrifuged at 5,000 rpm for 5 min, and the bottom phase containing recombinant RBP4 was collected. This step was repeated twice more with 1-h incubations of 40% butanol–60% diisopropyl ether. Endotoxin levels were remeasured after preparation of apo-RBP4 and no endotoxin was introduced with this procedure. Purified, recombinant human RBP4 was stored at −80 °C and protected from exposure to light. Holo-RBP4 and apo-RBP4 were used as indicated in the figure legends.

Reagents and Cell Culture. Unless otherwise indicated, all chemicals and reagents were from Sigma-Aldrich. The following cell lines were obtained through the Biodefense and Emerging Infections Research Resources Repository/National Institute of Allergy and Infectious Diseases and were originally deposited by Douglas Golenbock (University of Massachusetts, Worcester, MA): Macrophage cell line derived from WT mice, NR-9456; TLR4 KO mice, NR-9458; TLR2 KO mice, NR-9458; TLR4/TLR2 double KO mice, NR-9458; Myd88 KO mice, NR-9458; TRIF KO mice, NR-9458; and Myd88/TRIF double KO mice, NR-9458. Cultured macrophage cell lines and differentiated 3T3-L1 adipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). 3T3-L1 fibroblasts (ATCC) were cultured in DMEM containing 10% bovine calf serum (BCS). All cell culture media contained 1 mg/mL penicillin/streptomycin (Pen/Strep) and cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2. For 3T3L1 differentiation, 3T3-L1 fibroblasts were grown until confluent and maintained in this state for 2 d after which cells were incubated with induction media (0.25 μM dexamethasone, 0.5 mM isobutyl-1-methylxanthine (IBMX), 680 nM insulin, and 2 μM rosiglitazone). After 3 d, media were changed to DMEM containing 10% FBS. Media were changed every 2 d and cells were used at day 10 postinduction. For insulin stimulation studies, 3T3L1 adipocytes and primary adipocytes were serum starved for 3 h then stimulated with 100 nM insulin for 15 min. Primary adipocytes were treated with mouse IL1β (Cell Signaling) for 24 h prior to insulin stimulation. For conditioned media studies, cultured bone marrow-derived macrophages (BMDMs) were pretreated for 3 h with RBP4 (50 μg/mL) or dialysate as control and then stimulated overnight with adenosine 5′-triphosphate (ATP, 2.5 mM) or water (control). Media were harvested and cell debris was removed by centrifugation. For inflammasome activation studies, cultured BMDMs were pretreated for 3 h with 100 ng/mL Ultrapure *E. coli* O111:B4 lipopolysaccharide (InvivoGen) or 50 μg/mL RBP4 followed by 2.5 mM ATP, 250 μM palmitic acid, 100 μg/mL monosodium urate (MSU), 100 μM ceramide, or media (control) as indicated. Supernatants were also analyzed for cytokine secretion by ELISA. For cell-based TLR4 inhibition studies, macrophages were pretreated for 30 min with 1 μM TAK242 or dimethylsulfoxide (DMSO) control, followed by 4 h (gene expression) or 16 h (cytokine secretion) RBP4 treatment or dialysate as control.

Plasmids and Transfections. The following plasmids—pcDNA3-TLR2-YFP (Addgene plasmid 13016); pcDNA3-TLR4-YFP (Addgene plasmid 13018); pGL3-ELAM-luc (NFκB response element, Addgene plasmid 13029); pFlag-CMV1-hMD2 (Addgene plasmid 13028); pcDNA3-CD14 (Addgene plasmid 13645); pcDNA3-YFP (Addgene plasmid 13033)—were obtained from http://www.addgene.org/ and were originally deposited by Douglas Golenbock (30, 31). These plasmids were transfected into HEK293 cells, which do not normally express TLR2, TLR4, or MD-2. pRL-CMV was obtained from Promega. HEK293 cells were transfected with pRL-CMV and pGL3-ELAM-luc either alone (control) or in combination with the plasmids above using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen). The total DNA content per transfection was kept constant by adjusting the concentration of pcDNA3-YFP. After 48 h of transfections, cells were treated with RBP4 (50 μg/mL) or LPS (100 ng/mL) for 8 h, harvested, and the luciferase activity in lysates was measured using the Dual-Luciferase Reporter (DLR) Assay System following the manufacturer's (Promega) instructions.

Primary Murine Bone Marrow-Derived Macrophage Isolation and Differentiation. Primary murine BMDMs were isolated from the femur and tibia bone of WT, TLR2 KO, TLR4 KO, Caspase-1 KO, and NLRP3 KO mice (The Jackson Laboratory) as described (32). Mice were killed using CO_2. Hind legs were dissected and adherent tissues removed. Under sterile conditions, legs were separated at the knee joint. The top and bottom of the femur and tibia were cut and the bone marrow was flushed out with BMDM media (Advanced RPMI, Invitrogen), 10% FBS 1× amino acid solution (Gibco), 1× sodium pyruvate solution (Gibco), 1× vitamin solution (Gibco), 1× GlutaMAX (Gibco), 2 mM β-mercaptoethanol) using a 10-mL syringe with a 25-gauge needle. Bone marrow was resuspended in red blood cell lysis buffer (RBC lysis buffer, BioLegend) for 5 min to remove red blood cells. Afterward, the cell suspension was centrifuged (4 °C, 5 min, 650 × *g*) and resuspended in BMDM media supplemented with 30 ng/mL mouse macrophage colony stimulating factor (Cell Signaling). Cells were plated at 1×10^6 cells per well in 12-well plates. Media were changed after 4 d and cells were used on day 6.

Primary Stromal Vascular Fraction (SVF)-Derived Adipocyte Differentiation. Five-week-old C57BL6/J mice were killed and subcutaneous white adipose tissue (SQWAT) was dissected. Fat pads were then incubated with digestion buffer (PBS, 100 mg/mL Collagenase D (Roche) and 10 mM $CaCl_2$, 1× Dispase II (Roche) for 60 min with constant shaking at 37 °C. The resulting cell suspension was passed through a 100-μm cell strainer and centrifuged for 5 min at 600 × *g*, resuspended in SVF media (F12 media containing 15% FBS, 1× GlutaMAX, 5 μg/mL insulin). Cells were then passed through a 40-μm cell strainer, centrifuged for 5 min at 600 × *g*, and resuspended in SVF media and plated until confluent. Differentiation was induced in confluent cultures by incubating with F12 media, 10% FBS, 1× GlutaMAX, 1 mg/mL Pen/Strep, 5 μg/mL insulin, 1 μM dexamethasone, 0.5 mM IBMX, and 1 μM rosiglitazone. Adipocytes were cultured in SVF media before experimental use. Adipocytes were used 10 to 14 d postdifferentiation.

Western Blotting. Western blotting was performed as previously described (18). Briefly, cells were lysed with radioimmunoprecipitation assay buffer (Sigma) containing protease and phosphatase inhibitors (Complete mini, Roche), PhosStop (Roche), and 1 mM sodium orthovanadate followed by centrifugation at 4 °C for 15 min at 16,000 × *g*. Protein concentrations were determined by bicinchoninic acid assay (Sigma) and then boiled in 1× Laemmli loading buffer for 5 min. Gels were transferred to nitrocellulose membranes (Whatman) followed by blocking with 5% milk in Tris-buffered saline, 0.5% Tween-20 for 1 h. Membranes were incubated with primary antibodies overnight and horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. HRP-mediated chemiluminescence was detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer).

Real-Time PCR. RT-PCR was performed as previously described (17). Briefly, RNA was extracted using TRI-reagent (Molecular Research Center). After addition of 10% BCP-Phase Separation Reagent (bromochloropropane, Molecular Research Center), lysates were centrifuged for 15 min (16,000 × *g*, 4 °C). The clear RNA phase was incubated with an equal volume of 70% ethanol for 30 min at −20 °C. RNA was extracted and purified using the RNeasy Mini kit (Qiagen) following the manufacturer's protocol. Purified RNA was transcribed into cDNA using Advantage RT for PCR kit (Clontech) with random hexamer primers following the manufacturer's protocol. RT-PCR was performed with TaqMan primers (Life Technologies) and TaqMan Universal Master Mix (Life Technologies). Gene expression was normalized to 18S expression levels.

Measurement of Cytokine Secretion. Cell culture media was harvested and cell debris removed by centrifugation (5 min, 16,000 × *g*). Cytokines (TNF-α, IL-6, IL1β, and MCP-1) levels were measured by ELISA according to the manufacturer's instructions (BioLegend). For adipose tissue IL1β levels, tissue was lysed with 20 mM Tris·HCl (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 5 mM NaF, 5 mM β-glycerophosphate, and IL1β levels were measure by ELISA (BioLegend).

Flow Cytometry. AT SVF cells or BMDMs were resuspended in PBS supplemented with 2% flow cytometry buffer, and surface markers (CD45, CD11b, F4/80) were stained with monoclonal antibodies (BioLegend) for flow cytometry as described (17). For intracellular cytokine staining (IL1β, TNF), cells were stained with monoclonal antibodies (BioLegend) as previously described (33). The cells were acquired on an LSR II flow cytometer (BD Biosciences) at the Beth Israel Deaconess Medical Center Flow Cytometry Core and analyzed with FlowJo 9.5.3 software (Treestar).

In Vivo Studies. RBP4-Ox mice on a C57BL6/J background have been previously described (17). For TLR4 inhibition studies, male RBP4-Ox mice (36 wk old) received daily intraperitoneal (i.p.) injections of TAK242 (3 mg/kg body weight, InvivoGen) or dialysate buffer as control (95% saline with 5% DMSO) for 14 d. A baseline insulin tolerance test was performed at the beginning of the study before treatment commenced and repeated at days 7 and 14 of the study. For measurement of tissue cytokine levels, RBP4-Ox mice and their WT littermates were fed a standard diet (TD5008, LabDiet) or a high-fat diet (HFD) (55% fat calories, Harlan-Teklad 93075) for 18 wk. For RBP4 injection studies, 12-wk-old male FVB mice were injected intraperitoneally with holo-RBP4 (3 daily i.p. injections for a total daily dose of 0.3 mg/mouse for 5 wk) or apo-RBP4 (3 daily i.p. injections for a total daily dose of 0.3 mg/mouse for 3 wk) or dialysate (control mice). Insulin tolerance tests were performed after 3 wk of RBP4 treatment in awake mice 5 h after food removal. After 3 to 5 wk of treatment, the mice were killed and WAT depots harvested for cytokine measurement. Blood glucose was measured with a OneTouch Ultra 2 glucometer (LifeScan, Inc.) from blood extracted via a tail vein nick. Blood glucose was measured immediately before and 15, 30, 45, 60, and 90 min after an i.p. injection of 0.75 U/kg BW insulin (Humulin R, Eli Lilly). All animals were kept on a 14-h light/10-h dark schedule and fed ad libitum on regular chow diet (TD5008, LabDiet) unless otherwise specified. All studies were conducted in accordance with federal guidelines and approved by the Institutional Animal Care and Use Committee (Beth Israel Deaconess Medical Center, Boston, MA). All studies were performed on age- and sex-matched littermates.

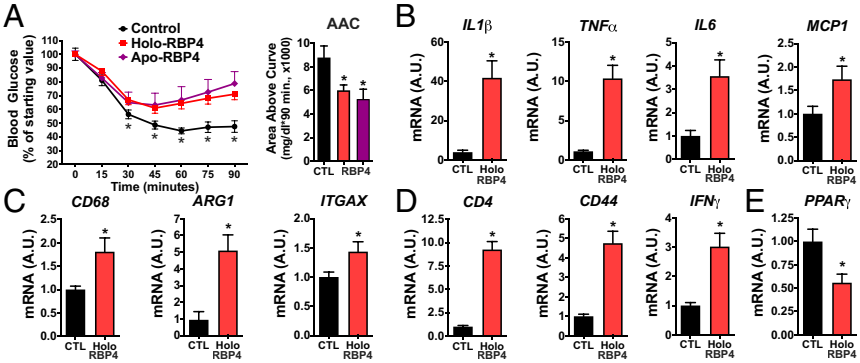

Fig. 1. Holo- or apo-RBP4 administration in WT mice induces PGWAT inflammation and whole-body insulin resistance. (*A*) Insulin tolerance in WT mice injected with recombinant holo-RBP4 for 5 wk and apo-RBP4 for 3 wk or dialysate (control). *$P < 0.05$ compared to holo- and apo-RBP4 injected mice at same timepoint (repeated-measures ANOVA). Area above the curve (AAC) for ITT. *$P < 0.05$ compared to dialysate control (CTL) (ANOVA). Expression levels of cytokine, chemokine (*B*), and macrophage markers (*C*), T cell markers (*D*), and *PPARγ* (*E*) in PGWAT of dialysate control (CTL) and holo-RBP4 (RBP4)-injected mice. $n = 8$ to 10/group, *$P < 0.05$ compared to dialysate control (*t* test).

Statistical Analysis. Statistical analysis was performed using GraphPad Prism 6.0. Sample size is indicated by "*n*." All values are given as means ± SEM. Differences between groups were compared using ANOVA for multiple comparisons or Student's *t* test as indicated. Differences were considered significant when $P < 0.05$.

Results

Administration of Holo- or Apo-RBP4 to WT Mice Induces at Inflammation and Insulin Resistance. To determine if the insulin resistance induced by RBP4 elevation in WT mice is associated with inflammation, WT mice were injected daily for 3 wk with apo-RBP4 and 5 wk with holo-RBP4 or dialysate. Insulin tolerance was markedly impaired in both apo- and holo-RBP4-treated chow-fed mice compared to dialysate treated mice (Fig. 1*A*) with no difference in body weight (26.00 ± 0.56, dialysate control; 26.63 ± 0.48, apo-RBP4; and 26.52 ± 0.37, holo-RBP4; mean ± SEM). Holo-RBP4 administration increased PGWAT mRNA expression levels of the proinflammatory cytokines *IL1β*, *TNFα*, and *IL6*, the chemokine *MCP1*, the macrophage cell marker *CD68*, the M2 macrophage-associated gene *ARG1*, and the proinflammatory or M1 macrophage-associated gene *ITGAX* (also called CD11c) compared to dialysate control (Fig. 1 *B* and *C*). The increased expression of both M1-and M2-related macrophage markers is consistent with previous data which showed that adipose tissue M2 macrophages from RBP4-overexpressing mice retain their M2 profile, but express higher levels of ARG1 along with proinflammatory cytokines (17). Holo-RBP4 administration also increased PGWAT mRNA expression levels of *CD4*, a T helper cell marker, *CD44*, a memory T cell marker, and *IFN-γ*, a T helper 1 (Th1) cytokine (Fig. 1*D*), in agreement with previous data in RBP4-Ox mice (17). Furthermore, holo-RBP4-treated mice had reduced mRNA levels of *PPARγ* (Fig. 1*E*), a transcription factor commonly found in alternatively activated adipose tissue macrophages and AT regulatory T cells (34). Administration of apo-RBP4 to WT mice had a similar effect to cause adipose tissue inflammation as measured by increased gene expression of *IL1β*, *TNFα*, *IL6*, *MCP1*, *CD68*, *ARG1*, *ITGAX*, *CD4*, *CD44*, *IFN-γ*, and decreased *PPARγ* expression in PGWAT (*SI Appendix*, Fig. S1*A*). This indicates that the immune-mediated effects of RBP4 in vivo are retinol independent. Thus, administration of apo- or holo-RBP4 to normal mice is sufficient to cause PGWAT inflammation and insulin resistance even in lean mice. In vitro experiments also showed that the proinflammatory effects of RBP4 are independent of retinol. Treatment of primary BMDMs from WT mice with apo-RBP4 increased the secretion of the proinflammatory cytokines MCP1, TNFα, and IL-6 similar to the effects of holo-RBP4 (*SI Appendix*, Fig. S1*B*).

TLR2, TLR4, and Their Downstream Pathways Are Necessary for RBP4-Induced Cytokine Secretion. Although we showed that TLR4 is partially necessary for RBP4-mediated macrophage activation (23), we did not report the TLR4-complex cell surface adaptor proteins or the intracellular adaptor proteins that initiate TLR2 and TLR4 signaling. In Fig. 2, we determined the role of TLR2 and TLR4-complex and TRIF and MyD88 signaling pathway in RBP4-induced macrophage activation. This is important because both these receptors sense extracellular DAMPs and RBP4 can be considered a DAMP because it initiates and perpetuates a noninfectious inflammatory response. RBP4-induced expression of *TNFα* (Fig. 2 *A*, *Left*) and *IL1β* (Fig. 2 *A*, *Right*) levels were reduced in both TLR4 and TLR2 KO cultured macrophages. Furthermore, RBP4-induced *TNFα* (*Left* panel) and *IL1β* (*Right* panel) levels were reduced to that of dialysate control in TLR2/4 double knockout macrophages (DKO) (Fig. 2*A*). These data demonstrate that TLR2 is involved in the proinflammatory actions of RBP4 and indicate that both TLR4 and TLR2 are necessary for the full proinflammatory effects of RBP4. Knocking out TRIF or MyD88 profoundly reduced RBP4-induced *IL1β* and *TNFα* expression in macrophages (Fig. 2*A*). MyD88/TRIF DKO completely blocked the proinflammatory effects of RBP4 (Fig. 2*A*, last bar in both panels) indicating that these TLR adaptor proteins are necessary for the proinflammatory effects of RBP4. Also, TLR2 and TLR4 KO markedly reduced RBP4-induced secretion of TNFα (*Left* panel) and ATP-induced release of IL1β (Fig. 2 *B*, *Right*). TLR2/TLR4 DKO completely blocked these RBP4-mediated effects (Fig. 2*B*). MyD88 or TRIF KO markedly reduced the secretion of proinflammatory cytokines, and MyD88 and TRIF DKO completely inhibited the effects of RBP4 on TNFα secretion and ATP-induced IL1β secretion (Fig. 2*B*). RBP4-induced signaling downstream of TLR2/TLR4 was also abolished in peritoneal (Fig. 2*C*) and bone marrow-derived macrophages (Fig. 2*D*) of MyD88 KO mice as showed by complete absence of TNFα and IL-6 secretion (Fig. 2 *C* and *D*).

RBP4 Activates TLR2 and a TLR4/MD2 Complex. Lipopolysaccharide (LPS) activation of TLR4 requires MD2 and cluster of differentiation 14 (CD14) coreceptors (5). To identify the receptor components required for RBP4 to signal through TLR4 and TLR2, we performed transfection studies in HEK293 cells which do not express TLR4 or TLR2 or any of their coreceptors. Transfection with TLR4, CD14, or MD2 alone was not sufficient for RBP4 to activate NFκB (Fig. 2*E*). However, cotransfection of TLR4 and MD2 together resulted in robust NFκB activation in response to RBP4 (Fig. 2*E*). In contrast, LPS required cotransfection of CD14, TLR4, and MD2 (Fig. 2*E*) to activate NFκB, indicating that RBP4 does not require the same complex as LPS to activate NFκB. Furthermore, a TLR4 neutralizing antibody blocked RBP4-induced IL8 secretion from TLR4/MD2 cotransfected cells (Fig. 2*F*). Transfection with TLR2 alone was also sufficient for RBP4 to induce NFκB activity (Fig. 2*G*). Taken together, these data show that RBP4 signals through the MD2 component of the cell surface TLR4 complex and through TLR2.

Pharmacological Inhibition of TLR4 In Vivo Improves Insulin-Resistance in RBP4-Ox Mice. To determine whether the inhibition of TLR4 is sufficient to improve insulin sensitivity in RBP4-Ox mice, we used TAK242 (resatorvid), a selective antagonist of TLR4. TAK242 markedly reduced RBP4-induced TNFα, IL-6, and MCP1 secretion from cultured macrophages (Fig. 3*A*). TAK242 did not affect body weight in RBP4-Ox mice during 2 wk of daily treatment. Insulin tolerance was not different between treatment groups (TAK242 vs. vehicle) at the beginning of the study. TAK242 treatment markedly improved insulin sensitivity in RBP4-Ox mice as soon as 7 d of treatment (*SI Appendix*, Fig. S2). The TAK242-mediated improvement in insulin sensitivity was sustained at least until 14 d of treatment (Fig. 3*B*). TAK242-improved insulin sensitivity in RBP4-Ox mice is evidenced by both increased area above the insulin tolerance test (ITT) curve (Fig. 3 *B*, *Right*) and also by the steeper slope of the fall in glucose in response to insulin (Fig. 3*C*). Furthermore, TAK242 treatment reduced the total number of adipose tissue macrophages (ATMs) and the percentages of $TNF\alpha^+$, $IL1\beta^+$, and TNFα/IL1β double positive ATMs in RBP4-Ox mice (Fig. 3*D*). Taken together, these data show that antagonizing TLR4 reduces RBP4-driven AT inflammation and improves insulin resistance in RBP4-Ox mice.

RBP4 Primes Bone Marrow-Derived Macrophages for IL1β Release. RBP4 markedly induced IL1β expression in PGWAT (Fig. 1*B*) and in cultured macrophages (Fig. 2*A*), indicating that RBP4 may be an endogenous inflammasome priming agent. In cultured BMDMs, RBP4 markedly increased the expression of several inflammasome components such as *NLRP3* and *Caspase 1* as well as *IL1β* and *TNFα* (Fig. 4*A*). To determine if RBP4-induced IL1β expression resulted in IL1β release in the presence of a "second signal," macrophages were treated with RBP4 and stimulated with

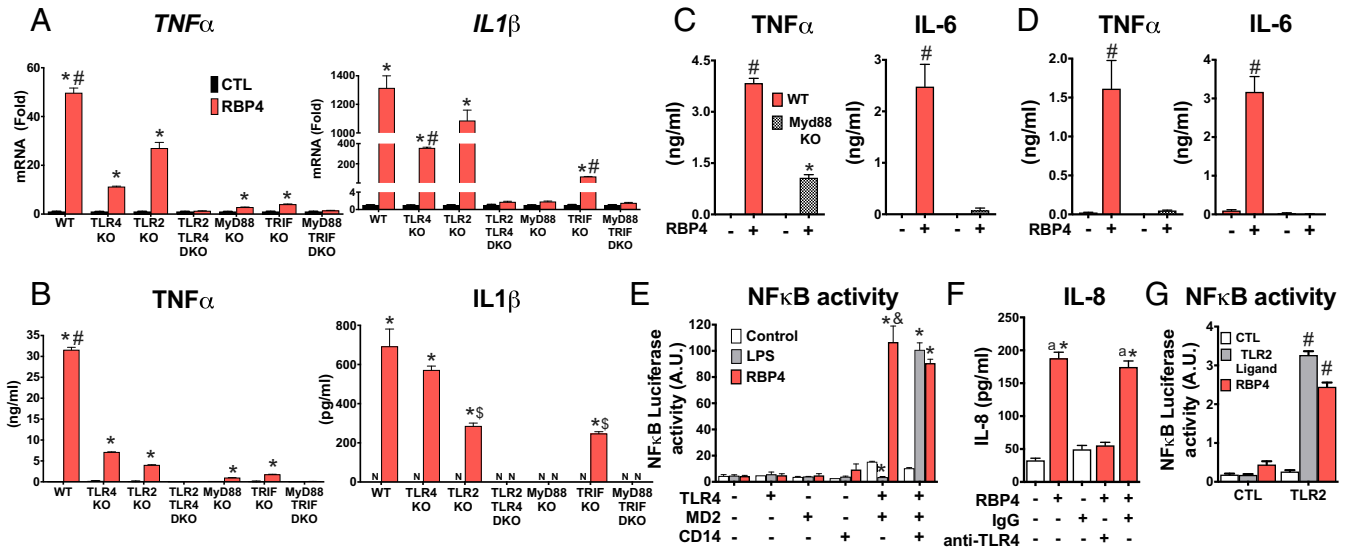

Fig. 2. RBP4 signals through TLR2- and TLR4-dependent pathways. (*A*) *TNFα* and *IL1β* gene expression in WT, TLR4, TLR2, TLR2/4, MyD88, TRIF, and MyD88/TRIF KO cultured macrophages treated with RBP4 or dialysate control (CTL). (*B*) TNFα secretion and IL1β release from WT, TLR4, TLR2, TLR2/4, MyD88, TRIF, and MyD88/TRIF KO macrophages treated with RBP4 or dialysate control and then stimulated with ATP (for IL1β release). (*C*) TNFα and IL6 secretion in primary WT and MyD88 KO peritoneal macrophages treated with RBP4 or dialysate control. (*D*) TNFα and IL6 secretion in primary WT and MyD88 KO BMDMs treated with RBP4 or dialysate control. (*E*) NFκB-driven luciferase reporter activity in HEK293 cells transfected with TLR4 and various coreceptors followed by treatment with RBP4 or dialysate control. (*F*) IL8 secretion in TLR4- and MD2-transfected HEK293 cells followed by RBP4 stimulation in the presence of a TLR4 neutralizing antibody or control IgG. (*G*) RBP4-induced NFκB-driven luciferase reporter activity in HEK293 cells transfected with TLR2. Holo-RBP4 was used in all experiments in this figure. $n = 3$/treatment, $^{*}P < 0.05$ compared to control. $^{\#}P < 0.05$ vs. all other groups. $^{\$}P < 0.05$ vs. RBP4 + ATP WT and TLR4KO. $^{\&}P < 0.05$ vs. LPS group expressing TLR4 and MD2. $^{a}P < 0.05$ compared to IgG and RBP4 + anti-TLR4 (ANOVA).

ATP. Cotreatment with both RBP4 and ATP resulted in robust IL1β release (Fig. 4*B*), but neither RBP4 nor ATP alone stimulated IL1β release (Fig. 4*B*). The Caspase1 inhibitor, ZVAD, markedly reduced RBP4/ATP-induced IL1β release but not TNFα secretion (Fig. 4*B*). Because RBP4 together with ATP induced IL1β release (Fig. 4*B*), we assessed NLRP3-dependent procaspase1

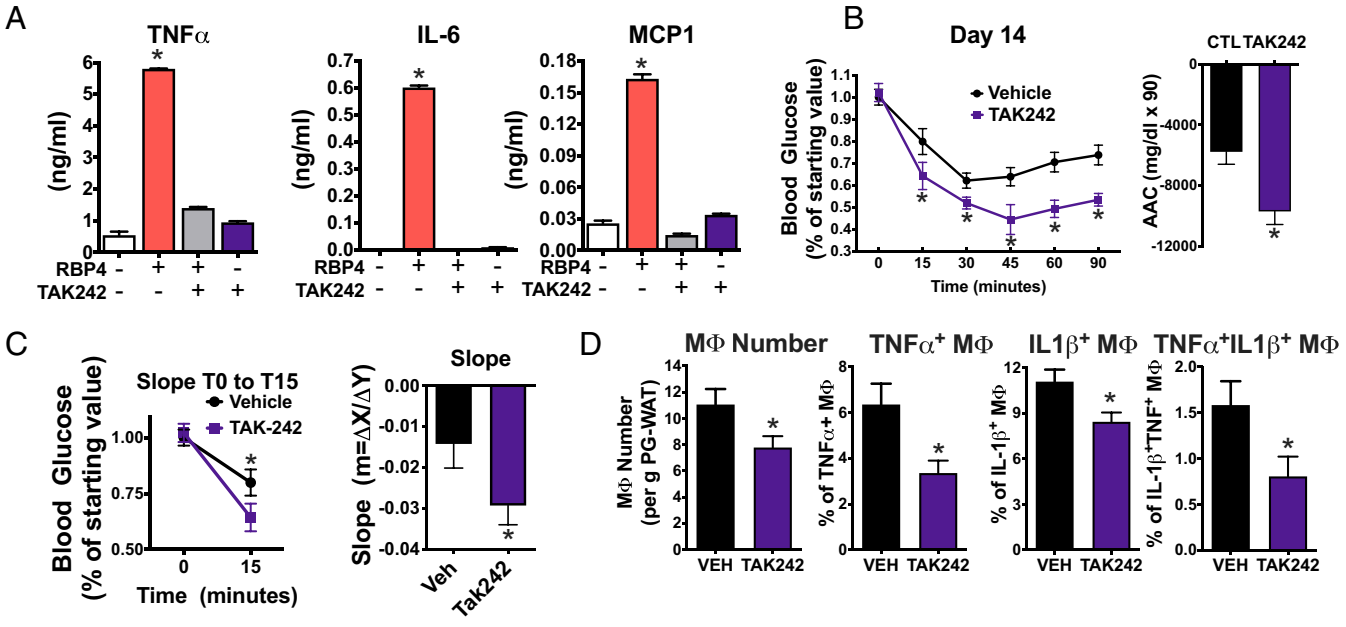

Fig. 3. Small molecule-mediated inhibition of TLR4 inhibits RBP4-induced proinflammatory effects and improves insulin sensitivity in RBP4-Ox mice. (*A*) Cytokine secretion from cultured BMDMs treated with holo-RBP4, the TLR4-specific inhibitor, TAK242 either alone or in combination. $n = 3$/treatment, $^{*}P < 0.05$ compared to all other groups by ANOVA. (*B*) RBP4-Ox mice were treated with TAK242 daily for 2 wk. Insulin sensitivity was assessed by ITT on day 14, $^{*}P < 0.05$ for TAK242-treated mice compared to control by repeated-measures ANOVA. (*C*) Slope of the ITT curve between 0 and 15 min at day 14, $^{*}P < 0.05$ by *t* test. (*D*) Macrophage number and % of adipose tissue macrophages expressing TNFα or IL1β or both in RBP4-Ox mice treated with vehicle or TAK242 for 14 d, $^{*}P < 0.05$ by *t* test. (*B–D*) $n = 10$/treatment.

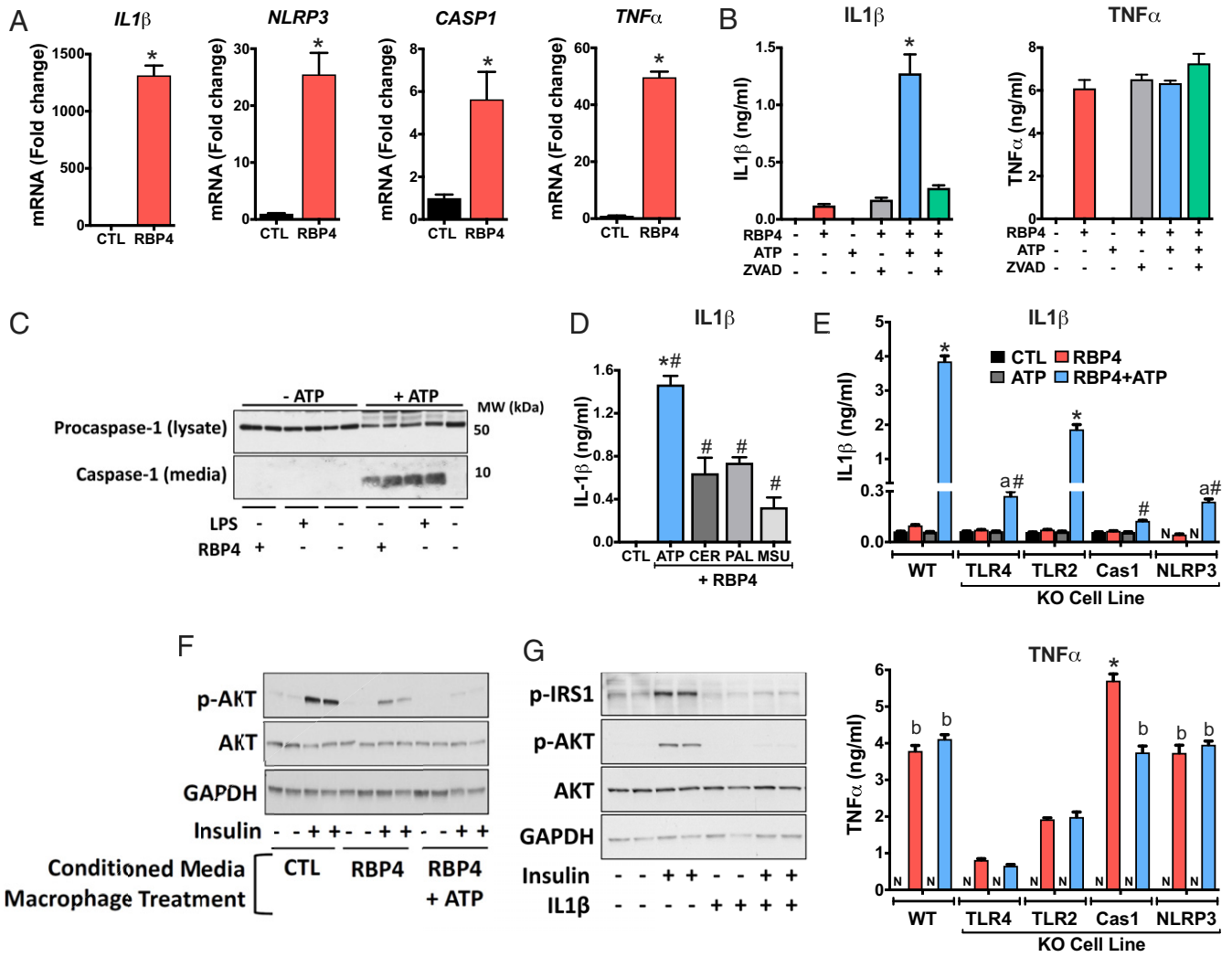

Fig. 4. RBP4 primes the NLRP3 inflammasome for activation. (*A*) *IL1β*, *NLRP3*, *CASP1* (Caspase 1), and *TNFα* expression in cultured BMDM treated with holo-RBP4 or dialysate control. (*B*) IL1β release and TNFα secretion from cultured BMDM treated with holo-RBP4, RBP4, and ATP or dialysate control in the presence or absence of the caspase 1 inhibitor ZVAD. (*C*) Procaspase 1 and caspase 1 protein levels in lysates or media (as indicated) from cells treated with RBP4 and LPS alone or in combination with ATP or respective controls. Data are representative of two independent experiments with similar results. (*D*) IL1β release from cultured BMDM-treated RBP4 in combination with ATP, ceramide (CER), palmitic acid (PAL), and monosodium urate (MSU). (*E*) IL1β release and TNFα secretion from primary BMDMs from WT, TLR4 KO, TLR2 KO, Caspase (Cas) 1 KO, or NLRP3 KO mice treated with RBP4, RBP4 and ATP or dialysate control. (*F*) p-AKT, total AKT, and GAPDH levels in primary SVF-derived adipocytes cultured with macrophage-conditioned media as indicated followed by serum starvation and insulin stimulation. CTL, dialysate control. Data are representative of two independent experiments with similar results. (*G*) p-IRS1, p-AKT, total AKT, and GAPDH levels in primary SVF-derived adipocytes treated with recombinant mouse IL1β as indicated followed by serum starvation and insulin stimulation. Data are representative of two independent experiments with similar results. Holo-RBP4 was used in all experiments in this figure. $n = 3$/treatment, $^{*}P < 0.05$ compared to all other groups. $^{\#}P < 0.05$ compared to dialysate control. $^{a}P < 0.05$ RBP4 + ATP compared to TLR2 and Cas1, same treatment. $^{b}P < 0.05$ compared to TLR2 and TLR4, same treatment. (*A*) *t* test; (*B–G*) ANOVA. N, not detectable.

cleavage, which is necessary for pro-IL1β cleavage and its subsequent release. RBP4 in combination with ATP induced pro-IL1β cleavage (Fig. 4*C*). LPS was used as a positive control in these studies (Fig. 4*C*). In addition, RBP4 primed macrophages for activation by ceramide, palmitic acid, and monosodium urate crystals (Fig. 4*D*), which are known obesity and metabolic disease-related inflammasome activators. Thus, RBP4 primes the macrophage inflammasome for activation in a Caspase1-dependent manner.

RBP4 Signals through TLR2 and TLR4 to Prime the NLRP3 Inflammasome. As the NLRP3 inflammasome is associated with metabolic diseases, we sought to determine if RBP4 primes the NLRP3 inflammasome activation. RBP4/ATP-induced IL1β release was markedly reduced in macrophages from both TLR4 KO and TLR2 KO mice compared to WT (Fig. 4*E*) and almost completely eliminated in macrophages from both Caspase1 and NLRP3 KO mice (Fig. 4*E*). RBP4/ATP-induced TNFα secretion was also reduced in both TLR4 and TLR2 KO BMDMs compared to WT (Fig. 4*E*), but was not affected by the knockdown of NLRP3 or Caspase1 (Fig. 4*E*). Thus, RBP4 signals through TLR2 and TLR4 to induce *IL1β* expression and TNFα secretion. The induction of *IL1β* expression is necessary for NLRP3/Caspase1-dependent IL1β release.

NLRP3 Activation Potentiates RBP4-Induced Insulin Resistance in Primary SVF-Derived Adipocytes. SVF-derived adipocytes incubated with conditioned media from macrophages treated with RBP4 had a marked reduction in insulin-induced AKT phosphorylation (Fig. 4*F*). Conditioned media from macrophages treated with ATP alone had no effect on insulin-induced AKT phosphorylation.

However, SVF-derived adipocytes incubated with conditioned media from macrophages treated with RBP4 and ATP displayed more severe insulin resistance than those incubated with conditioned media from macrophages treated with RBP4 alone (Fig. 4*F*). Furthermore, recombinant IL1β alone was sufficient to induce resistance to insulin-induced AKT phosphorylation in primary SVF-derived adipocytes (Fig. 4*G*). Thus, RBP4-mediated inflammasome priming and the subsequent IL1β release from macrophages may mediate the insulin-desensitizing effects of RBP4 on adipocytes.

RBP4-Mediated IL1β Expression and Release Is Glucose Dependent. Since T2D is associated with hyperglycemia and inflammasome activation is highly dependent on glycolysis (35–37), we sought to determine if glucose levels affect RBP4-induced IL1β expression and release. Cultured BMDMs were incubated with increasing glucose concentrations and stimulated with RBP4 or dialysate control. RBP4-induced *IL1β*, *TNFα*, and *MCP1* expression were increased at 5 mM compared to 1 mM glucose (Fig. 5*A*). A total of 25 mM glucose further induced *IL1β* but not *TNFα* or *MCP-1* expression (Fig. 5*A*). Similar to the effects on *IL1β*, RBP4 induced expression of *GLUT1*, the major glucose transporter in macrophages, in a glucose-dependent manner from 1 to 25 mM glucose (Fig. 5*A*), which further indicates that RBP4 primes the inflammasome and may increase glucose uptake. To determine if glucose is required for RBP4-mediated inflammasome priming, BMDMs were incubated with 2-deoxy-D-glucose (2DG, 1 mM), a nonmetabolizable glucose analog, or D-glucose (1 mM) prior to RBP4 stimulation. The 2DG treatment obliterated RBP4-induced *IL1β* expression but had no effect on *TNFα* expression, supporting the conclusion that the effect on *IL1β* and not on *TNFα* is glucose dependent (Fig. 5*B*). RBP4-induced *MCP-1* but not *GLUT1* expression was also glucose dependent (Fig. 5*B*). RBP4 induced IL1β release, but not TNFα secretion in a glucose-dependent manner (Fig. 5*C*). These effects on IL1β release are likely due in part to the effects of glucose on RBP4-induced *IL1β* transcription (Fig. 5 *A* and *B*). Thus, these results on IL1β release most likely reflect glucose dependence of RBP4-mediated pro-IL1β induction and NLRP3-inflammasome activation (Fig. 5).

IL1β Is Elevated in RBP4-Overexpressing Mice. To determine if overexpression of RBP4 in vivo is associated with elevated IL1β, we measured IL1β levels in the adipose tissue of WT and RBP4-Ox mice fed chow or HFD. We observed that RBP4-Ox mice on chow diet express higher levels of IL1β than WT chow-fed mice with levels similar to WT mice fed HFD (Fig. 6, *Upper Left*). Compared to chow-fed WT mice, chow-fed RBP4-Ox mice and HFD-fed WT mice had a higher percentage of IL1β and TNFα positive ATMs (Fig. 6, *Lower Left* and *Upper Right*). This was increased even further in HFD-fed RBP4-Ox mice compared to HFD-fed WT mice (Fig. 6, *Upper Right* and *Lower Left*). Thus, RBP4 overexpression is sufficient to increase the percentage of IL1β and TNFα positive ATMs in mice fed on either chow or HFD. HFD-fed WT mice had higher AT IL1β and TNFα levels than chow-fed WT mice and levels were similar to those in chow-fed RBP4-Ox mice (Fig. 6, *Upper Left*). Serum IL1β levels were not different in chow-fed WT mice compared to chow-fed RBP4-Ox mice. However, serum IL1β levels were elevated in HFD-fed RBP4-Ox mice compared to HFD-fed WT mice (Fig. 6, *Lower Right*). Since serum levels reflect secreted IL1β, these data reinforce our in vitro data showing that RBP4 is sufficient to prime the inflammasome for activation (signal 1) but cannot serve as signal 2 to activate the inflammasome.

Discussion

In obesity, AT expansion ultimately becomes exhausted, which leads to adipocyte dysfunction, localized inflammation, and insulin resistance (4, 34). However, there is not enough information on the endogenous proteins and metabolites which can activate sterile inflammation in obesity and T2D. Our work describes how a native serum retinol carrier, RBP4, acts as a DAMP to elicit proinflammatory effects. Elevated RBP4 is associated with obesity and T2D in humans and this correlates with proinflammatory cytokine expression (38). Genetic overexpression of RBP4 leads to PGWAT inflammation and insulin resistance (17). Here we determine the mechanisms underlying these effects.

TLRs play an important role in inflammation-induced insulin resistance. TLR2- or TLR4-deficient mice or mice with spontaneous inactivating TLR4 mutations are protected from obesity-induced insulin resistance (6). These TLRs mediate crosstalk between the innate immune system and whole-body metabolism (39). Previous studies show that TLR4 mediates the secretion of some but not all of the RBP4-induced cytokines (23). In this study, we show that RBP4-induced cytokine secretion is markedly reduced in macrophages with genetic knockout of TLR2 or TLR4 individually and completely inhibited by double knockout of these receptors. We do not know if the binding of RBP4 to these receptors occurs in a coordinated, sequential fashion or simultaneously. In addition, we identify the critical components of the TLR complex required for RBP4-mediated insulin resistance. RBP4-mediated TLR4 activation requires the coreceptor MD2. Consistent with the roles of MyD88 and TRIF as the sole adaptors downstream of TLR2 and TLR4, knockout of MyD88 and TRIF in macrophages markedly reduced TNFα and IL1β expression and release. Double knockout of both MyD88 and TRIF completely inhibited RBP4-induced cytokine expression and secretion. Thus, in addition to TLR4, TLR2, and their downstream intracellular adaptor MyD88, and TRIF, are critical mediators of the proinflammatory effects of elevated RBP4 in obesity and T2D (Fig. 7).

Our findings provide further mechanistic insights into the inflammatory effects of RBP4 on immune cells and how this results in impaired insulin signaling in adipocytes (Fig. 7). We show that RBP4 is an endogenous NLRP3-inflammasome priming agent which induces the expression of IL1β and other proinflammatory cytokines through TLR2 and TLR4/MD2. In macrophages, RBP4 induced expression of pro-IL1β, Caspase 1, and NLRP3. These effects are retinol independent since they are elicited by either apo- or holo-RBP4. NLRP3 priming and subsequent activation potentiated RBP4-mediated insulin resistance in adipocytes. We propose that in obesity, the NLRP3 inflammasome can be primed by RBP4, an endogenous protein. Elevated IL1β expression and secretion parallels elevated RBP4 serum levels. IL1β is elevated in serum of HFD-fed RBP4-Ox mice compared to HFD-fed WT mice, which reflects its cleavage and release. This is consistent with our in vitro findings showing that RBP4 primes the NLRP3 inflammasome but requires a second signal for IL1β release. The requirement for two signals is further highlighted by the fact that HFD-fed NLRP3 KO mice, but not chow-fed mice, are more insulin sensitive compared to their respective WT controls (27).

In humans, the NLRP3 inflammasome and IL1β are rapidly emerging as a critical link between inflammation and metabolic diseases, including obesity and T2D (27, 40). Both IL1β and NLRP3 are up-regulated in humans with T2D compared to normal controls (27, 40). In obese T2D humans, weight loss is associated with reduced AT NLRP3 expression, decreased inflammation, and improved insulin sensitivity (27). Additionally, in humans, Caspase 1 activity is increased in visceral compared to SQWAT (41) and Caspase 1 knockout mice are protected against HFD-induced insulin resistance (42). Taken together, these data suggest that the NLRP3 inflammasome plays an important role in the pathogenesis of insulin resistance. Since RBP4 primes the NLRP3 inflammasome, it may play a critical role in NLRP3-induced insulin resistance in obesity and T2D.

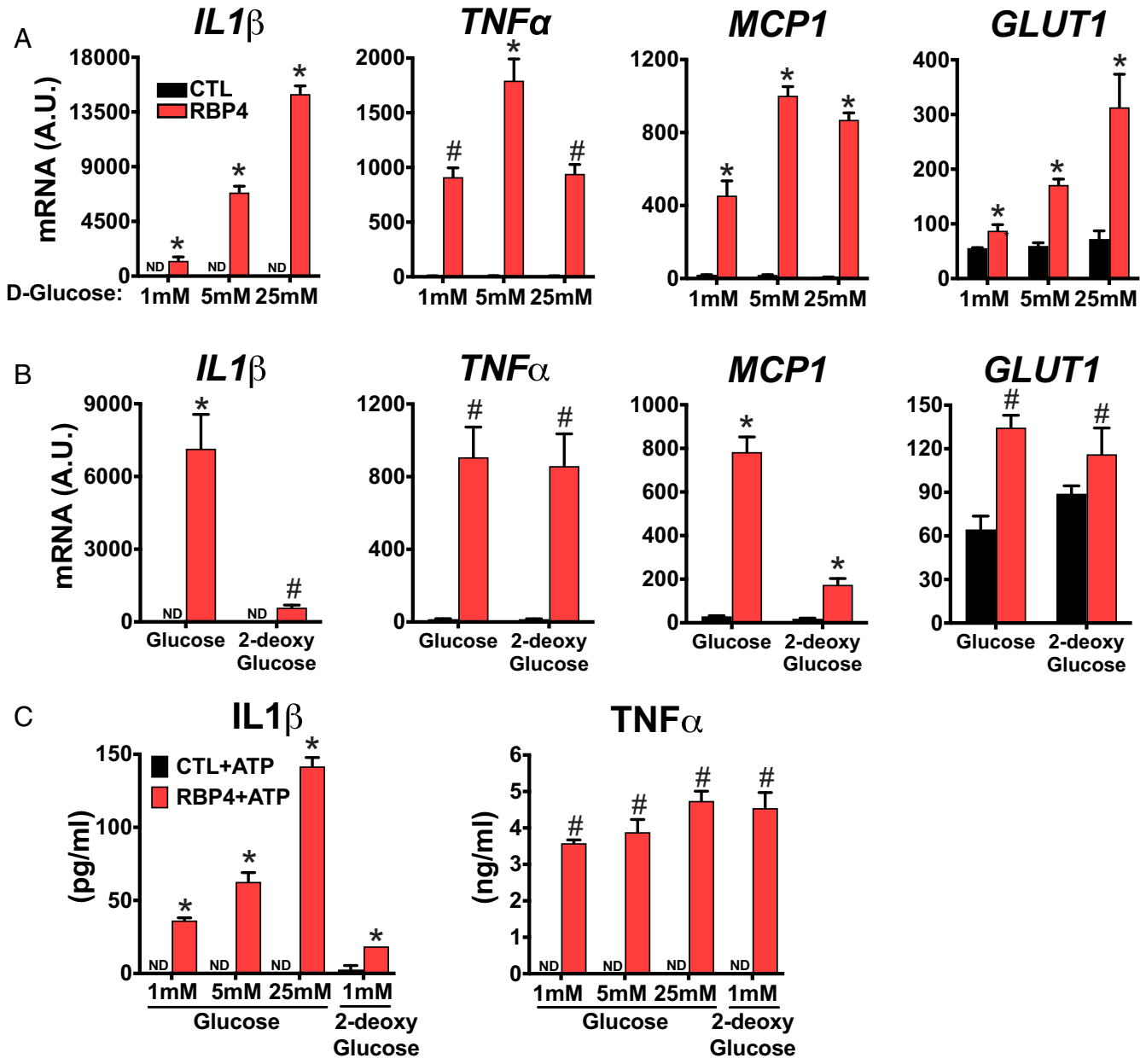

Fig. 5. RBP4-induced inflammasome priming and IL1β release is glucose and glycolysis dependent. (*A*) *IL1β*, *TNFα*, *MCP1*, and *GLUT1* expression in cultured BMDM treated with RBP4 or dialysate control in different D-glucose concentrations. (*B*) *IL1β*, *TNFα*, *MCP1*, and *GLUT1* expression in cultured BMDMs treated with RBP4 or dialysate control in the presence of D-glucose or 2-deoxy-D-glucose. (*C*) IL1β release and TNFα secretion from cultured BMDMs treated with RBP4 or dialysate control in different D-glucose concentrations or in the presence of D-glucose or 2-deoxy-D-glucose. Holo-RBP4 was used in all experiments in this figure. $n = 3$/treatment, $^{*}P < 0.05$ compared to all other groups and $^{\#}P < 0.05$ compared to dialysate control (CTL) by ANOVA. ND, not detectable.

Hyperglycemia is a hallmark of T2D and can contribute to AT inflammation, including NLRP3-inflammasome activation (43). Both *IL1β* and *NLRP3* mRNA levels are elevated in visceral compared to s.c. ATMs from obese compared to nonobese human subjects (40). IL1β levels are increased even further in ATMs from obese diabetic humans compared to obese nondiabetic patients (40). These data suggest that AT IL1β in humans may be regulated by glycemic control. Our present study shows that RBP4-mediated inflammasome priming and IL1β release are glucose dependent, which is consistent with the hypothesis that the hyperglycemia in T2D may potentiate the effects of elevated RBP4 on NLRP3-inflammasome priming, thereby contributing to T2D pathogenesis and hyperglycemia-induced inflammation.

Many studies show that serum RBP4 levels correlate with insulin resistance although some studies do not agree, as recently reviewed in ref. 13. Examples supporting a strong relationship include a study of 3,289 people living in Beijing and Shanghai which showed that elevated circulating RBP4 levels were strongly associated with body mass index, waist circumference, and insulin resistance (21). Many other studies found similar results (21, 44–48). However, some studies failed to find a relationship between increased RBP4 levels and insulin resistance (discussed in ref. 13 and refs. 49–53). Potential confounding factors may include the methodology used to measure RBP4 levels (49, 50) and analyzing associations only with circulating RBP4 levels and not adipose tissue levels (13). Also, some studies propose that serum RBP4 levels in T2D individuals may

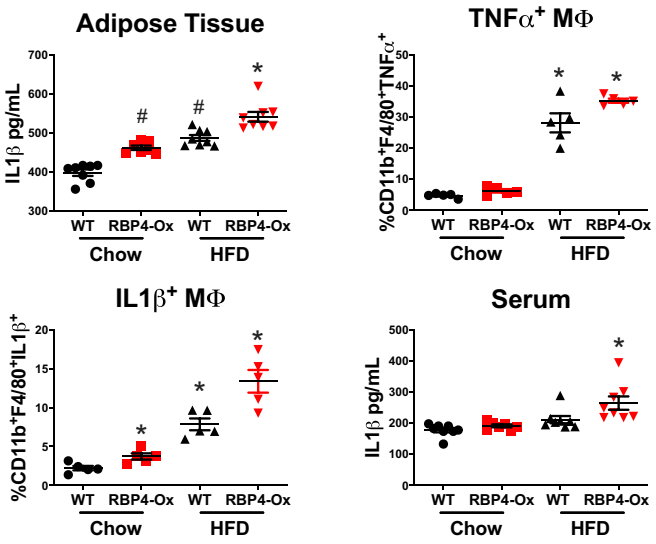

Fig. 6. IL1β and TNFα are elevated in RBP4-Ox mice. (*Upper Left*) PGWAT IL1β protein levels in WT and RBP4-Ox mice fed on chow and HFD diet, measured by ELISA. (*Upper Right* and *Lower Left*) % of TNFα or IL1β positive $CD11b^+F4/80^+$ PGWAT macrophages in WT and RBP4-Ox mice fed on chow and HFD diet were measured by flow cytometry. (*Lower Right*) Serum IL1β levels in WT and RBP4-Ox mice fed on chow and HFD diet were measured by ELISA. $n = 5$ for flow cytometry studies and $n = 8$/group for ELISA. $^*P < 0.05$ compared to all other groups. $^{\#}P < 0.05$ compared to WT chow-fed group and RBP4-Ox HFD fed group. All comparisons were done by ANOVA.

be directly affected by incipient nephropathy (51–53), although this is not a factor in other studies (13, 16, 49, 50). Importantly, a causative role for RBP4 in T2D is strongly supported by the genetic and epidemiological data showing that a gain-of-function polymorphism in the RBP4 promoter which increases adipose RBP4 expression is associated with an 80% increased risk of T2D in humans (19, 22). Other genetic studies also support a causative role as reviewed in ref. 13.

The current study demonstrates that TLR4 inhibition in vivo is sufficient to markedly improve insulin sensitivity in the setting of elevated RBP4 in mice. This inhibition decreases PGWAT macrophage infiltration and proinflammatory cytokine levels, including IL1β levels in AT macrophages. Our data are consistent with studies in the literature showing that pharmacological inhibition of TLR4 with TAK242 protects mice against LPS- and nonesterified fatty acid-induced inflammation and insulin resistance, and also protects rats from acute and chronic HFD-induced insulin resistance (54, 55). Our findings serve as proof of concept that inhibition of TLR4 activation reduces RBP4-driven AT inflammation, including IL1β release and the subsequent insulin resistance.

In conclusion, we show that RBP4 primes the NLRP3 inflammasome for IL1β release through both TLR2 and TLR4/MD2 cell surface receptors and the downstream MyD88 and TRIF intracellular adaptor proteins in macrophages. This mediates RBP4-induced insulin resistance in adipocytes. IL1β is also elevated in adipose tissue macrophages of chow-fed RBP4-Ox mice and increased further in HFD-fed RBP4-Ox. Pharmacological inhibition of TLR4 in RBP4-Ox mice decreases AT inflammation, including IL1β expression and release, and improves insulin sensitivity (Fig. 7). Administration of RBP4 alone is sufficient to induce adipose tissue inflammation, including IL1β expression and the subsequent insulin resistance. These studies advance our understanding of the cellular mechanisms by which RBP4 causes adipose tissue inflammation and insulin resistance and could lead to new preventative and therapeutic approaches for obesity-induced resistance.

Data Availability. All study data are included in the article and supporting information.

ACKNOWLEDGMENTS. We thank Dr. Odile D. Peroni and Abha Dhaneshwar for critical experimental assistance and Dr. Anna Santoro for comments on the manuscript. This work was supported by grants from the NIH R01DK43051 (B.B.K.), P30DK57521 (B.B.K.), 5T32DK007516 (B.B.K. and M.M.Y.), F32 DK091041 (J.N.), a grant from the JPB Foundation (B.B.K.), a grant from the Harvard Training Program in Nutrition and Metabolism, 2T32HD052961 (M.M.Y.), and a grant from Fundação de Amparo a Pesquisa do Estado de São Paulo 2015/15626-8 and 2014/02218-6 (A.C.). A.S.-P. was a visiting diploma student from the Department of Medicine, University of Freiburg, Germany. A.C. was a visiting PhD student from the Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, SP, Brazil. We also acknowledge the BioRender software used to generate Fig. 7.

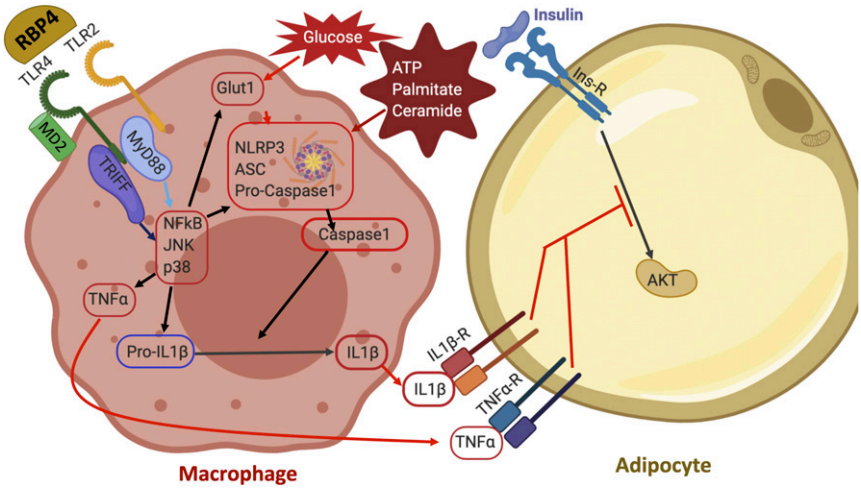

Fig. 7. Schematic representation of RBP4 effects on macrophages and the resulting consequences on insulin signaling in adipocytes. RBP4 signaling through TLR2 and TLR4 is required for TNFα secretion and IL1β expression and release. RBP4 primes the NLRP3 inflammasome in macrophages through a TLR4/MD2 receptor complex and through TLR2 and the downstream pathways MyD88 and TRIF. The effects of RBP4-mediated IL1β release are glucose dependent and triggered by NLRP3-inflammasome activation, which can be induced by ATP, ceramide, or palmitate. RBP4-mediated macrophage activation results in the blockade of insulin signaling in adipocytes.

1. L. M. Jaacks *et al*., The obesity transition: Stages of the global epidemic. *Lancet Diabetes Endocrinol.* **7**, 231–240 (2019).
2. A. W. Ferrante, Jr, Macrophages, fat, and the emergence of immunometabolism. *J. Clin. Invest.* **123**, 4992–4993 (2013).
3. L. Monteiro, J. A. D. S. Pereira, L. Palhinha, P. M. M. Moraes-Vieira, Leptin in the regulation of the immunometabolism of adipose tissue-macrophages. *J. Leukoc. Biol.* **106**, 703–716 (2019).
4. A. W. Ferrante, Jr, The immune cells in adipose tissue. *Diabetes Obes. Metab.* **15**, 34–38 (2013).
5. K. Newton, V. M. Dixit, Signaling in innate immunity and inflammation. *Cold Spring Harb. Perspect. Biol.* **4**, a006049 (2012).
6. H. Shi *et al*., TLR4 links innate immunity and fatty acid-induced insulin resistance. *J. Clin. Invest.* **116**, 3015–3025 (2006).
7. R. W. Himes, C. W. Smith, Tlr2 is critical for diet-induced metabolic syndrome in a murine model. *FASEB J.* **24**, 731–739 (2010).
8. R. Ahmad *et al*., Elevated expression of the toll like receptors 2 and 4 in obese individuals: Its significance for obesity-induced inflammation. *J. Inflamm. (Lond.)* **9**, 48 (2012).
9. F. Ursini *et al*., Abatacept improves whole-body insulin sensitivity in rheumatoid arthritis: An observational study. *Medicine (Baltimore)* **94**, e888 (2015).
10. F. Ursini, D. Mauro, S. Naty, D. Gagliardi, R. D. Grembiale, Improvement in insulin resistance after short-term treatment with abatacept: Case report and short review. *Clin. Rheumatol.* **31**, 1401–1402 (2012).
11. G. S. Hotamisligil, R. J. Davis, Cell signaling and stress responses. *Cold Spring Harb. Perspect. Biol.* **8**, a006072 (2016).
12. A. C. Könner, J. C. Brüning, Toll-like receptors: Linking inflammation to metabolism. *Trends Endocrinol. Metab.* **22**, 16–23 (2011).
13. W. S. Blaner, Vitamin A signaling and homeostasis in obesity, diabetes, and metabolic disorders. *Pharmacol. Ther.* **197**, 153–178 (2019).
14. S. A. Lee, J. J. Yuen, H. Jiang, B. B. Kahn, W. S. Blaner, Adipocyte-specific overexpression of retinol-binding protein 4 causes hepatic steatosis in mice. *Hepatology* **64**, 1534–1546 (2016).
15. C. Meisinger *et al*., Retinol-binding protein 4 is associated with prediabetes in adults from the general population: The cooperative health research in the region of augsburg (KORA) F4 study. *Diabetes Care* **34**, 1648–1650 (2011).
16. Q. Yang *et al*., Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* **436**, 356–362 (2005).
17. P. M. Moraes-Vieira *et al*., RBP4 activates antigen-presenting cells, leading to adipose tissue inflammation and systemic insulin resistance. *Cell Metab.* **19**, 512–526 (2014).
18. P. M. Moraes-Vieira *et al*., Antigen presentation and T-cell activation are critical for RBP4-induced insulin resistance. *Diabetes* **65**, 1317–1327 (2016).
19. M. van Hoek *et al*., An RBP4 promoter polymorphism increases risk of type 2 diabetes. *Diabetologia* **51**, 1423–1428 (2008).
20. Q. Sun *et al*., Plasma retinol-binding protein 4 (RBP4) levels and risk of coronary heart disease: A prospective analysis among women in the nurses' health study. *Circulation* **127**, 1938–1947 (2013).
21. Q. Qi *et al*., Elevated retinol-binding protein 4 levels are associated with metabolic syndrome in Chinese people. *J. Clin. Endocrinol. Metab.* **92**, 4827–4834 (2007).
22. L. Munkhtulga *et al*., Regulatory SNP in the RBP4 gene modified the expression in adipocytes and associated with BMI. *Obesity (Silver Spring)* **18**, 1006–1014 (2010).
23. J. Norseen *et al*., Retinol-binding protein 4 inhibits insulin signaling in adipocytes by inducing proinflammatory cytokines in macrophages through a c-Jun N-terminal kinase- and toll-like receptor 4-dependent and retinol-independent mechanism. *Mol. Cell. Biol.* **32**, 2010–2019 (2012).
24. R. Fedders *et al*., Liver-secreted RBP4 does not impair glucose homeostasis in mice. *J. Biol. Chem.* **293**, 15269–15276 (2018).
25. S. J. Thompson *et al*., Hepatocytes are the principal source of circulating RBP4 in mice. *Diabetes* **66**, 58–63 (2017).
26. R. Stienstra, C. J. Tack, T. D. Kanneganti, L. A. Joosten, M. G. Netea, The inflammasome puts obesity in the danger zone. *Cell Metab.* **15**, 10–18 (2012).
27. B. Vandanmagsar *et al*., The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat. Med.* **17**, 179–188 (2011).
28. P. M. Moraes-Vieira, E. J. Bassi, R. C. Araujo, N. O. Câmara, Leptin as a link between the immune system and kidney-related diseases: Leading actor or just a coadjuvant? *Obes. Rev.* **13**, 733–743 (2012).
29. H. Wen *et al*., Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nat. Immunol.* **12**, 408–415 (2011).
30. J. C. Chow, D. W. Young, D. T. Golenbock, W. J. Christ, F. Gusovsky, Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* **274**, 10689–10692 (1999).
31. K. A. Fitzgerald, D. C. Rowe, D. T. Golenbock, Endotoxin recognition and signal transduction by the TLR4/MD2-complex. *Microbes Infect.* **6**, 1361–1367 (2004).
32. L. de Brito Monteiro *et al*., M-CSF- and L929-derived macrophages present distinct metabolic profiles with similar inflammatory outcomes. *Immunobiology* **225**, 151935 (2020).
33. P. M. Moraes-Vieira *et al*., Leptin deficiency impairs maturation of dendritic cells and enhances induction of regulatory T and Th17 cells. *Eur. J. Immunol.* **44**, 794–806 (2014).
34. A. Castoldi, C. Naffah de Souza, N. O. Câmara, P. M. Moraes-Vieira, The macrophage switch in obesity development. *Front. Immunol.* **6**, 637 (2016).
35. J. S. Moon *et al*., mTORC1-Induced HK1-dependent glycolysis regulates NLRP3 inflammasome activation. *Cell Rep.* **12**, 102–115 (2015).
36. M. Xie *et al*., PKM2-dependent glycolysis promotes NLRP3 and AIM2 inflammasome activation. *Nat. Commun.* **7**, 13280 (2016).
37. A. C. Codo *et al*., Elevated glucose levels favor SARS-CoV-2 infection and monocyte response through a HIF-1alpha/glycolysis-dependent axis. *Cell Metab.* **32**, 437–446.e5 (2020).
38. A. Yao-Borengasser *et al*., Retinol binding protein 4 expression in humans: Relationship to insulin resistance, inflammation, and response to pioglitazone. *J. Clin. Endocrinol. Metab.* **92**, 2590–2597 (2007).
39. C. N. S. Breda, G. G. Davanzo, P. J. Basso, N. O. Saraiva Câmara, P. M. M. Moraes-Vieira, Mitochondria as central hub of the immune system. *Redox Biol.* **26**, 101255 (2019).
40. E. Dalmas *et al*., T cell-derived IL-22 amplifies IL-1β-driven inflammation in human adipose tissue: Relevance to obesity and type 2 diabetes. *Diabetes* **63**, 1966–1977 (2014).
41. T. B. Koenen *et al*., The inflammasome and caspase-1 activation: A new mechanism underlying increased inflammatory activity in human visceral adipose tissue. *Endocrinology* **152**, 3769–3778 (2011).
42. R. Stienstra *et al*., The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity. *Cell Metab.* **12**, 593–605 (2010).
43. T. B. Koenen *et al*., Hyperglycemia activates caspase-1 and TXNIP-mediated IL-1beta transcription in human adipose tissue. *Diabetes* **60**, 517–524 (2011).
44. E. Goodman *et al*., The relationship of retinol binding protein 4 to changes in insulin resistance and cardiometabolic risk in overweight black adolescents. *J. Pediatr.* **154**, 67–73.e1 (2009).
45. N. Hoggard, A. Agouni, N. Mody, M. Delibegovic, Serum levels of RBP4 and adipose tissue levels of PTP1B are increased in obese men resident in northeast Scotland without associated changes in ER stress response genes. *Int. J. Gen. Med.* **5**, 403–411 (2012).
46. J. W. Lee *et al*., Retinol binding protein 4 and insulin resistance in apparently healthy elderly subjects. *Clin. Chim. Acta* **400**, 30–32 (2009).
47. G. K. Pandey *et al*., Altered circulating levels of retinol binding protein 4 and transthyretin in relation to insulin resistance, obesity, and glucose intolerance in asian Indians. *Endocr. Pract.* **21**, 861–869 (2015).
48. J. Ram *et al*., Retinol binding protein-4 predicts incident diabetes in Asian Indian men with prediabetes. *Biofactors* **41**, 160–165 (2015).
49. T. E. Graham, C. J. Wason, M. Blüher, B. B. Kahn, Shortcomings in methodology complicate measurements of serum retinol binding protein (RBP4) in insulin-resistant human subjects. *Diabetologia* **50**, 814–823 (2007).
50. Q. Yang *et al*., Quantitative measurement of full-length and C-terminal proteolyzed RBP4 in serum of normal and insulin-resistant humans using a novel mass spectrometry immunoassay. *Endocrinology* **153**, 1519–1527 (2012).
51. A. Henze *et al*., Evidence that kidney function but not type 2 diabetes determines retinol-binding protein 4 serum levels. *Diabetes* **57**, 3323–3326 (2008).
52. J. Raila *et al*., Microalbuminuria is a major determinant of elevated plasma retinol-binding protein 4 in type 2 diabetic patients. *Kidney Int.* **72**, 505–511 (2007).
53. M. Murata *et al*., An increase in serum retinol-binding protein 4 in the type 2 diabetic subjects with nephropathy. *Endocr. J.* **56**, 287–294 (2009).
54. N. Zhang *et al*., Pharmacological TLR4 inhibition protects against acute and chronic fat-induced insulin resistance in rats. *PLoS One* **10**, e0132575 (2015).
55. S. E. Hussey *et al*., TAK-242, a small-molecule inhibitor of Toll-like receptor 4 signalling, unveils similarities and differences in lipopolysaccharide- and lipid-induced inflammation and insulin resistance in muscle cells. *Biosci. Rep.* **33**, 37–47 (2012).