RBP4 Activates Antigen-Presenting Cells, Leading to Adipose Tissue Inflammation and Systemic Insulin Resistance

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http://dx.doi.org/10.1016/j.cmet.2014.01.018

SUMMARY

Insulin resistance is a major cause of diabetes and is highly associated with adipose tissue (AT) inflammation in obesity. RBP4, a retinol transporter, is elevated in insulin resistance and contributes to increased diabetes risk. We aimed to determine the mechanisms for RBP4-induced insulin resistance. Here we show that RBP4 elevation causes AT inflammation by activating innate immunity that elicits an adaptive immune response. RBP4-overexpressing mice (RBP4-Ox) are insulin resistant and glucose intolerant and have increased AT macrophage and CD4 T cell infiltration. In RBP4-Ox, AT CD206+ macrophages express proinflammatory markers and activate CD4 T cells while maintaining alternatively activated macrophage markers. These effects result from direct activation of AT antigen-presenting cells (APCs) by RBP4 through a JNK-dependent pathway. Transfer of RBP4-activated APCs into normal mice is sufficient to induce AT inflammation, insulin resistance, and glucose intolerance. Thus, RBP4 causes insulin resistance, at least partly, by activating AT APCs that induce CD4 T cell Th1 polarization and AT inflammation.

INTRODUCTION

The immune system plays an important role in obesity-related insulin resistance, which is a major pathogenic factor in type 2 diabetes (T2D) and the related cardiovascular disease (Moraes-Vieira et al., 2012; Olefsky and Glass, 2010; Weisberg et al., 2003; Xu et al., 2002). Large epidemiologic studies demonstrate that elevated circulating RBP4 levels are a biomarker for insulin resistance, prediabetes, the metabolic syndrome (Meisinger et al., 2011; Qi et al., 2007), and myocardial infarction (Sun et al., 2013). Genetic studies indicate that elevated RBP4 markedly increases diabetes risk and may play a causative role in the disease (van Hoek et al., 2008). Emerging evidence suggests a possible role for proinflammatory pathways in RBP4-induced insulin resistance. RBP4 levels in serum (Balagopal et al., 2007) and AT (Yao-Borengasser et al., 2007) strongly correlate with subclinical inflammation, including proinflammatory cytokine levels. RBP4 impairs insulin signaling in adipocytes indirectly by inducing proinflammatory cytokine production from macrophages through Toll-like receptor 4 (TLR4) and c-Jun N-terminal protein kinase (JNK) pathways (Norseen et al., 2012). Also, the RBP4/retinol complex stimulates JAK2/STAT5 signaling and expression of suppressor of cytokine signaling 3, which has also been implicated in insulin resistance (Berry et al., 2011).

Several immunologic cell types regulate AT inflammation and insulin sensitivity (Odegaard and Chawla, 2013). Macrophages and dendritic cells (DC), which are both APCs (Steinman et al., 2005), are present in healthy AT throughout the lifespan (Bertola et al., 2012; Morris et al., 2013). Although leptin (Lord et al., 2005), fatty acids (Nguyen et al., 2007), and other factors have been implicated in APC activation, the molecular drivers of AT inflammation are not well understood. AT macrophages (ATM) in healthy AT express anti-inflammatory markers typical of alternatively activated or M2 macrophages, which generally promote tissue repair (Lumeng et al., 2007; Odegaard and Chawla, 2013). Obesity triggers the accumulation of F4/80+ macrophages in AT (Weisberg et al., 2003), which coexpress the DC marker CD11c and proinflammatory genes typical of classically activated or M1 macrophages (Lumeng et al., 2007; Odegaard and Chawla, 2013; Weisberg et al., 2003). Recently, AT APCs have been implicated in insulin resistance (Bertola et al., 2012; Cipolletta et al., 2012; Morris et al., 2013). In addition to macrophages, lymphocytes in AT are also regulated by metabolic status (Cipolletta et al., 2011; Goossens et al., 2012; Lynch et al., 2012; Nishimura et al., 2009; Strissel et al., 2010; Winer et al., 2011).

Depending on the signals during the interactions with APCs, CD4 T cells can differentiate into pro- (T helper 1, Th1 and Th17) or anti-inflammatory (Th2 and regulatory T cells, Treg) cells (Zhu and Paul, 2008). Obesity is associated with a progressive bias toward a proinflammatory Th1 phenotype in AT, which is linked to insulin resistance (Winer et al., 2009). Moreover, insulin sensitivity is improved in Th1 lineage-defining transcription factor (T-bet)-deficient mice on high-fat diet (HFD) despite increased visceral adiposity (Stolarczyk et al., 2013). The mechanisms that regulate the inflammatory fate of AT T cells are unknown. CD4 T cells undergo polyclonal expansion within AT in obesity (Feuerer et al., 2009; Ilan et al., 2010; Yang et al., 2010). This implies that AT CD4 T cell expansion and polarization are an adaptive immune response to obesity and insulin resistance. Classically, APCs shape CD4 T cell activation by three
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signals which define the differentiation of naive CD4 T cells into pro- or anti-inflammatory CD4 T cell subsets: (1) presentation of peptide antigens on major histocompatibility complex class II (MHCII), (2) expression of T cell costimulatory molecules (CD40, CD80, and CD86), and (3) production of cytokines. Because RBP4 is elevated in many insulin-resistant states (Balagop et al., 2007; Quadro et al., 2002; Yang et al., 2005) and therefore closely represent the serum elevation of RBP4 in insulin-resistant ob/ob mice, diet-induced obese mice (Mody et al., 2005; van Hoek et al., 2008), and because RBP4 induces inflammatory responses in vitro (Norseen et al., 2012), we proposed that RBP4 triggers AT inflammation in vivo. We observed that elevated RBP4 induces APC activation through the JNK pathway, which results in proinflammatory CD4 T cell proliferation and Th1 polarization. This is sufficient to promote systemic insulin resistance.

RESULTS

RBP4 Overexpression Results in AT Inflammation and Systemic Insulin Resistance

Serum human RBP4 (hRNP4) levels in RBP4-Ox mice were 2- to 3-fold higher than endogenous mouse RBP4 (data not shown) (Episkopou et al., 1993; Quadro et al., 2002; Yang et al., 2005) and therefore closely represent the serum elevation of RBP4 in insulin-resistant ob/ob mice, diet-induced obese mice (Mody et al., 2005; van Hoek et al., 2008), and insulin-resistant human subjects (Graham et al., 2006; Norseen et al., 2012; Yang et al., 2005) compared to their normal counterparts. RBP4-Ox mice fed on chow diet are glucose intolerant and insulin resistant (Figures 1A and 1B) with normal body weight, fat mass, serum triglycerides, free fatty acids, and adiponectin (Figures 1C–1F). Previously, we showed that RBP4 has a proinflammatory effect in vitro (Norseen et al., 2012). Because chronic inflammation is a major factor contributing to insulin resistance, we investigated the inflammatory status of the visceral AT of RBP4-Ox mice compared to WT mice. Visceral AT of RBP4-Ox shows more inflammation than WT AT (Figures 1G–1I). To characterize visceral AT inflammation, we selected CD45+ cells from AT (see Figure S1A available online) and analyzed the expression of surface markers for specific subtypes of immune cells. First, AT cells were divided in Ly6C-Ly6G-cells (monocytes) and Ly6C-Ly6G+ cells (monocyte myeloid cells) (Figure S1A). RBP4-Ox mice have higher numbers of AT monocytes (Figure S1B) which produce higher levels of proinflammatory cytokines including TNF and IL-6 (Figure S1B). Next, we evaluated the population of ATM+ by selecting F4/80+CD11b+ cells that were negative for the monocytic marker Ly6C. Back gate analysis of these CD11b+/F4/80+AT cells revealed that the majority of ATM+ were Ly6G-Ly6C (Figure S1C). Hence, we considered ATM+ to be the cells with the phenotype of CD45+Ly6C-Ly6G+CD11b+/F4/80+. RBP4-Ox mice displayed more ATM+ than WT littermates (Figure 1G). Because the population of Ly6G+ cells (enriched with ATM+) is also increased in RBP4-Ox, this results in a 70% increase in ATM+ compared with WT (Figure 1G). These ATM+ produced higher levels of TNF and IL-1β (Figure 1H), indicating that RBP4 overexpression results in increased AT inflammation. Since Ly6G is also a neutrophil marker, we evaluated the number of neutrophils present in these AT samples. Because neutrophils do not express MHCII, we selected cells that are negative for this marker. To exclude macrophages from this MHCII null population, we further selected for cells negative for the macrophage markers, F4/80 and CD68, and subsequently, cells negative for CD11c and CD206. After these gate exclusions, neutrophils were identified by Gr-1 and CD11b expression (Figure S1D). RBP4-Ox mice do not have increased numbers of neutrophils compared to WT littermates (Figure S1E). To further understand the inflammatory changes in RBP4-Ox mice, we evaluated ATM+ subpopulations. Proinflammatory macrophages have increased CD11c and reduced CD206 expression and anti-inflammatory macrophages have the opposite (Ferrante, 2013; Lumeng et al., 2007; Nguyen et al., 2011) (Figure 1I, top panel). RBP4-Ox mice displayed a 5-fold increase of CD11c+ and a 60% increase in CD206+ macrophages (Figure 1I, bottom panel). However, CD206+ macrophages were still more abundant in AT of both genotypes. Together, our data indicate that RBP4-Ox have increased numbers of macrophages of two distinct subtypes, CD11c+ and CD206+.

RBP4 Overexpression Enhances Antigen Presentation Capacity in Adipose Tissue Macrophages

Because RBP4-Ox ATM+ produce more CD4 T cell-activating cytokines (Figure 1H), we evaluated the population of these cells in RBP4-Ox AT and found increased numbers of CD4 T cells (Figure 2A).

Although obesity and insulin resistance are usually associated with reduction in Treg cells (Cipolletta et al., 2011), lean RBP4-Ox mice did not show any difference in AT Treg numbers and percentages (Figure 2B). However, there were more memory (CD49CD44+) and activated (CD49CD69+) T cells in AT of RBP4-Ox mice (Figure 2C). RBP4-Ox had increased percentage and number of CD49 T cells producing IFN-γ (Th1) (Figure 2D). No differences in the population of Th2 (CD49IL-4+) and Th17 (CD49IL-17+) cells were observed (Figures S2A and S2B). Next, we evaluated MHCII and costimulatory molecules (CD86 and CD40) required for ATM+ mediated CD4 T cell activation and found they were significantly increased in RBP4-Ox ATM+ (Figure 2E). This elevation was specific to CD206+ ATM+ (Figure 2G), as no elevation in MHCII, CD86, or CD40 was observed in CD11c+ ATM+ (Figure 2F). Thus, RBP4-Ox mice not only have a higher number of CD11c+ and CD206+ ATM+, but also more of the CD206+ ATM+ are likely to induce CD4 T cell activation compared with WT CD206+ ATM+. This is further demonstrated in Figure 4.

RBP4 Overexpression Results in Mild Liver Inflammation but Not Systemic Inflammation

To determine whether increased ATM+ and CD4 T cell numbers in RBP4-Ox mice are localized (specific to perigonadal/visceral AT) or systemic, we analyzed the spleen, mesenteric lymph node (data not shown), subcutaneous AT, and liver of RBP4-Ox mice compared to WT littermates. The increased number of AT CD49 T cells producing IFN-γ (Th1) was specific to visceral AT, as no increase in Th1 cells was observed in the spleen and lymph node of RBP4-Ox mice compared to WT littermates (Figures S2C and S2D). Moreover, no difference was observed in the numbers or percentages of total CD4 T cells, Treg cells (data not shown), monocytes, macrophages, or DCs (Figures S3A–S3E). Furthermore, no differences in the expression of MHCII or costimulatory molecules were found in splenic DCs or macrophages (Figures S3F and S3G). We next evaluated whether the
subcutaneous AT of RBP4-Ox mice displayed signs of inflammation. No increase in the inflammatory markers Cd11c, Tnf, and Il-6 or in Arg-T was observed in subcutaneous AT (Figure S4A). Moreover, no change in the total number of CD11c+ or CD206+ macrophages or CD4+ T cells was observed (Figures S4B–S4D). However, liver of RBP4-Ox mice displayed a modest increase in Tnf expression compared to control littermates (Figure 3A). Flow cytometry analysis of these liver samples showed elevated numbers of total liver macrophages (Figure 3B) consisting of three different phenotypes, CD11c+, CD206+, and CD11c+CD206+ (Figure 3C), and of macrophages expressing Tnf, but not Il-1β (Figure 3D). In contrast to ATMφ (Figures 2E–2G), liver macrophages from RBP4-Ox mice did not display increased levels of MHCII and CD40 compared to WT macrophages (Figure 3E). In agreement with these results, no difference in the total number of CD4 T cells or in the percentage or total number of Th1 cells was observed (Figure 3F). To understand whether the differences in liver and AT inflammation could be due to different RBP4 levels in these tissues, we measured transgenic human RBP4 in RBP4-Ox mice. Human RBP4 was 6-fold higher in perigonadal

Figure 1. Elevated Serum RBP4 Levels Cause Glucose Intolerance, Insulin Resistance, and AT Inflammation
(A) Glucose tolerance test (left panel) and insulin tolerance test (right panel) (n = 13–15/group).
(B) Serum insulin levels (n = 13–15/group).
(C and D) Body weight and (D) fat mass (n = 13–15/group).
(E) Serum triglyceride and free fatty acid (FFA) levels (n = 11–13/group).
(F) Serum adiponectin levels (n = 11–13/group).
(G) Flow cytometry representation of gated ATMφ (CD11b+F4/80+). ATMφ numbers in perigonadal fat (right panel) (n = 8/group).
(H) TNF and IL-1β intracellular staining in ATMφ (n = 5/group).
(I) Flow cytometry representation (top panel) and number (bottom panel) of CD11c+ and CD206+ ATMφ (n = 8/group). Studies were performed at 12–16 weeks of age. Values are means ± SEM, *p < 0.05. AUC, area under the curve. AAC, area above the curve. Pg, perigonadal.
AT compared to liver (Figure 3G). The endogenous mouse RBP4 levels in serum, liver, and AT were not altered by hRBP4 overexpression (Figure 3H). These data indicate that increased RBP4 levels promote the accumulation of both CD11c+ and CD206+ macrophages in perigonadal AT and liver, but not in peripheral lymphoid tissues or subcutaneous fat, and that RBP4-induced CD4 T cell responses take place preferentially in perigonadal AT, possibly due to increased RBP4 levels in this adipose tissue depot compared to liver.

RBP4 Overexpression Causes CD206+ Adipose Tissue Macrophages to Express Proinflammatory Molecules

Since our data indicate that RBP4 overexpression promotes CD11c+ and CD206+ macrophage accumulation (Figure 1I), we...
Figure 3. Elevated Serum RBP4 Levels Increase Liver Macrophages with No Effect on CD4 T Cell Activation

(A) Liver mRNA expression of Tnf, Il-6, Cd11c, and Arg-1 was determined by qPCR.
(B) Flow cytometry representation of liver macrophage (CD11b+F4/80+) gating and macrophage numbers in liver (right panel).
(C) Flow cytometry representation (left panel) and number (right panel) of CD11c+, CD11c+CD206+, and CD206+ liver macrophages.
(D) Flow cytometry representation of TNF and IL-1β intracellular staining in liver macrophages (left panel) and percentage and total number of TNF+ macrophages (right panel).
(E) MHCII and costimulatory molecule (CD40) expression in gated CD11c+, CD206+ or CD11c+CD206+ liver macrophages.
(F) Flow cytometry representation of gated AT CD4 T cells stained with IFN-γ and IL-17 (upper panel). Percentage and number of CD4+IFN-γ+ T cells (bottom panel).
(G) Western blot of transgenic human RBP4 (hRBP4) in AT and liver (left panel) and quantification of blot (right panel).
(H) Levels of endogenous mouse RBP4 (mRBP4) in serum, AT, and liver of RBP4-Ox and WT mice using an antiserum that does not crossreact with human RBP4. n = 4–6/group. Values are means ± SEM. *p < 0.05. Pg, perigonadal. MFI, median fluorescence intensity.
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RBP4 overexpression increased the expression of classically activated macrophage markers in CD206+ ADM to levels similar to levels similar (Tnf) and even higher (Il-1Î³ and Il-12) than in CD11c+ ADM (Figure 4A) without decreasing the expression of alternatively activated macrophage-related markers (Figure 4B). These data confirm that RBP4 overexpression upregulates proinflammatory cytokines in ADM that express alternatively activated macrophage markers.

Adipose Tissue CD206+ Macrophages of RBP4-Ox Are Potent Inducers of Th1 T Cells ADM are sufficient to induce CD4 T cell proliferation (Morris et al., 2013). Thus, we next measured the capacity of purified AT CD11b+ cells (enriched with ADM) to trigger CD4 T cell proliferation. CD11b+ cells from RBP4-Ox mice induced greater proliferation of CD4 T cells than WT CD11b+ cells (Figure S5A). To determine whether both CD11c+ and CD206+ ADM induce CD4 T cell proliferation, we FACS sorted ADM and cocultured them with syngeneic WT splenic CD4 T cells. CD11c+ and CD206+ macrophages from both WT and RBP4-Ox mice induced greater proliferation of CD4 T cells compared with total myeloid (CD11b+) cells (Figure S5B), CD11c+ and CD206+ ADM from RBP4-Ox mice induced greater proliferation of CD4 T cells compared to WT ADM (Figures 4C and 4D). Importantly, CD206+ ADM from RBP4-Ox mice induced greater CD4 T cell proliferation than CD11c+ ADM from WT and RBP4-Ox mice and CD206+ ADM from WT mice (Figures 4C and 4D).

To determine whether these ADM also affect CD4 T cell polarization, the intracellular cytokine content of cocultured CD4 T cells was measured. Although neither CD11c+ nor CD206+ ADM from RBP4-Ox and WT mice was able to induce Th17 polarization (Figure S5C), they induced Th1 T cell polarization (Figures 4E and 4F). No difference in the induction of Th1 cells was observed between CD4 T cells cocultured with CD11c+ ADM from WT compared to RBP4-Ox mice. In contrast, CD206+ ADM from RBP4-Ox mice induced higher percentage of Th1 cells compared to WT CD206+ ADM (Figures 4E and 4F) and higher proliferation of Th1 cells (Figure S5D). IFN-Î½ production, which is characteristic of Th1 cells, by CD4 T cells was higher when they were cocultured with CD206+ ADM from RBP4-Ox mice compared with CD206+ ADM from WT mice (Figure 4G). No polarization to Th2 or Th17 was observed in the coculture assay as indicated by lack of IL-4 or IL-17 secretion (data not shown). To further confirm Th1 polarization, we measured CD4 T cell lineage transcription factor (Tbx21) expression in cocultured cells. Tbx21 was upregulated in the coculture of CD206+ ADM from RBP4-Ox with CD4 T cells compared to CD206+ ADM from WT mice and compared to CD11c+ ADM from both genotypes (Figure 4H). This was reinforced by increased AT Tbx21 expression in RBP4-Ox compared to WT mice (Figure S5E). Th2 (Gata-3), Th17 (Rorc), and Treg (Foxp3) transcription factor expression was unchanged. These results indicate that CD206+ ADM from RBP4-Ox mice retain their alternatively activated macrophage phenotype but potentially activate and promote a proinflammatory Th1 profile.

RBP4 Directly Activates Antigen-Presenting Cells We wanted to determine whether treatment of APCs with RBP4 is sufficient to cause insulin resistance in vivo. Because (1) both macrophages and DCs are professional APCs, (2) the yield of bone marrow-derived dendritic cells (BMDCs) is much greater than bone marrow-derived macrophages (BMDMs), and (3) DC transfer systems are far better worked out than macrophage transfer systems (Quintana et al., 2010; Ruffner and Robbins, 2010; Sivaganesh et al., 2013), we activated BMDC with RBP4 for in vivo transfer experiments. RBP4 potently activated BMDC in a dose-dependent manner, as evidenced by upregulation of MHCII, costimulatory molecules (Figures 5A and S5F), and of proinflammatory cytokines (Figure 5B). The induction of IL-12 was confirmed by intracellular staining in CD11c+ MHCII+ BMDC (Figure 5C). Moreover, RBP4-activated BMDC induced CD4 T cell proliferation (Figures 5D and 5E). RBP4-activated BMDCs cocultured with CD4 T cells resulted in increased IFN-Î½ secretion (Figure 5F) and IFN-Î½ and TNF intracellular staining in CD4 T cells (Figure 5G), indicative of Th1 T cell polarization. There was no induction of other CD4 T cell subtypes indicated by reduced IL-4 and IL-17 intracellular staining (Figure 5G). These results were confirmed by increased expression of Tbx21 and not of Gata-3, Rorc, and Foxp3 (Figure 5H). Thus, RBP4 directly activates BMDCs and causes Th1 polarization. The RBP4 effects on CD4 T cells are mediated by activation of APCs, as RBP4 has no direct effect on splenic CD4 T cells in either WT or RBP4-Ox mice (Figure S5G). Proteomic and lipidomic mass spectrometry analysis of our RBP4 preparation confirmed its purity and showed no contaminating endotoxin (LPS), other lipopolysaccharides, lipoproteins, lipids, or additional proteins (Figures S6A and S6B; Norseen et al., 2012).
Figure 5. RBP4 Directly Activates Dendritic Cell, which Induce CD4 T Cell Proliferation and Polarization

DCs were generated from bone marrow (BMDCs) of 8-week-old male WT mice.

(A) DC activation was demonstrated by increased expression of CD40, CD80, CD86, and MHCII determined by flow cytometry.

(D) CD4 T cell proliferation was demonstrated by Cell Trace Violet staining.

(G) Flow cytometry for cytokine production (IL-4, IL-17, IFN-γ, TNF).

(H) mRNA expression levels of Tbx21 (Th1), Gata-3 (Th2), Rorc (Th17), and Foxp3 (Treg).

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Absence of potential lipopolysaccharide contamination is further demonstrated by the fact that boiling, which would denature the RBP4 protein, removed the effect of RBP4 to stimulate TNF secretion from BMDC, but not the effect of LPS (Figure S6C). If LPS was present, the proinflammatory effect of RBP4 would persist after boiling. In addition, recombinant RBP4 generated from mammalian cells (Figure S6D) and hRBP4 purified from human blood (data now shown), where the potential exposure to LPS is minimal, had the same inflammatory effect as bacterially derived RBP4. A summary of the data confirming the purity of our RBP4 preparation is shown in Table S1.

Transfer of RBP4-Activated APCs into WT Mice Leads to Adipose Tissue Inflammation and Insulin Resistance

To determine whether RBP4 activation of APCs is sufficient to cause AT inflammation and insulin resistance, we performed APC transfer experiments. Control (not activated) immature dendritic cells (iDCs) and RBP4-activated mature dendritic cells (mDCs) were transferred into lean WT mice. Transfer of mDC but not iDC or PBS alone resulted in insulin resistance (Figures 6A and 6B). Transfer of RBP4-activated BMDC (mDC) also resulted in elevated fasting glucose levels (Figure 6C), glucose intolerance (Figures 6D and 6E), and increased insulin levels (Figure 6F) with no change in body weight or adiponectin levels (Figure 6G). Moreover, mDC transfer resulted in increased ATM- and ATM- producing proinflammatory cytokines (Figure 6H). A switch from a predominant CD206+ phenotype to a CD11c+ phenotype occurred when mice received mDCs (Figure 6I). Because RBP4 activates APCs and increases their capacity to activate CD4 T cells, we analyzed the AT CD4 T cell profile. Transfer of mDC resulted in increased numbers of total CD4 T cells and CD4 T cells producing IFN-γ and TNF (Figures 6J and 6K). Thus, BMDC activation by RBP4 is sufficient to cause AT inflammation and insulin resistance in normal mice.

RBP4-Induced Activation of Macrophages and Antigen Presentation to CD4 T Cells Are Partially Dependent on JNK Signaling

Previously we showed that the JNK pathway is important for RBP4-induced macrophage activation in vitro (Norseen et al., 2012). JNK1 and JNK2 in macrophages are required for induction of AT inflammation and insulin resistance in obesity (Han et al., 2013). Therefore, we analyzed the activation status of JNK in CD45+CD11b+ AT cells (myeloid cells enriched in macrophages) in both RBP4-Ox and WT mice. RBP4-Ox mice display increased phosphorylation of JNK in CD45+CD11b+ cells compared to WT mice (Figure 7A). In addition, RBP4 effects on macrophages and DCs are STRA6 independent, since these cells do not express STRA6 (Figure S7A). Furthermore, RBP4-induced cytokine/chemokine (TNF, IL-6, IL-12, IL-1β, IFN-γ, and MCP1), MHCII, and costimulatory molecule levels were significantly reduced in both JNK1/2 KO BMDM compared to WT BMDM and in WT BMDM in the presence of JNK inhibitor (Figures S7B and S7C).

To assess JNK involvement in AT CD11c+ and CD206+ macrophages, we FACS sorted CD11c+ and CD206+ macrophages from WT mice and stimulated them in vitro with RBP4 in the presence or absence of JNK inhibitor. RBP4 equally upregulated TNF, IL-6, IL-12, and IL-1β in both CD11c+ and CD206+ macrophages (Figure 7B), indicating activation of both populations of cells. Treatment with JNK inhibitor reduced the RBP4-induced upregulation of cytokines in both CD11c+ and CD206+ macrophages (Figure 7B), indicating that RBP4 activation of both populations of macrophages is partially JNK dependent. Next, we sorted CD11c+ and CD206+ macrophages from JNK1/2 KO and control WT mice and treated them in vitro with RBP4. RBP4 induced the secretion of TNF, IL-12, and IL-1β in both CD11c+ and CD206+ ATM- from WT mice to similar levels (Figure 7C). RBP4-induced IL-6 secretion was lower in CD206+ than in CD11c+ AT macrophages from WT mice. JNK1/2 KO DC11c+ and CD206+ ATM- displayed decreased secretion of these cytokines (Figure 7C), with a complete block in RBP4-induced IL-6 and IL-1β secretion.

Because JNK ablation inhibited RBP4-induced activation of AT macrophages, we next investigated the outcome of this inhibition on CD4 T cell proliferation and Th1 polarization. RBP4-activated CD11c+ and CD206+ ATM- induce CD4 polarization and Th1 polarization (Figures 7D and 7E). RBP4-activated CD11c+ and CD206+ ATM- from JNK1/2 KO mice displayed reduced capacity to induce CD4 T cell proliferation (Figures 7D and 7E) and Th1 polarization compared to WT (Figures 7D and 7E). Thus, RBP4 activation of both CD11c+ and CD206+ ATM- and induction of CD4 T cell activation by both CD11c+ and CD206+ ATM- depend on JNK signaling.

DISCUSSION

The mechanisms for RBP4-induced insulin resistance in vivo are unknown. Here we show that RBP4 directly activates AT APCs in a JNK-dependent manner, induces CD206+ macrophages to secrete proinflammatory cytokines, and triggers CD4 T cell polarization (Th1) and proliferation. Elevation of RBP4 results in greater inflammation in AT than in liver. In both tissues, the innate immune system (macrophages) is activated, but the adaptive immune system (CD4 T cells), which amplifies the inflammation, is activated only in visceral AT. APC activation by RBP4 is sufficient

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(B) Dose–response effect of RBP4 on secretion of TNF, IL-6, and IL-12 from BMDCs.
(C) Increased production of IL-12 by RBP4+ or LPS-activated BMDC was confirmed by intracellular staining of CD11c+ MHCII+ (BMDCs).
(D) RBP4- or LPS-activated BMDCs were cocultured with splenic syngeneic cell-trace-labeled CD4 T cells, and CD4 T cell proliferation was demonstrated by cell-trace dilution.
(E) Expansion index representing the degree of CD4 T cell proliferation.
(F) IFN-γ secretion on day 5 of coculture.
(G) RBP4-activated BMDCs induce IFN-γ and TNF production by CD4 T cells, visualized by intracellular staining using flow cytometry.
(H) mRNA expression in the coculture assay of CD4 T cell lineage transcription factors. Data represent three experiments performed in triplicate, each using pools of bone marrow cells from two to six mice. Values are means ± SEM. *p < 0.05 versus dialysate. †p < 0.05 versus LPS. MFI, median fluorescence intensity. Dia, dialysate control.

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**Figure A**: Glycemia (mg/dL) over Time (min). PBS, iDC, mDC (RBP4).

**Figure B**: % of baseline AUC (mg/dL) over Time (min). PBS, iDC, mDC (RBP4).

**Figure C**: Glycemia (mg/dL) over 0-120 min. PBS, iDC, mDC (RBP4).

**Figure D**: 0-120 Min Insulin (mIU/L). PBS, iDC, mDC (RBP4).

**Figure E**: Body weight. PBS, iDC, mDC (RBP4).

**Figure F**: Adiponectin (µg/mL). PBS, iDC, mDC (RBP4).

**Figure G**: CD11c^+ ATM^Φ CD206^+ ATM^Φ. PBS, iDC, mDC (RBP4).

**Figure H**: CD11b^+ F4/80^+. PBS, iDC, mDC (RBP4).

**Figure I**: ATM^Φ IL1^+^β^+. PBS, iDC, mDC (RBP4).

**Figure J**: ATM^Φ TNF^+. PBS, iDC, mDC (RBP4).

**Figure K**: CD4^+ T cells (µg/Pg AT). PBS, iDC, mDC (RBP4).

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to cause insulin resistance, since transfer of RBP4-activated APCs into normal mice induces AT inflammation and impairs glucose tolerance and insulin sensitivity.

In obesity and other insulin-resistant states, AT macrophage polarization spans a spectrum, and the optimal constellation of markers to define pro- and anti-inflammatory macrophages is still evolving. Prolinflammatory ATMΦ which express CD11c+ (used as a marker for classically activated, also called M1, macrophages) are generally increased leading to inflammation-induced insulin resistance (Lumeng et al., 2007). This results in a reduction in the relative number of CD206+ macrophages (alternatively activated or M2 macrophages) which are predominantly anti-inflammatory (Fujisaka et al., 2009; Han et al., 2013; Lumeng et al., 2007; Stein et al., 1992). In AT of RBP4-Ox mice, not only CD11c+ macrophages but also CD206+ macrophages are increased. In addition, in RBP4-Ox AT, CD206+ macrophages unexpectedly produce proinflammatory cytokines and express elevated amounts of costimulatory molecules required for CD4 T cell activation, which are typical of CD11c+ macrophages (Lumeng et al., 2007; Stein et al., 1992). Importantly, although these CD206+ macrophages have increased proinflammatory cytokine expression, they retain the expression of molecules that are usually associated with alternatively activated macrophages (Lumeng et al., 2007; Odegaard and Chawla, 2011). Because CD206+ macrophages are 20 times more abundant than CD11c+ macrophages in AT of RBP4-Ox mice, the changes in CD206+ macrophages may play a significant role in AT inflammation.

Accumulation of ATMΦ is only part of the immune response that causes insulin resistance. T cells are also recruited to AT during the development of obesity-related insulin resistance (Feuerer et al., 2009; Nishimura et al., 2009; Winer et al., 2009). We show in RBP4-Ox mice and with RBP4 treatment of immune cells in vitro that RBP4-activated CD11c+ and CD206+ ATMΦ induce CD4 T cell proliferation and Th1 polarization. Thus, RBP4 increases the capacity of ATMΦ to induce a CD4 T cell immune response. Moreover, transfer of RBP4-activated APCs into normal mice is sufficient to increase CD4 T cell numbers, Th1 polarization, and systemic insulin resistance. Th1 cells play a critical role in AT inflammation, as the knockdown of these cells (Tbet−/−) or their product (IFN-γ) reduces AT inflammation and insulin resistance during obesity (Stolarczyk et al., 2013; Strissel et al., 2010; Winer et al., 2009; Yang et al., 2010).

Although a reduction in anti-inflammatory Treg cells has been implicated in obesity-related insulin resistance (Feuerer et al., 2009), lean insulin-resistant RBP4-Ox mice do not display reduced Treg numbers, indicating that the role of Treg cells may differ in lean versus obese insulin-resistant states.

JNK expression in macrophages is required for both AT inflammation and obesity-induced insulin resistance (Han et al., 2013). We previously demonstrated that activation of macrophages in vitro by RBP4 was dependent on the JNK pathway (Norseen et al., 2012). Here we show that JNK signaling is required in vivo for RBP4-mediated proinflammatory cytokine secretion and for expression of MHCII and costimulatory molecules by ATMΦ. Moreover, we show that JNK signaling in RBP4-treated APCs is required for activation of CD4 T cells. In agreement with our results, the JNK pathway has been implicated not only in the accumulation and activation of AT macrophages (Han et al., 2013) but also in Th1 immune responses in obesity (Davis, 2000; Dong et al., 1998, 2000; Wang et al., 1994).

RBP4 undoubtedly is not the only AT-derived factor that is important for AT inflammation and T2D. For example, leptin and free fatty acids also induce proinflammatory cytokine production (Lord et al., 1998; Nguyen et al., 2007). However, our data identify RBP4 as a key endogenous protein that contributes to AT inflammation and insulin resistance by triggering an interplay of the innate and adaptive immune systems (Figure 7F). These findings conceptually advance our understanding of the integration of different inflammatory pathways in AT in insulin-resistant states and may have therapeutic implications for type 2 diabetes.

**EXPERIMENTAL PROCEDURES**

**Animal Studies and Measurement of Metabolic Parameters**

Male RBP4-overexpressing (RBP4-Ox) mice on a C57BL6 background were bred with female C57BL6/J mice (Jackson Laboratories) to generate RBP4-Ox mice and control littermates. The RBP4-Ox mice express human-RBP4 under the control of mouse muscle creatine kinase (MCK) promoter and were extensively characterized (Quadro et al., 2003). Male mice 14–19 weeks of age were used for all studies. Male macrophage-specific JNK1/2 knockout mice (8 weeks old) were provided by Dr. Roger J. Davis and were previously described (Han et al., 2013). Body composition was measured by NMR (Echo Medical Systems). Insulin (ITT) and glucose (GTT) tolerance tests were performed in awake mice after a 5 hr fast and a 12 hr fast, respectively. Blood glucose was determined using a One Touch Basic glucometer (LifeScan). Mouse studies were conducted in accordance with federal guidelines. The
Figure 7. RBP4-Induced Activation of Adipose Tissue Macrophages and Resulting CD4 T Cell Proliferation and Th1 Polarization Are JNK Dependent

(A) Histograms representing pJNK staining (pT183/pY185) (upper panel) and normalized pJNK levels (bottom panel) in CD45+CD11b+ AT cells from RBP4-Ox and WT mice.

(B) CD11c+ and CD206+ ATM were sorted from WT mice and stimulated with RBP4 (50 μg/mL for 24 hr) in the presence or absence of JNK inhibitor (5 μM). Levels of TNF, IL-6, IL-12, and IL-1β were measured by ELISA.

(C) CD11c+ and CD206+ ATM were sorted from macrophage-specific JNK1/2 knockout (JNK KO) and WT mice and treated with RBP4 (50 μg/mL for 24 hr). Levels of TNF, IL-6, IL-12, and IL-1β were measured by ELISA.

(D) CD11c+ and CD206+ ATM were sorted from JNK KO and WT mice and treated with RBP4 (50 μg/mL for 24 hr). Next, WT CD4 T cells plus anti-CD3 antibody were added, and CD4 T cell proliferation and IFN-γ+CD4+ T cells were measured. Flow cytometry representation of CD4 T cell proliferation (left panel) and IFN-γ and IL-17 intracellular staining in gated CD4 T cells (right panel).

(E) Expansion index representing the degree of CD4 T cell proliferation (left panel) and IFN-γ secretion in the coculture assay (right panel) (n = 4/group). Values are means ± SEM. *p < 0.05 versus dialysate control, **p < 0.05, versus WT macrophages treated with RBP4 and no JNK inhibitor (B) or versus WT macrophages treated with RBP4 (C and E). Pg, perigonadal.
RBP4 or LPS) were cocultured with cell-trace violet-labeled bead-or BMDC from WT C57B6/J (not activated, dialysate or activated with Biosystems).

Gene Expression Analysis
RNA from CD11c+ and CD206+ cells from the perigonadal AT of RBP4-Ox or WT mice were purified by magnetic beads as described by the manufacturer (Milenyi). Liver immune cells and AT stromal vascular fractions (SVFs) were isolated as previously described (Nguyen et al., 2007; Pien and Biron, 2000). AT SVFs were stained with fluorescence-labeled antibodies for CD45, CD11b, Ly6C, F4/80, CD11c, and CD206 (Biolegend) and sorted at high speed (BD FACSAria II, Beth Israel Deaconess Medical Center Flow Cytometry Core). After sorting, cells purities were higher than 98%.

Flow Cytometry of Surface Markers, Intracellular Cytokine, and Foxp3 Transcription Factor
The AT SFV cells or BMDCs were resuspended in PBS supplemented with 2% FCS, and surface markers were stained with monoclonal antibody for multicolor flow cytometry. For intracellular cytokine staining, cells were stained as previously described (Moraes-Vieira et al., 2013a). To determine the frequency of Tregs (CD4+CD25+Foxp3+) in vivo, cells were stained intraacellular for Foxp3 using a PE anti-mouse/rat Foxp3 antibody kit (eBioscience). The cells were acquired on a specially ordered five-laser 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).

Statistical Analyses
We thank Dr. Terry Strom for stimulating discussions; Dr. Diane Mathis and Dr. Aldo Rossaini for helpful comments on the manuscript; and Dr. Odile D. Peroni, Kenny Wellenstein, Abha Dhaneshwar, and BIDMC Flow Cytometry Core Facility for technical assistance. We thank Dr. Roger J. Davis and Dr. Myoung S. Han for the macrophage-specific JNK1/2 knockout mice. We thank the Small Molecule Mass Spectrometry Facility at Harvard University, Dr. Sunia Trauger, and Dr. Alan Saghatelian for the mass spectrometry analysis of RBP4. This work was supported by National Institutes of Health grants R37 DK 43051 and P30 DK57521 (to B.B.K.), 5T32DK07516-29 (to B.B.K. and M.M.Y.), Harvard Training Program in Nutrition and Metabolism, 2T32HD052961-06A1 (to M.M.Y.), and a grant from the JPB foundation (to B.B.K.); B.B.K. is an inventor on a patent related to RBP4.

ACKNOWLEDGMENTS
Received: August 30, 2013
Revised: December 5, 2013
Accepted: January 22, 2014
Published: March 4, 2014

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