Adipocyte Inflammation Is Essential for Healthy Adipose Tissue Expansion and Remodeling

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SUMMARY

Chronic inflammation constitutes an important link between obesity and its pathophysiological sequelae. In contrast to the belief that inflammatory signals exert a fundamentally negative impact on metabolism, we show that proinflammatory signaling in the adipocyte is in fact required for proper adipose tissue remodeling and expansion. Three mouse models with an adipose tissue-specific reduction in proinflammatory potential were generated that display a reduced capacity for adipogenesis in vivo, while the differentiation potential is unaltered in vitro. Upon high-fat-diet exposure, the expansion of visceral adipose tissue is prominently affected. This is associated with decreased intestinal barrier function, increased hepatic steatosis, and metabolic dysfunction. An impaired local proinflammatory response in the adipocyte leads to increased ectopic lipid accumulation, glucose intolerance, and systemic inflammation. Adipose tissue inflammation is therefore an adaptive response that enables safe storage of excess nutrients and contributes to a visceral depot barrier that effectively filters gut-derived endotoxin.

INTRODUCTION

Adipose tissue expansion in response to excess caloric intake is an important systemic response to avoid the lipotoxic side effects exerted by excess lipid and fatty acid (FA) deposition in cells other than adipocytes. The basic mechanisms, leading to a gradual and “healthy” expansion of fat pads, are starting to be elucidated. Healthy expansion is associated with appropriate angiogenesis and vascular and extracellular matrix (ECM) remodeling.

Increased adiposity is more often than not associated with an increased risk for a number of chronic diseases, including diabetes, cardiovascular disease, and some types of cancers (Park et al., 2011). The underlying mechanisms for the link between obesity and these diseases are not fully understood but are likely to involve a state of chronic systemic low-grade inflammation.

The causality between local adipose tissue inflammation, systemic inflammation, and metabolic dysfunction has, however, not been studied. Therefore, we developed three distinct but complementary mouse models to investigate the role of adipose tissue inflammation in high-fat-diet (HFD)-induced metabolic disturbances. By design, two models express the anti-inflammatory factors in adipose tissue constitutively, while one model is inducible.

Analysis of these three mouse models reveals that the inability to mount an appropriate local proinflammatory response at the level of the adipocyte reduces adipose tissue expansion under normal physiological as well as under HFD-fed conditions. This inability to expand adipose tissue is associated with ectopic lipid deposition and a deteriorated metabolic profile. Furthermore, we demonstrate that mesenteric adipose tissue (MWAT, a visceral fat depot) plays an important role for proper intestinal barrier function. An ineffective response of MWAT to proinflammatory stimuli with respect to its expansion is associated with a “leaky gut,” colitis, and metabolic dysfunction. Thus, these mouse models demonstrate that a local inflammatory response derived from the adipocyte is an adaptive response and an important preemptive factor for ensuing obesity-associated systemic inflammation.

RESULTS

We have recently developed a mouse model (the “adipochaser mouse”) in which we can permanently activate β-galactosidase expression in all preexisting adipocytes by a short bout of doxycycline treatment. Removal of doxycycline enables the detection of newly differentiated adipocytes that are negative for the blue X-Gal-LacZ staining (Wang et al., 2013). Repeated local LPS injections into adipose tissue stimulate adipogenesis without affecting overall weight gain (Sadler et al., 2005). Exploiting our adipochaser mouse, we are able to confirm the findings by Sadler and colleagues. New adipocyte formation was evident in the LPS-injected inguinal WAT (IWAT) depot, but not in the control depot (Figure 1A and data not shown). These observations suggest that the induction of acute inflammation in the
Figure 1. Reduced Fat Mass and Glucose Tolerance in dnTNF tg Mice

(A) X-Gal-LacZ stained LPS-injected Adipochaser IWAT (blue, preexisting adipocytes; white, new adipocytes).

(B and C) Leptin and body weight change after i.p. injection with 0.3 mg/kg LPS in dnTNF tg and wild-type female mice.

(D) IWAT and GWAT weight in relation to body weight in chow (top) and HFD-fed (bottom) male dnTNF tg and wild-type controls.

(E–H) Body weight, glucose tolerance test, serum adiponectin, and SAA levels in male dnTNF tg and littermate controls after 11 weeks of HFD feeding.

(I) Representative Trichrome stain of IWAT in male dnTNF tg and littermate controls after 11 weeks of HFD feeding.
context of intact adipose tissue stimulates adipogenesis in vivo. We therefore hypothesized that acute inflammation within adipose tissue may play an essential role for adipose tissue expansion, remodeling, and overall homeostasis by stimulating ECM degradation and angiogenesis. To study the role of inflammation in adipose tissue, we developed a mouse model expressing a dominant-negative version of the potent proinflammatory cytokine TNF-α (dnTNF) (Steed et al., 2003) under the control of the ap2 promoter (“dnTNF tg”). This is a version of TNF-α that effectively heterotrimersize with wild-type TNF-α subunits, but the presence of a mutant subunit engages the TNF receptors nonproductively. ap2 promoter-driven constructs are predominately transcribed in adipocytes, but expression has been reported in macrophages and other tissues, such as the heart and the brain, though at much lower levels. Our dnTNF tg mice express the transgene specifically in adipose tissue, but also to a minor extent in isolated peritoneal macrophages (see Figure S1A available online). The low level of dnTNF expression in macrophages does not affect the inflammatory state of thioglycollate-activated peritoneal macrophages, since a number of M1/2 markers (e.g., TNF-α, IL-1β, TGF-β, Arg1, and CD11c) remained unaltered compared to macrophages isolated from littermate controls (Figure S1B).

As expected, unchallenged chow-fed dnTNF tg do not display significant differences with respect to genes involved in inflammation or macrophage polarization, e.g., TNF-α, F4/80, CD11c, NOS2, CD206, and CD301 in adipose tissue (data not shown). However, the dnTNF tg mice display a reduced NFkB activation in adipose tissue (as judged by reduced IκB phosphorylation) in response to an intraperitoneal injection with TNF-α (Figure S1C). Furthermore, LPS induces a highly specific response on adipocytes in adipose tissue, leading to an acute increase in serum leptin levels. In the presence of dnTNF, this LPS-mediated increase in serum leptin levels is significantly blunted (Figure 1B). This indicates that an autocrine TNF-α loop is part of the LPS-mediated effects on leptin release. Despite the increase in serum SAA and α1-acid-glycoprotein (Figure S1D and data not shown), the body weight recovery is slightly faster in the dnTNF tg mice (Figure 1C). Combined, these results confirm that the dnTNF transgene is indeed functional, and that it exerts its effects primarily at the level of adipose tissue, while leaving the inflammatory response in other tissues, such as the liver, fully intact.

Reduced Body Weight, Fat Mass and Glucose Tolerance in HFD-Fed dnTNF tg Mice

In young chow-fed mice, the body weights trend to be lower in the dnTNF mice. The HFD weight is reduced, while the gonadal WAT (GWAT) weight is similar between genotypes (Figure 1D). This pattern changes upon HFD feeding. The degree of HFD-induced obesity is reduced in the dnTNF mice (Figure 1E). This is particularly apparent in GWAT, which is much more prominently affected than the IWAT depot in HFD-fed mice (Figure 1F). In absolute terms, both GWAT and IWAT are of reduced size in HFD-fed dnTNF tg mice compared to littermates. The reduced IWAT weight is, however, proportional to the lower body weight in the dnTNF mice. Brown adipose tissue (BAT) and MWAT are, however, of similar sizes in dnTNF and littermate controls, regardless of diet (data not shown). Similar results were seen when the dnTNF transgene was bred into the genetically obese ob/ob background, indicating that there are leptin-independent mechanisms responsible for the reduced body and GWAT mass weight in dnTNF mice (Figure S1E).

We went on to test whether the reduced body weight translates into the expected improvements in metabolic parameters. To our surprise, HFD-fed dnTNF tg mice are severely glucose intolerant and have lower adiponectin levels, despite lower levels of circulating SAA compared to littermate controls (Figures 1F–1H).

These observations suggest that adipose tissue TNF-α signaling is relevant for adipose tissue remodeling and expansion. Consistent with this hypothesis, we found increased amounts of ECM deposits in IWAT of HFD-fed dnTNF mice compared to littermate controls (Figure 1I). This result is consistent with a need for TNF-α for successful ECM remodeling in the context of wound healing (Heo et al., 2011; Saika et al., 2006). The HFD-induced adipocyte hypertrophy in IWAT is similar between genotypes (relative adipocyte sizes 1.00 ± 0.3 and 0.94 ± 0.1 for, respectively, wild-type and dnTNF tg IWAT; p = 0.84), despite about 60% reduced IWAT depot weight in the dnTNF tg mice (Figure 1D), suggesting that inhibition of TNF-α-signaling either causes a reduced capacity for adipogenesis in vivo or may simply be secondary to the increased fibrosis. Disruption of this fibrotic state, such as in the context of HFD-exposed mice lacking collagen VI (khan et al., 2009), reduces adipocyte apoptosis. In contrast, enhanced fibrosis increases the rate of adipocyte death (Halberg et al., 2009). In line with fibrosis-induced adipocyte death, prolonged HFD-feeding (22 weeks) leads to an increased presence of crown-like structures (CLSs) in the GWAT of dnTNF tg mice. This is apparent when examining infiltrating macrophages (as judged by Mac2 staining) surrounding dead adipocytes (as judged by perilipin-negative staining) (Figure 1J). We did not, however, detect a difference in adipocyte death rates in IWAT, and no difference was observed in GWAT at earlier stages of HFD feeding (data not shown). Thus, a reduction in adipogenesis is more likely to explain the enlarged adipocytes in IWAT and the reduced GWAT size in HFD-fed dnTNF tg mice.

Essential Role for Acute Inflammation for Adipose Tissue Functionality

Given the many studies showing the negative impact of TNF-α on insulin sensitivity as well as on adipocyte differentiation (Engelman et al., 2000; Gustafson and Smith, 2006; Hotamisligil et al., 1993), the metabolic and adipose tissue dysfunction seen in the dnTNF tg mice is rather surprising at first sight. However, our observations do not contradict a model in which chronic inflammation is an important contributor toward the metabolic syndrome. Rather, though, “immunologic fitness” as we have previously defined it (Asterholm et al., 2012), seems to be an
Figure 2. Reduced Fat Mass and Reduced Glucose Tolerance in RID tg Mice

(A) LPS (0.3 mg/kg)-induced body weight change in male RID tg and wild-type controls.
(B) Gene expression in GWAT harvested from male RID tg and wild-type controls 6 hr after LPS injection.

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important component for tissue homeostasis in general and for adipose tissue in particular. To further explore this concept of “physiological adipose tissue inflammation,” we wanted to test an additional model to strengthen the general validity of our initial findings in these dnTNF tg mice. To do so, we generated a more potent adipose-specific anti-inflammatory model. This second mouse model takes advantage of RIDΔ/β (RID), an adenosiv protein complex that suppresses the local host immune response by potently inhibiting a number of proinflammatory signaling pathways (e.g., TLR4-, TNF-α - and IL-1β-mediated signaling) (Lichtenstein et al., 2004; Delgado-Lopez and Horwitz, 2006). Similar to the dnTNF tg mice, we put the expression of RID under the control of the αP2 promoter (RID tg). Just as for the dnTNF tg mouse model, we detected a high transgene expression in adipocytes with low level expression also seen in macrophages, but not in other tissues (Figure S2A). Upon isolating fresh peritoneal thioglycollate-stimulated macrophages from wild-type and RID mice, we did not detect any difference in macrophage function, suggesting that the trace levels of expression do not amount to an effective suppression of inflammation in macrophages (Figure S2B). Consistent with a potent action within the adipocyte, the RID tg mice display a significantly blunted response to LPS in adipose tissue, associated with a reduced response to LPS-induced body weight loss as well (Figures 2A and 2B). Moreover, and also similar to the dnTNF tg mice, fat pad weights of the RID tg mice are reduced. The more effective anti-inflammatory effects in the RID tg lead to a significant reduction in both IWAT and GWAT depot sizes, even in young chow-fed mice (Figure 2C). HFD feeding causes an even more dramatic difference with respect to the visceral GWAT and MWAT depots, while the subcutaneous IWAT depot no longer differs in size from the wild-type controls under these conditions (Figure 2D). BAT weights are similar between genotypes, with or without HFD challenge (data not shown). Despite the reduced overall amounts of white adipose tissue, the RID tg mice do not display altered overall body weight (Figure 2C).

Body composition, as assessed by NMR, confirmed a slightly reduced fat mass in both the dnTNF and the RID tg mice compared to wild-type controls, and as little as 2 days of HFD feeding enhances this difference between genotypes (Figures S2D and S2E). Lean body mass was however unaltered in both mouse models (Figures S2D and S2E). We also investigated whether these transgenic mice display an altered energy balance, but neither food intake nor oxygen consumption differed from their respective controls (data not shown). Thus, the reduced body weight in the dnTNF must be caused by an alteration in energy balance that is too small to be detected with available methods. In contrast, the respiratory quotient (RQ = VO2/ VCO2) was significantly altered. HFD-fed dnTNF tg mice display a lower RQ in the dark phase, and the RID tg mice display a lower dark phase-RQ on both chow and HFD. This reduction in RQ in both dnTNF and RID tg mice compared to their controls indicates a heavier reliance on FA oxidation for their energy need (Figures S2F and S2G). Typically, healthy mice burn carbohydrates during the dark phase. At this time of day, they have the highest food intake and hence the highest insulin levels. Thus, a lower RQ during the dark phase, reflective of reduced carbohydrate use, is an indication of systemic insulin resistance and metabolic inflexibility in both transgenic strains.

The inhibition of adipogenesis (as gauged by the estimated number of adipocytes) is even more pronounced in the RID tg mice (Figure 2E). Chow-fed RID tg mice display 17% ± 2% larger adipocytes in IWAT (p < 0.05) and a trend toward larger adipocytes in GWAT (27% ± 11% larger, p = 0.14) despite the fact that these depots are 25% (IWAT) and 30% (GWAT) smaller than in wild-type controls (Figure 2C). The circulating SAA levels are, to our surprise, higher in chow-fed RID tg mice, but this difference disappears on HFD; i.e., the HFD-induced increase in SAA is more pronounced in wild-type mice (Figure S2H). Similar to the dnTNF tg mice, the RID tg adipose phenotype is associated with both reduced adiponectin levels and glucose intolerance (Figures 2F and 2G). In fact, RID tg mice have a substantial degree of glucose intolerance with mild hyperinsulinemia already under unchallenged, chow-fed conditions at a young age. It could very well be that the lower adiponectin levels contribute to this impaired insulin sensitivity. HFD feeding aggravates the metabolic phenotype further, and the RID tg mice continue to display reduced glucose tolerance despite severe hyperinsulinemia relative to the wild-type controls (Figures 2H and 2I). Upon closer analysis of the adipose tissue, we found elevated levels of collagen in HFD-fed RID tg IWAT. Interestingly, in these experiments, we noticed that HFD induces a rapid and dramatic reduction of septa in IWAT. These septa are easily detectable with a Picrosirius red stain, while somewhat less apparent with Trichrome stain, and correspond to collagen streaks that compartmentalize adipose tissue “units” in young wild-type mice (Figures 3A and 3A). There is no description in the literature of either the developmental origin or the relevance of these functional “miniunits” in adipose tissue. The reduction in septa can be quantified by total collagen measurements. HFD-fed wild-type, but not RID tg, mice have a reduced total amount of collagen per milligram adipose tissue compared to the levels in chow-fed controls (Figure 3B). Thus, adipose tissue fibrosis in obesity may, at least in some cases, be the consequence of reduced ECM degradation, rather than an increase in ECM production. We should also note that total collagen content measurements of adipose tissue do not necessarily reflect states of pathological fibrosis, since healthy chow-fed mice have higher

(C) IWAT and GWAT in relation to body weight in chow-fed male RID tg and wild-type mice.
(D) IWAT, GWAT, and MWAT in relation to body weight in 15-week HFD-fed male RID tg and wild-type mice.
(E) Representative H&E stain of IWAT and GWAT in male RID tg and wild-type mice on chow.
(F) Adiponectin levels in male RID tg and wild-type mice on chow and after 12 weeks of HFD.
(G and H) Glucose tolerance in (G) chow-fed and (H) 12-week HFD-fed male RID tg and wild-type mice.
(I) Serum-insulin levels in 3 hr fasted male RID tg and wild-type mice. One-way ANOVA analysis shows that both diet (F = 8.1, p = 0.013) and genotype (F = 12.3, p = 0.004) contribute significantly to collagen levels. Error bars represent SEM; p < 0.05 according to Student’s t test was considered significant and is indicated by “∗,” “∗∗,” “∗∗∗,” /p < 0.01; “∗∗∗∗,” /p < 0.001 (difference in between genotype, /difference from initial weight). p values in red indicate difference between groups during time courses according to repeated-measurement ANOVA.
Figure 3. Increased HFD-Induced Steatosis and Delayed Adipose Tissue Development in dnTNF and RID tg Mice
(A) Representative Picrosirius red stain of IWAT from 8-week-old C57B6 males on chow and after 9 days with HFD.
(B) Representative Trichrome stain and collagen levels in IWAT of HFD-fed male RID tg and wild-type mice.
(C) Representative H&E stain of liver sections from 11-week HFD-fed male dnTNF tg and littermate control.
(D) Liver fat quantified by CT and liver weight in 11-week HFD-fed male RID tg and wild-type controls.
(E and F) Representative H&E stain of IWAT and GWAT sections and endomucin immune stain of IWAT (bottom panels) and (with quantification shown in F) from 10-day-old male RID tg and wild-type pups.
(G) Body weight, IWAT weight, and adipocyte sizes in 10-day-old dnTNF tg and littermate controls. Error bars represent SEM; p < 0.05 according to Student’s t test was considered significant and is indicated by *; **p < 0.01; ***p < 0.001 (*significantly different from WT, **significantly different from untreated controls of same genotype).
levels of collagen in IWAT than metabolically challenged obese mice. Instead, these data suggest that, beyond biochemical measurements, a histological examination also has to be performed to accurately assess whether a pathological condition prevails (e.g., the collagen deposits show up as irregular streaks or in association with CLSs) or whether the collagen deposits are in the form of well-organized septa, as seen in the lean animals.

**Reduced HFD-Induced Adipose Tissue Expansion in dnTNF and RID tg Mice Is Associated with Hepatic Steatosis**

These initial observations support the hypothesis that the ability to mount a proinflammatory response is intimately coupled to adipose tissue expansion, proper remodeling, and the resolution of inflammation. We therefore wanted to determine whether the reduced storage capacity in adipose tissue leads to increased ectopic lipid deposition. Indeed, both the dnTNF and the RID tg mice display an increased degree of HFD-induced hepatic steatosis (Figures 3C and 3D). Given the blunted HFD-induced weight gain in the dnTNF tg mice and the higher anti-inflammatory potency of the RID transgene, it is not surprising that the effects on the liver are more severe in the RID tg mice. In addition to the increased amounts of liver fat, RID tg mice also display a high level of hepatomegaly during HFD-fed conditions (Figure 3D). Thus, the increased ectopic lipid deposition in dnTNF and RID tg mice can, at least in part, explain the more severe degree of HFD-induced metabolic dysfunction compared to controls in these mouse models.

**Adipocyte Inflammation Is Important for Early Postnatal Adipogenesis**

The observations so far argue that the reduced adipose tissue mass in the dnTNF and the RID tg mice is the consequence of a decreased adipogenesis rate, partially offset by a more pronounced hypertrophy of existing adipocytes. Moreover, the reduced overall fat mass cannot solely be the consequence of an altered energy balance, since only the dnTNF, but not the RID transgenic, mice display lower body weights; and adipose depot sizes in both the dnTNF and the RID tg mice are disproportionately reduced beyond what is expected from the body weight differentials to wild-type mice. We were therefore prompted to further explore the phenomenon of reduced adipose expansion and remodeling in these anti-inflammatory models. First, we explored whether adipogenesis is already negatively affected during postnatal development. Immediately at birth, wild-type mice rapidly expand their IWAT depots. IWAT pads grow substantially within 1–2 weeks, while the visceral adipose depots remain almost undetectable at this young age. We measured the differences in adipose tissue size in 10-day-old pups. Indeed, the RID tg pups display fewer, but larger, adipocytes in developing IWAT, while body weights are unaltered compared to wild-type controls (Figures 3E and 3F and data not shown). This difference is associated with a decrease in vascular density as judged by IHC stains of endomucin of IWAT (Figures 3E and 3F). The dnTNF mice display a similar, albeit less dramatic, phenotype. The 10-day-old dnTNF tg pups have a slightly reduced total body weight, though the IWAT weight is reduced both in relation to body weight and in absolute terms compared to littermate controls (Figure 3G). The dnTNF adipocytes in GWAT are larger, while the adipocytes in IWAT are of similar size compared to wild-type adipocytes on postnatal day 10 (Figure 3G). Even the dnTNF tg mice have therefore a reduced number of adipocytes, since the adipose depots are overall smaller. There is no evidence of dead adipocytes in either GWAT or IWAT in any of the genotypes (Figure S3B for RID tg; data not shown for dnTNF tg).

A concern in this context is that both RID tg and dnTNF could lead to inhibition of adipogenesis due to cytotoxic effects or due to unspecific interference with the differentiation program. To investigate this possibility, we isolated stromal vascular cells from IWAT of wild-type, dnTNF, and RID tg mice; propagated them in culture; and then subjected the cells to an in vitro differentiation protocol and assessed the degree of differentiation by several different criteria (Figures S3C–S3E). All three cell preparations differentiated to the same extent as judged by appearance of lipid droplets in bright field microscopy (Figure S3C), oil red O staining (Figure S3D), and immunoblotting for the adipocyte marker adiponectin (Figure S3E). Thus, the reduced adipogenic potential in the transgenic mice is truly a function of the reduced ability of these cells to respond to external proinflammatory stimuli in the context of an intact adipose tissue depot, rather than a cell-autonomous differentiation defect due to transgene expression.

The first few postnatal days are associated with an increased exposure to many new antigens due to microbial colonization of the gut, concomitant with a sudden intake of large quantities of milk. A large intake of lipids has been shown to acutely cause inflammatory responses in various tissues, including adipose tissue (Asterholm et al., 2012; Magne et al., 2010). We hypothesize that the likely increase in the systemic levels of bacterial toxins further elevates the lipid-induced proinflammatory response, and facilitates angiogenesis that in turn is permissive for adipogenesis (Figure S4A). Hence, we suggest that gut bacteria play a major role in proper storage of excess nutrients during this early postnatal stage. To test this hypothesis, we exposed wild-type and RID transgenic mice for 5 weeks prior to mating to a Chow diet supplemented with antibiotics that effectively deplete the commensal microflora (Rakoff-Nahoum et al., 2004). The antibiotics were removed from the food between E10 and E16 and were reintroduced again at day E17 to avoid potentially harmful effects of these drugs on fetal development. There was no effect on the offspring’s body weight, neither by antibiotics nor by genotype, and all pups survived and appeared healthy regardless of treatment group. We found that antibiotic-treated wild-type offspring display reduced amounts of adipose tissue at 10 days of age compared to the untreated control mice. The RID tg pups display a reduced amount of adipose tissue relative to wild-type mice, regardless of whether they were on antibiotics or not (Figure 4A). Thus, the presence or absence of bacterially derived toxins in the RID tg pups has no effect on adipose tissue growth, supporting the notion that a local inflammatory response is an important component of normal adipose tissue expansion. Furthermore, the male breeders used in this study were sacrificed after 5 weeks of treatment, and their MWAT was collected for histological analysis. We found that antibiotic-treated wild-type mice have a reduced capillary density in their MWAT compared to controls (Figure 4B). This observation provides additional support for the idea
that bacterially induced inflammation leads to increased angiogenesis, at least locally in MWAT (Figure S4B). RID tg mice had a reduced capillary density, even in the absence of antibiotic treatment (Figure 4B). Furthermore, the average MWAT adipocyte size is 20% ± 5% (p < 0.05) larger, while the MWAT depot weight is 73% ± 7% reduced, indicating a dramatic reduction in the number of MWAT adipocytes in RID tg mice compared to wild-type controls. Antibiotic treatment did not have an impact on adipocyte size, but tended to reduce the capillary density even further in the RID tg MWAT, suggesting that additional cells...
Beyond adipocytes play a role in the response to microbial toxins in adult MWAT (Figure 4B).

**Reduced Adipogenesis and HFD-Induced Glucose Intolerance in an Inducible Adipocyte-Specific Anti-Inflammatory Model**

Since the effects seen in adipose tissue expansion and metabolic health were quite striking in the RID tg and dnTNF mice, we wanted to test whether we could see a similar phenomenon in an adipocyte-specific inducible model. To that end, we generated a mouse that expresses a mutated human IkB-BZ(S32G-S36A) version under the control of a tet-responsive element (TRE-IxB tg). IkB-BZ(S32G-S36A) is an effective inhibitor of the NFκB pathway. This TRE-IxB tg model was crossed with transgenic mice expressing the “tet-on” transcription factor rtTA under the control of the highly adipocyte-specific adiponectin promoter (Ad-rtTA tg) (Wang et al., 2010). Upon exposing Ad-rtTA-TRE-IxB tg mice to doxycycline, IkB-BZ(S32G-S36A) mRNA gets selectively induced in adipocytes, whereas no expression is observed in other tissues, such as the liver (Figure S4C). When we analyzed the impact of the expression of this anti-inflammatory protein during late gestation and the first 10 days of the postnatal period by exposing both wild-type and transgenic dams to doxycycline, the pups expressing IkB-BZ(S32G-S36A) displayed a reduced IWAT weight, while no effect on body weight was observed (Figure 4C). The adipocyte sizes were, however, similar between genotypes (data not shown), arguing for lower total number of inguinal adipocytes in 10-day-old Ad-rtTA-TRE-IxB tg pups. Similar to the other models, there was no evidence of any dead adipocytes, as judged by perilipin stain (data not shown).

We investigated the metabolic phenotype of Ad-rtTA-TRE-IxB tg mice. We found no difference between genotypes in either body weight or glucose tolerance on doxycycline-supplemented chow (data not shown). Challenging the mice with HFD for 8 weeks, however, revealed a significant difference between genotypes. Ad-rtTA-TRE-IxB tg mice were more glucose intolerant than littermate controls, despite comparable body weights (Figure 4D). Similar to the dnTNF and the RID tg mice, the GWAT weight was reduced, while the liver weights were increased in the HFD-fed Ad-rtTA-TRE-IxB tg mice (Figure 4E). In contrast, there was no difference in IWAT, MWAT, and BAT weights (Figure 4E; data not shown). To assess whether the Ad-rtTA-TRE-IxB tg mice are more susceptible to develop HFD-induced hepatic steatosis, livers were harvested from a second cohort of mice after 1, 6, and 10 weeks on doxycycline-supplemented HFD. We found a strong trend toward increased HFD-induced hepatic steatosis in relation to body weight (Figure S4D). These findings confirm, in a third independent system, that the inability to mount a proinflammatory response in adipose tissue impairs adipose tissue expansion associated with metabolic dysfunction.

**Impaired β3-Adrenergic Receptor Agonist-Induced Adipose Tissue Remodeling in RID tg Mice**

The adipose tissue response to chronic β3-adrenergic receptor (AR) agonist involves a transient acute bout of inflammation and, over time, triggers “browning” of the IWAT as defined by an increased number of multilocular cells expressing uncoupling protein (UCP)-1 (Granneman et al., 2005; Mottillo et al., 2010). There is also evidence for increased adipogenesis in GWAT in response to chronic β3-AR-agonist treatment (Lee et al., 2012; Wang et al., 2013). We aimed to investigate whether adipose tissue “beiging” depends on a proinflammatory response. We examined one of our models, the RID tg mice and exposed them and their wild-type controls to daily β3-AR-agonist and bromoalcohol (BrDu) injections for 10 days. Rather dramatic differences were apparent with respect to the response to this chronic β3-AR-agonist treatment. Wild-type adipose tissues displayed a much “browner” color than the RID tg adipose tissue (Figure 5A), and IWAT contained a large number of multilocular adipocytes, while RID IWAT only displays a modest response in this respect (Figure 5B). In line with these observations, the resulting UCP-1 mRNA levels are also reduced in both IWAT and GWAT in the RID tg mice (Figure S5A). Furthermore, very few BrdU-positive adipocytes were found in GWAT after chronic β3-AR-agonist in RID tg mice, while several positive cells are found in the wild-type GWAT (Figure 5C). We measured β3-AR mRNA expression as well as the acute lipolytic response to β3-AR agonist treatment and found no difference in β3-AR expression (Figure S5A). The β3-AR agonist-induced serum FFA increase is just marginally affected in the RID mice (Figure S5B). This eliminates receptor abundance and activity as a trivial explanation for these findings. Along the same rationale, receptor abundance and signaling are therefore unlikely to explain the differences in β3-AR agonist-induced “beiging” between genotypes. We have previously reported that inducible adipocyte-specific expression of VEGF-A leads to a “browning” of adipose tissue, similar to the effects reported for chronic β3-AR agonist treatment (Sun et al., 2012). Therefore, we speculated that the lack of β3-AR agonist-induced “beiging” in the RID tg mice may relate to a reduced ability to induce proangiogenic mediators. Indeed, upon examining the gene expression response in IWAT within 3 hr of a single dose of β3-AR agonist, we find a blunted induction of several proinflammatory cytokines in the RID tg mice (Figure 5D). Furthermore, the mRNA expression levels of classical proangiogenic mediators, such as VEGF-A, angiopoietin-1, and angiopoietin-2 (Angpt1 and Angpt2) are also reduced in the β3-AR agonist-treated RID tg mice (Figure 5D). Notably, we did not detect an acute β3-AR agonist-mediated induction of these proangiogenic mediators in the wild-type mice. Rather, the difference between genotypes relates mainly to a downregulation of these particular genes in response to β3-AR agonist in the RID tg mice. Thus, in the context of acute β3-AR agonist stimulation, it seems that a potent inflammatory response is necessary to maintain the expression of VEGF-A, Angpt1, and Angpt2 in IWAT. A comparable gene expression pattern was also seen in GWAT, albeit the individual variation is larger than in IWAT (Figure S5C).

The Reduced MWAT Expansion in the RID tg Mice Is Associated with a “Leaky Gut”

The RID tg mice have a more severe metabolic phenotype than the dnTNF tg and the Ad-rtTA-TRE-IxB tg mice. It caught our attention that the dnTNF tg and the Ad-rtTA-TRE-IxB tg mice display a normal HFD-induced MWAT expansion and mesenteric adipocyte size (data not shown). In contrast, MWAT expansion is dramatically reduced in the RID tg mice (Figure 2D). This indicates...
Figure 5. Reduced \( \beta_3 \)-AR-Agonist-Induced Browning of White Adipose Tissue in RID \( \beta_3 \) Mice

(A) Photo of IWAT and GWAT harvested from male \( \beta_3 \) and wild-type mice after chronic \( \beta_3 \)-AR-agonist treatment (10 days with daily i.p injection with 1 mg/kg in PBS).

(B) Representative H&E stain of IWAT from male \( \beta_3 \) and wild-type mice after chronic \( \beta_3 \)-AR-agonist treatment.

(C) Representative BrdU immunostain of GWAT harvested after chronic \( \beta_3 \)-AR-agonist treatment (coadministered with 10 mg/kg BrdU) in male \( \beta_3 \) and wild-type mice. Orange arrows point toward BrdU-positive nuclei.

Figure legend continued on next page.
that inhibition of inflammatory signaling in our mouse models is
compensated for by an increase in other inflammatory signaling
pathways in the mesenteric area. These compensatory mecha-
nisms are, however, not present or effective if GWAT or IWAT.
Moreover, the glucose intolerance seen in young unchallenged
RID tg mice cannot be explained by hepatic steatosis, since
comparably low levels of hepatic lipids are seen between
chow-fed wild-type and RID tg mice, at least while the body
weights are <30 g (Figure 6A). We noted, however, that SAA levels
are increased and both the liver weights and the average size of the
individual hepatocytes are enlarged in the chow-fed RID tg
mice, even in the absence of steatosis (Figures S2H, 6B, and
6C). Furthermore, the spleens of the RID tg mice are also enlarged
(Figure 6D). Taken together, these observations argue for a state
of elevated hepatic stress and increased systemic immune acti-
vation in the RID tg mice. A likely explanation for these phenom-
ena is an increased exposure to bacterial toxins that are leaking
out from the gut. In line with this hypothesis, we found elevated
levels of anti-LPS IgGs and increased intestinal permeability as
judged by the circulating levels of FITC-dextran after an oral
load in young RID tg mice (6–8 weeks old with a body weight of
≤20 g) on a chow diet (Figures 6E and 6F). Treatment with 2%
dextran sulfate sodium (DSS) through drinking water, which
damages the colonic epithelium, increases permeability and
causes colitis, leads to a further elevation of anti-LPS IgGs levels
and serves as a positive control for the plasma anti-LPS IgG
assay (Figure 5E). The colons of the DSS-treated RID tg mice
display increased pathological changes, such as an increased
degree of colon hyperplasia, more severe crypt disruption and
leukocyte infiltration, and further increased spleen size compared
to the DSS-treated wild-type mice (Figures 6G and 6H). Fur-
thermore, the DSS-treated RID tg mice have increased expression
of CD14, TRIF, TNF-α, MCP-1, MIP-1α, F4/80, and MPO mRNA in
colon, which supports the histological findings and indicates that
there is a higher degree of inflammation, with enhanced recruit-
ment/infiltration of macrophages and neutrophils, compared to
the DSS-treated wild-type mice (Figure 7A). It should also be
noted that even the non-DSS-treated RID tg mice display mild
colon hyperplasia and elevated colon expression of CD14 and
SAA3 mRNA (Figures 6G and 7A).

Despite the lack of an effect on body weight, DSS treatment
does, however, impair glucose tolerance in regular wild-type
mice (Figure 6A). This suggests that impaired intestinal barrier
function alone (even in the absence of HFD) can have a negative
impact on systemic metabolic regulation. This also suggests that
at least some of the RID tg phenotype may be improved if expo-
sure to bacterial toxins is limited. We put this hypothesis to the
test and found that indeed, a 5-week antibiotics treatment nor-
malizes spleen size and insulin levels in the transgenic mice (Fig-
ures 7B and 7C). To a large extent, the expression of the liver
acute phase reactants SAA1 and SAA2 is also lowered to levels
seen in the untreated wild-type mice (Figure 7D). These data
suggest that RID tg mice suffer from impaired intestinal barrier
function, leading to “leaky gut” and colitis. This also provides
an explanation for their hepatomegaly, spleen enlargement,
and glucose intolerance, even in the absence of an exogenous
inflammatory challenge.

In contrast to the RID tg mice, the dnTNF tg and the Ad-rtTA-
TRE-IκBtg mice, with their associated normal MWAT expansion,
do not display hepatomegaly, enlarged spleen, or colon hyper-
plasia (data not shown). This leads us to conclude that MWAT
inflammation and its subsequent expansion is an adaptive
response that plays a significant role in sustaining proper intesti-
nal barrier function and healthy symbiosis between the
commensal microflora and the host.

The intestinal barrier is, however, not only preventing bacte-
rial toxins from leaking out, but it also contributes to a healthy
symbiosis with colonizing bacteria. An impaired intestinal bar-
rier and/or chronic inflammation may lead to unfavorable shifts
in microbial composition. This is based on the observation that
gut flora can be transferred from mouse to mouse and affect
the pathogenesis of obesity and hepatic steatosis (Garrett
et al., 2010; Henao-Mejia et al., 2012). As expected, we
found a difference in bacterial composition as judged by qPCR anal-
ysis of eight different bacterial strains of DNA isolated from
cecal content from wild-type and RID tg mice housed in
different cages. Cecal levels of Lactobacillus murinus, Mucis-
pirillum Schaedleri, and Eubacterium Pleimcaudatum were
increased, and there was a trend for reduced levels of
Firmicutes sp. In contrast, the levels of Lactobacillus sp.,
Clostridium sp., and B. Distasonis-Porphyromonas were similar
between RID tg and wild-type mice (Figure S6B). Differences in
bacterial composition are not necessarily a reflection of a direct
negative effect of the RID tg transgene on intestinal barrier
function but can also be secondary to metabolic disturbances
and also differ between litters and cages. Importantly, the
metabolic dysfunction in RID tg is apparent regardless of
whether the mice under comparison are littermates or not.
Furthermore, there is no difference in either HFD-induced
body weight gain, glucose tolerance, insulin levels, or hepatic
steatosis in wild-type mice cohoused either with unrelated
wild-type mice or with RID tg (data not shown). Thus, the
potential negative effect of an altered bacterial composition in
the RID tg mice is not potent enough to have an impact on
metabolic health in the context of a normal immune defense
in wild-type mice. We can also conclude that the wild-type
gut flora does not offer a significant protective effect on the
metabolic phenotype in the RID tg mice.

DISCUSSION

The data presented here argue that a reduced ability to sense
and respond to proinflammatory stimuli at the level of the
adipocyte decreases the capacity for healthy adipose tissue
expansion and remodeling. This inability results in increased
HFD-induced hepatic steatosis and metabolic dysfunction.
Interestingly, while the ability to sense proinflammatory cues
that trigger expansion is of importance for all fat pads, a

(D) Gene expression analyses of IWAT 3 hr (acute) after 12-AR-agonist injection and after chronic treatment (IWAT harvested 24 hr after last injection) in male RID
tg and wild-type mice. Error bars represent SEM; *p < 0.05 according to Student’s t test was considered significant and is indicated by */##p < 0.01
(significantly different from WT, “significantly different from untreated controls of same genotype). Additional one-way ANOVA analyses have been performed for
Figure 4D (Table S3).
Figure 6. Leaky Gut and Colitis Associated with Signs of Systemic Inflammation in RID tg Mice
(A) Liver fat quantified by CT in chow-fed male RID tg and wild-type mice. (B and C) Liver weight and representative H&E stain of liver sections to show hepatocyte size in chow-fed male RID tg and wild-type mice with a body weight < 30 g. (D) Spleen size in chow-fed RIDs and wild-type mice. (E) Serum levels of anti-LPS IgG in young chow-fed versus DSS-treated male RID tg and wild-type mice. One-way ANOVA analysis shows that both treatment (F = 78.8, p < 0.001) and genotype (F = 26.4, p < 0.001) contribute significantly to anti-LPS IgG levels. (F) Serum levels of FITC-dextran after an oral load in young chow-fed female RID tg and wild-type mice. One-way ANOVA analysis shows that both treatment (F = 153.6, p < 0.001) and genotype (F = 27.4, p < 0.001) contribute significantly to colonic permeability. (G and H) Colon weight/length ratios, representative H&E images of colon, and spleen weight in untreated, and in response to DSS-treated, male RID tg and wild-type mice. One-way ANOVA analysis shows that both treatment (F = 153.6, p < 0.001) and genotype (F = 27.4, p < 0.001) contribute significantly to both colon thickness and spleen size. Error bars represent SEM; p < 0.05 according to Student’s t test was considered significant and is indicated by */#; **/##p < 0.01; ***/###p < 0.001 (*significantly different from WT, #significantly different from untreated controls of same genotype).
Figure 7. Antibiotics Treatment Improves the RID tg Mouse Phenotype

(A) Gene expression analysis of proximal colon in untreated, and in response to three bouts of DSS treatments, in male RID tg and wild-type mice.

(B–D) (B) Spleen, (C) fasting serum-insulin, and (D) SAA1 and SAA2 mRNA levels in liver in control or antibiotic-treated male RID tg and wild-type mice. One-way ANOVA analysis shows that both treatment (F = 15.8/8.7/12.1/5.8, p = 0.001/0.003/0.004/0.03) and genotype (F = 19.3/17.1/23.6/31.8, p < 0.001/0.001/ < 0.001/ < 0.001) contribute significantly to spleen size, insulin levels, liver SAA1, and liver SAA2 levels.

(E) Summary and proposed model: Acute inflammation is essential for healthy adipose tissue expansion and proper remodeling. Inability of adipose tissue to accurately sense and respond to inflammatory stimuli leads to reduced adipose tissue expansion and an increased risk for microbial translocation. Error bars represent SEM; p < 0.05 according to Student’s t test was considered significant and is indicated by */#; **/##p < 0.01; ***p < 0.001 (*significantly different from WT, #significantly different from untreated controls of same genotype).
deficiency in this regard has particularly profound consequences for the functionality of the visceral MWAT depot. We observe that the lack of MWAT expansion is associated with increased intestinal permeability and colitis, resulting in chronic systemic inflammation and metabolic dysfunction, even in the absence of HFD.

The Underlying Mechanism for Inflammation-Induced Adipose Tissue Expansion

Adipose tissue expands either through hypertrophy of existing adipocytes or adipogenesis, i.e., differentiation of new adipocytes from adipogenic precursor cells. All models studied here establish a reduced number of adipocytes in most depots examined. Chronic HFD feeding leads to a more pronounced GWAT depot weight difference, while the IWAT depot weight differences between tg and wild-type mice are reduced. Nevertheless, both GWAT and IWAT depots are at all times populated by fewer adipocytes in the transgenic mice than in the wild-type mice, reflecting a reduced rate of adipogenesis. Thus, the ability to mount an acute inflammatory response is playing a more important role in facilitating adipogenesis than in adipocyte hypertrophy.

The concept of inflammation-driven adipogenesis may seem contradictory at first, since proinflammatory cytokines, such as TNF-α, are lipolytic and block adipocyte differentiation in vitro (Gustafson and Smith, 2006). The situation in vitro is, however, substantially different from the situation in vivo, since there is neither a need for angiogenesis nor ECM remodeling under in vitro conditions. In fact, several older studies support a role of local inflammation in increased adipogenesis. For instance, Sadler and colleagues show that low-dose LPS leads to adipocyte hyperplasia at the site of administration (Sadler et al., 2002), and there is a selective increase in the amount of MWAT in Crohn’s disease as well as in experimentally induced colitis (Gambero et al., 2007; Sheehan et al., 1992). Moreover, a very recent study on proliferation and differentiation of PDGFRα+ adipocyte progenitors in vivo demonstrates that different adipogenic conditions are associated with an upregulation of different sets of inflammatory and macrophage-associated genes in white adipose tissue (Lee et al., 2013).

It is likely that several distinct mechanisms contribute toward the inflammation-driven adipogenic response, with several interconnected processes triggering adipose tissue growth. Here, we found that HFD feeding acutely (within days) leads to a reduction of the total levels of collagen in IWAT in wild-type mice, but not to the same extent in the dnTNF and the RID tg mice. Thus, healthy adipose tissue expansion is associated with a net loss of adipose tissue collagen, while dysfunctional adipose tissue in more advanced obesity displays an increased number of ECM deposits, along with chronic inflammation and hypoxia. The inability to effectively degrade ECM limits the capacity for healthy adipose tissue expansion. RID tg adipose tissue also has a reduced capillary density. In line with this finding, the capillary density of wild-type MWAT gets reduced by ablation of gut microbiota. These data demonstrate that proinflammatory responses in adipose tissue are essential for both proper ECM remodeling and angiogenesis, two processes known to facilitate adipogenesis in vivo, and therefore are likely mediators of the inflammation-induced adipose tissue expansion phenomenon (Cao, 2007; Cristancho and Lazar, 2011).

Chronic systemic inflammation interferes with optimal metabolic fitness. However, in light of our findings in three independent adipocyte anti-inflammatory models (summarized in Table S1), the view of adipose inflammation as a driving force for systemic inflammation and metabolic dysfunction is an oversimplification. Rather, we postulate that a potent acute inflammatory response is essential for adipose tissue protection, remodeling, and expansion (Figure 7E). This facilitates the return to a healthy equilibrium with metabolic homeostasis that subsequently allows the inflammation to reach resolution as opposed to becoming chronic.

EXPERIMENTAL PROCEDURES

Animals

Adipochaser mice were described in Wang et al. (2013), dP2-dntNF, dP2-RID, and TRE-lk-Bx(S32G-S36A) transgenic mice were used in a pure C57BL/6J (dnTNF and TRE-lk) or on a FVB background (RID). Mice were maintained on a 12 hr dark/light cycle and housed in groups of four to five with unlimited access to water, chow (number 5058, Lab-Diet) or HFD (number D12492, Research Diets Inc.) as indicated for the individual experiments. In all experiments, littermate controls were used unless specifically stated otherwise. The Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center, Dallas, has approved all animal experiments.

LPS-Induced Adipogenesis

Adipochaser mice were fed doxycycline-supplemented chow (600 mg/kg) for 1 week and were thereafter switched to regular chow 3 days prior the experiment. The right IWAT depot of adipochaser mice was injected twice a week for 2 weeks with 20 μg lipopolysaccharide (LPS, Sigma, USA) in 50 μl PBS, while the left IWAT depot remained untreated. Five weeks later, tissues were harvested for X-Gal-LacZ staining (performed as previously described [Wang et al., 2013]).

LPS and TNFα Response In Vivo

The mice received one intraperitoneal injection with 0.3 mg/kg LPS in PBS or 0.5 μg/mouse TNFα (recombinant mouse TNFα, Biolegend, USA) in PBS supplemented with 1% BSA. Blood was collected from the tail at indicated time points.

Oral Glucose Tolerance Test

The mice were fasted for 3 hr during the light phase, and blood samples were drawn from the tail vein before and 15, 30, 60, and 120 min after an intragastric load with 2.5 g/kg glucose in PBS.

Adipose Tissue Collagen Levels Measurements

Pieces of IWAT (~50 mg) were snap frozen in N2 (l) until analysis. The total collagen content was measured, using a commercial kit (QuickZyme Biosciences, Netherlands).

Hepatic Steatosis Measurements

Quantification of hepatic steatosis was performed by computerized tomography (CT) or standard biochemical tissue triglyceride analysis. The CT analysis was performed as previously described (Asterholm and Scherer, 2010). In brief, mice were anesthetized with isoflurane, and a CT scan was performed at a resolution of 93 μm using the short scan mode (180°) on an eXplore Locus in vivo MicroCT Scanner from GE Healthcare. Liver lipid content was estimated by obtaining the average CT value in multiple regions well within the liver, as validated in Asterholm and Scherer (2010).

Intestinal Permeability Assay In Vivo

The mice were fasted for 5 hr; thereafter they were given an oral load with 0.6 mg/g FITC-labeled dextran (average mole weight 4000, Sigma). Blood samples were collected at indicated time points and the serum was diluted two times in PBS and loaded together with known standards diluted...
in 50/50 PBS/control serum on a 96-well plate and analyzed (485 nm exc., 535 nm em.) on a POLARstar Optima Analyzer.

**Statistical Analysis**
Data are in generally expressed as mean ± SEM. The Student’s t test and one- or two-way ANOVA (repeated measurement) were used for comparisons between groups; log transformation was performed as necessary to obtain normal distribution. SPSS software (version 21) was used for these statistical calculations; *p < 0.05 was considered significant; **p < 0.01; ***p < 0.001.

**SUPPLEMENTAL INFORMATION**
Supplemental information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2014.05.005.

**AUTHOR CONTRIBUTIONS**
I.W.A. designed the study, carried out the research, interpreted the results, and wrote the manuscript. C.T., T.S.M., Q.A.W., F.D.-L., and Z.V.W. assisted in study design, performed research, and reviewed the manuscript. P.E.S. designed the study, analyzed the data, and revised the manuscript, and is responsible for the integrity of this work. All authors approved the final version of the manuscript.

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