



HHS Public Access

Author manuscript

Immunity. Author manuscript; available in PMC 2017 September 20.

Published in final edited form as:

Immunity. 2016 September 20; 45(3): 583–596. doi:10.1016/j.immuni.2016.08.002.

CD301b⁺ mononuclear phagocytes maintain positive energy balance through secretion of resistin-like molecule (RELM) alpha

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Summary

Mononuclear phagocytes (MNPs) are a highly heterogeneous group of cells that play important roles in maintaining the body's homeostasis. Here, we found CD301b (also known as MGL2), a lectin commonly used as a marker for alternatively-activated macrophages (M2), was selectively expressed by a subset of CD11b⁺CD11c⁺MHCII⁺ MNPs in multiple organs including adipose tissues. Depleting CD301b⁺ MNPs *in vivo* led to a significant weight loss with increased insulin sensitivity and a marked reduction in serum RELM α , a multifunctional cytokine produced by MNPs. Reconstituting RELM α in CD301b⁺ MNP-depleted animals restored body weight and normoglycemia. Thus, CD301b⁺ MNPs play crucial roles in maintaining glucose metabolism and net energy balance.

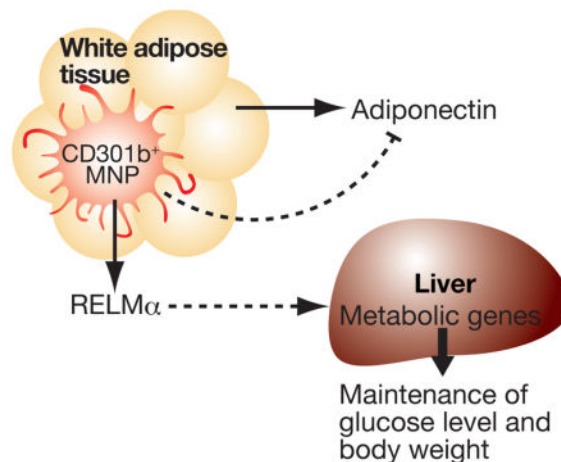
Graphical Abstract

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Author Contributions

Y.K. and A.I. designed research. Y.K., M.J., J.G.C. and M.S. performed experiments. Y.K., T.H., G.S. and A.I. analyzed data. Y.K. and A.I. wrote the manuscript.

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Keywords

Alternatively activated macrophage; glucose metabolism; adipose tissue macrophage

Introduction

Maintaining the whole body energy balance is vital to our lives. Mononuclear phagocytes (MNP), including monocytes, macrophages and dendritic cells (DCs) are present in most organs throughout the body and play an important role in maintaining whole body homeostasis. MNPs respond to and counteract metabolic stresses such as those imposed by high fat diet (HFD) feeding in mouse models (Biswas and Mantovani, 2012; Chawla et al., 2011; Olefsky and Glass, 2010). However, little is known regarding their role in maintaining metabolic homeostasis in metabolically normal conditions under regular chow feeding.

Macrophages are thought to adopt distinct phenotypes depending on the external stimuli. M1, or classical activation, refers to the activation status whereby MNPs are stimulated with pro-inflammatory type 1 cytokines or inflammatory stimuli such as interferon- γ or lipopolysaccharide (LPS). M2, or alternative activation, in contrast, refers to the activation status of MNPs that are stimulated with type 2 cytokines such as interleukin (IL)-4 or IL-13 (Martinez et al., 2009). In general, M1 MNPs are considered to play pro-inflammatory roles, while M2 MNPs are generally specialized on homeostasis and tissue repair. Although the concept of M1 and M2 activation has come originally from *in vitro* observations (Stein et al., 1992), it is now widely applied to *in vivo* MNP populations that have similar gene expression profile to those activated *in vitro* (Martinez et al., 2009).

Chronic low-grade inflammation has long been linked to metabolic complications associated with obesity such as insulin resistance. Accordingly, several types of immune cells within the adipose tissue have been linked to obesity-associated metabolic disease (Mathis, 2013). Contributions of MNPs in metabolic disease have been well documented (Hotamisligil, 2006; Weisberg et al., 2003; Xu et al., 2003). Early during the development of obesity, MNPs in the adipose tissue 'switch' their phenotype from an M2-like status in the lean conditions to an M1-like phenotype (Lumeng et al., 2007). Importantly, genetic and

pharmacological reduction in the number of M1 MNPs has been shown to result in a marked improvement in insulin sensitivity (Feng et al., 2011; Kanda et al., 2006; Patsouris et al., 2008; Weisberg et al., 2006). In contrast, blocking M2 activation cascade by deleting macrophage expression of IL-4 receptor downstream components, such as PPAR γ , PPAR δ and KLF4, results in exacerbated insulin resistance and, in some models, increase in weight gain upon HFD feeding (Kang et al., 2008; Liao et al., 2011; Odegaard et al., 2007; Odegaard et al., 2008). However, MNP populations *in vivo* are highly heterogeneous, and do not necessarily conform to either the M1 or M2 phenotypes (Shaul et al., 2010; Wentworth et al., 2010). Therefore, dissection of the role of MNP subsets beyond the simple M1-*versus*-M2 classification is needed to understand the role of various MNPs in the context of metabolic diseases.

The *Mgl2* gene (encoding the CD301b protein) is often considered to be a prototypical M2 marker both *in vivo* and *in vitro* (Auffray et al., 2007; Byles et al., 2013; Fujisaka et al., 2009; Han et al., 2013; Kang et al., 2008; Li et al., 2011; Lumeng et al., 2007; Raes et al., 2005). Importantly, a marked reduction of *Mgl2* expression in the adipose tissue MNPs has been reported in MNP-specific PPAR δ -deficient mice, in which exacerbated insulin resistance with more weight gain upon HFD feeding has also been observed (Kang et al., 2008). These studies suggest that CD301b⁺ MNPs play a protective role against metabolic stress and that depletion of CD301b⁺ MNPs would result in a similar phenotype to the above mentioned M2-deficient models.

Here, to characterize the role of CD301b⁺ MNPs in whole body metabolism, we examined the expression pattern of the CD301b protein among MNP subsets in the adipose tissue and other organs. In a mouse model expressing diphtheria toxin receptor (DTR) under the control of the *Mgl2* gene (*Mgl2*-DTR mouse) (Kumamoto et al., 2013), we examined the metabolic consequences of acute depletion of CD301b⁺ MNPs by diphtheria toxin (DT) treatment in both regular chow fed mice as well as those on high fat diet. Contrary to previous studies that have suggested an anti-diabetogenic role of CD301b⁺ MNPs, our results reveal their requirement for the maintenance of positive energy balance under both steady state and high fat diet metabolic conditions, and identify RELM α as a key downstream effector molecule.

Results

CD301b is expressed in a subset of MNPs *in vitro* and *in vivo*

Mgl2 mRNA, encoding the CD301b protein, has been shown to be highly expressed in interleukin-4 (IL-4)-stimulated M2 macrophages *in vitro* and M2-like MNPs *in vivo* (Raes et al., 2005). However, the proportion of CD301b⁺ cells out of all M2 macrophages *in vivo*, or the phenotype of CD301b⁺ cells in metabolic organs is unknown. To this end, we investigated the expression of CD301b in MNPs isolated from various non-lymphoid tissues of WT mice fed regular chow. CD301b was found on a subset of MNPs in most of the peripheral organs tested, including the epididymal fat, colon, and the peritoneum (Figure 1A). Little if any CD301b⁺ cells were found in the blood, consistent with a previous observation that *Mgl2* expression is upregulated in a fraction of circulating MNPs after their entry into the peripheral tissue (Figure 1A) (Auffray et al., 2007). A previous study has

shown that adipose tissue macrophages express a high amount of *Mgl2* mRNA particularly in lean mice (Lumeng et al., 2007). Indeed, a fraction of CD11b⁺F4/80^{int to high} macrophage-like cells in the visceral adipose tissue, as well as other metabolic organs, expressed CD301b protein (Figure 1B). Importantly, as was observed previously with the CD301b⁺ cells in other sites of the body (Denda-Nagai et al., 2010; Kumamoto et al., 2009; Kumamoto et al., 2013), the majority of CD301b⁺ cells in the peripheral organs uniformly expressed a high amount of MHC class II and CD11b, an intermediate to high amount of CD11c, and only a moderate amount of F4/80 (Figure 1A, B).

Since *Mgl2* mRNA expression has often been used as a marker for adipose tissue-resident M2-like MNPs *in vivo* (Fujisaka et al., 2009; Han et al., 2013; Li et al., 2011; Lumeng et al., 2007), we next compared the expression of CD301b and two other M2 markers, Arginase1 and CD206. Cells expressing *Arg1* mRNA were analyzed using the YARG reporter mice in which expression of Arginase 1 is monitored by YFP expression from the *Arg1* locus (Reese et al., 2007). Expression of CD206 (also known as macrophage mannose receptor), another commonly-used M2 marker, was assessed by a specific antibody. In the epididymal fat, the expression of CD301b was observed selectively in the cells expressing CD206. In contrast, *Arg1* expression was observed in two clearly distinct populations in the adipose tissue: those expressing common MNP markers including CD11b, F4/80, MHCII, CD11c, CD206 and CD301b, and those that were negative for all MNP markers tested (Figure 1C). In the peritoneal cavity, the expression of CD301b showed distinct patterns from that of *Arg1*, though it still partially overlapped with that of CD206. In the peritoneum, *Arg1* expression was confined to CD11b⁺ F4/80⁺ MHCII⁻ CD11c⁻ CD206⁻ cells. In both organs, CD301b was specifically expressed in CD11c⁺ CD11b⁺ MHCII^{hi} F4/80^{int} CD206⁺ MNPs (Figure 1). These results indicate that not all M2 markers examined (CD301b, CD206, Arg1) are expressed in the same MNP subset *in vivo* and that CD301b⁺ MNPs represent a relatively consistent subset throughout different organs. Therefore, the “M2” macrophage markers delineate multiple distinct cell subsets in different organs, and CD301b⁺ MNPs do not clearly fit either M1 or M2 macrophage definition.

Depletion of CD301b⁺ MNPs results in weight loss in regular chow and HFD fed mice

Next, we examined the role of CD301b⁺ MNPs in possible regulation of *in vivo* metabolism by using *Mgl2*^{+/DTRcGFP} (*Mgl2*-DTR) mice, in which CD301b⁺ MNPs can be specifically and inducibly depleted by injecting diphtheria toxin (DT) (Kumamoto et al., 2013). Quantitative real-time PCR experiments in the epididymal white adipose tissue revealed that the DT treatment successfully abolished expression of *Mgl2* but not *Mrc1* (encoding CD206) (Figure 2A). Consistent with the mRNA expression data, flow-cytometric experiments revealed that DT treatment of *Mgl2*-DTR mice did not affect the rest of the MNPs that did not express this marker (Figure 2B). After DT injections given every-third-day for 10 days, the regular chow-fed *Mgl2*-DTR mice had a significant reduction in body weight compared to the wild-type (WT) animals treated with DT or *Mgl2*-DTR mice treated with phosphate-buffered saline (PBS) (Figure 2C,D and Supplementary Figure 1B). The weight loss was not due to the loss of CD301b protein induced by the DT treatment but rather to the loss of CD301b-expressing cells and was generally observed in both males and females, as indicated by the significant weight loss after the DT-induced cell depletion in

Mgl2^{DTR^{eGFP}/DTR^{eGFP}} homozygotic mice of both sexes that are deficient for the CD301b protein (DTR-eGFP is genetically targeted into the *Mgl2* locus) (Figure 2E).

It was formally possible that DT injection led to either an off target effect or that depletion of MNPs in general accounts for our observation. DT treatments did not deplete leukocyte subsets other than MNPs including eosinophils, natural killer and natural killer T cells, or type 2 innate lymphoid cells that are important in regulating various metabolic pathways (Supplementary Figure 1A) (Brestoff et al., 2015; Ji et al., 2012; Lee et al., 2016; Lynch et al., 2012; Molofsky et al., 2013; Ohmura et al., 2010; Satoh et al., 2012; Schipper et al., 2012; Strodtz et al., 2013; Wensveen et al., 2015; Wu et al., 2011; Wu et al., 2012). However, we noted previously that DT treatment of *Mgl2*-DTR mice results in deletion of skin Langerhans cells and dermal DCs in addition to internal tissue MNPs (Kumamoto et al., 2013). Thus, to address the contribution of Langerhans cell loss in the observed weight loss, we compared weight loss in another DTR based system, *CD207*-DTR transgenic mice, in which DT treatment results in the selective depletion of skin Langerhans cells (Bobr et al., 2010). The weight loss was not induced in *CD207*-DTR transgenic mice treated with DT (Figure 2C and Supplementary Figure 1B). In addition, as opposed to the *Itgax*-DTR mice (Jung et al., 2002; Patsouris et al., 2008), *Mgl2*-DTR mice showed no apparent sickness or lethality even with multiple DT injections (Kumamoto et al., 2013). These data indicate that depletion of CD301b⁺ MNPs, but not Langerhans cells, results in weight loss.

To determine whether this phenotype is recapitulated in obese animals, we depleted CD301b⁺ MNPs after short term or long term HFD feeding. Similarly to the regular chow-fed animals, *Mgl2*-DTR mice fed with HFD either for 3 weeks or for 4 months gained significantly less weight, or lost more weight, respectively, upon DT treatment (Figure 2F and Supplementary Figure 1C). The DT treatment did not affect the fat ratio in the total body mass as determined by nuclear magnetic resonance, suggesting that the depletion leads to a reduction in both lean and fat mass (Figure 2G). These data indicate that depletion of CD301b⁺ MNPs results in weight loss even in animals that are already obese.

Depletion of CD301b⁺ MNPs alters whole body energy balance

Next, we probed the mechanism of weight loss seen following CD301b⁺ MNP depletion. Behavioral and calorimetric analyses in metabolic cages revealed a significant reduction in food intake per body weight as well as per mouse in both regular chow- and HFD-fed *Mgl2*-DTR mice treated with DT (Figure 3A and Supplementary Figure 2A,B). There was also a trend for reduction in energy expenditure in regular chow-fed DT-treated animals during the night, but it did not reach statistical significance as a whole-day average when normalized by body weight (Figure 3A,B). At the whole animal level, the reduction in energy expenditure was evident throughout the day when mice were fed with regular chow (Supplementary Figure 2A,B). Consistent with the weight loss, the net energy balance (energy expenditure subtracted from the food intake) was significantly lower in the DT-treated animals compared to the PBS-treated animals (Figure 3B and Supplementary Figure 2B). In regular chow-fed groups, the depletion significantly lowered the respiratory exchange ratio (RER), indicating that they relied more on lipid oxidation rather than carbohydrate oxidation compared to the PBS-treated controls (Figure 3C). Reduction in RER is often observed in calorically

restricted animals (Bruss et al., 2010; Wueest et al., 2014), and may reflect the reduction in food intake in CD301b⁺ MNP-depleted mice (Figure 3A, B).

Feeding behavior is regulated by the hunger-satiety circuit in the central nervous system. Since c-Fos is transiently expressed in recently activated neurons, immunohistochemical staining for c-Fos provides a reliable method to label activated neurons. Thus, we predicted that, if the feeding behavior is controlled centrally, we should see increased c-Fos expression in the proopiomelanocortin (POMC) neurons in the arcuate nucleus (ARC), which inhibit food intake. Furthermore, we predicted that neurons in the parabrachial nucleus (PBN) which when activated trigger profound anorexia, would also express higher amounts of c-Fos. However, we did not see significant changes in c-Fos expression in either the POMC neurons in the ARC or neurons within the PBN following depletion of CD301b⁺ MNPs (Supplementary Figure 2C). In addition, we did not find CD301b⁺ cells in the hypothalamus. Nevertheless, the alteration in the net energy balance was accompanied by increased sympathetic output, as indicated by the upregulation of *Ucp1* gene expression in the brown adipose tissue induced by the depletion of CD301b⁺ MNPs but not by DT treatment alone (Figure 3D). Again, increased expression of *Ucp1* gene was only seen after depletion of CD301b⁺ MNP but not Langerhans cells. Although the enhanced sympathetic output could directly explain the reduced food intake, chemical sympathectomy by intraperitoneal 6-hydroxydopamine injection did not rescue the weight loss in DT-treated animals, suggesting that the sympathetic output in the periphery alone cannot account for the weight loss phenotype (Figure 3E). Collectively, these results suggest a role for CD301b⁺ MNPs in active maintenance of positive energy balance, and suggest that these cells do so without directly interacting with cells of the feeding control center of the central nervous system.

Depletion of CD301b⁺ MNPs causes hypoglycemia in both regular chow- and HFD-fed animals

We next explored the role of CD301b⁺ MNPs in the regulation of glucose metabolism. The CD301b⁺ MNP depletion did not affect plasma glucose concentrations in lean animals that were fed *ad libitum* (Figure 4A). In a standard glucose-tolerance test (GTT) in overnight fasted animals, however, the plasma glucose concentrations were significantly reduced in DT-treated animals in both regular chow and HFD feeding conditions (Figure 4B–D). In DT-treated regular chow-fed animals, the plasma insulin concentrations were also lower than the PBS-treated control animals in the fasting state and during the GTT, suggesting that the depletion of CD301b⁺ MNPs enhanced insulin sensitivity (Figure 4E–G). To test this possibility, we performed hyperinsulinemic-euglycemic clamp studies on DT- or PBS-treated *Mgl2-DTR* mice that had been on HFD for 3 weeks, in which glucose-reducing effect of DT was most prominent in our GTTs. Consistent with the GTT results, the DT-treated animals displayed improved whole-body insulin sensitivity as demonstrated by the higher glucose infusion rate required to maintain euglycemia under experimental conditions in which plasma insulin concentrations were matched (Figure 4H–J). The higher glucose infusion rate in the depleted mice was largely explained by the suppression of hepatic glucose production compared with controls, whereas insulin-stimulated glucose uptake was not different between these groups (Figure 4K–M).

Depletion of CD301b⁺ MNPs alters metabolic gene expression patterns and increases serum adiponectin

Our data thus far indicated the requirement for CD301b⁺ MNPs in maintaining normal weight and glucose metabolism. We next examined how the CD301b⁺ MNPs maintain such metabolic balance. To this end, we examined circulating concentrations of two key adipokines, leptin and adiponectin, which are known to affect body weight and insulin sensitivity through distinct mechanisms (Ouchi et al., 2011; Rosen and Spiegelman, 2006). Measurement of concentrations of these factors in the sera showed no difference in serum leptin concentrations between CD301b⁺ MNP-intact and depleted animals (Figure 5A). In contrast, serum adiponectin was significantly increased in CD301b⁺ MNP-depleted lean mice (Figure 5B). The elevation of circulating adiponectin concentration was not observed in *CD207*-DTR mice, further confirming the subset specificity of this phenomenon (Figure 5B). Comparison of serum adiponectin concentration and the body weight revealed inverse correlation in mice depleted of CD301b⁺ MNPs (Figure 5C). In parallel, in the liver, the expression of adiponectin receptor *Adipor1* was significantly elevated in CD301b⁺ MNP-depleted mice, suggesting that adiponectin signaling may be selectively enhanced by the depletion of CD301b⁺ MNPs (Figure 5D). To test whether the increased adiponectin reflects the cause or the consequence of metabolic imbalance, we crossed the *Mgl2*-DTR mice to *Adipoq*^{-/-} mice. Our results demonstrated clearly that weight loss and better glucose clearance upon DT treatment in both regular chow- and HFD-feeding conditions were preserved in the absence of *Adipoq* gene following CD301b⁺ MNP depletion (Figure 5E–I). The expression of CD301b protein in the *Adipoq*^{-/-} mice was comparable to that in WT mice (Figure 5J), and CD301b⁺ MNP depletion was complete (Figure 5K). Collectively, these results indicate that the elevated serum adiponectin concentrations in CD301b⁺ MNP-depleted mice are the consequence, and not the cause, of weight loss in these animals.

Depletion of CD301b⁺ MNPs alters distinct gene sets in the white adipose tissue and the liver

To understand the molecular basis for the altered metabolism in the CD301b⁺ MNP-depleted mice, we next examined the transcriptome profiles in the visceral and subcutaneous white adipose tissues (WAT) and the liver. Among genes that were highly significantly downregulated by the depletion ($p < 0.01$, more than 50% reduction), 42 genes were shared between the inguinal and epididymal WAT, many of which were myeloid cell markers that were likely expressed in CD301b⁺ MNPs themselves (Figure 6A). In contrast, we found a very different set of genes downregulated in the liver, compared to WAT (Figure 6A). Consistent with the improved hepatic insulin sensitivity, many of the 28 genes that were specifically downregulated in the liver had metabolic function. Gene set enrichment analysis (Subramanian et al., 2005) also revealed significant reduction in ‘immune-related’ pathways in the WAT and in metabolic pathways in the liver, but little overlap between these two organs (Supplementary Figure 3). The top differentially expressed genes in the WAT belonged to the genes expressed by the CD301b⁺ MNPs themselves (*Mgl2*, *Cd209g*, *Cd209f*, *Clec4b1*, *Clec4a3*, *Clec10a*, *Csf1r*). Further quantitative analyses by real-time PCR in the liver identified several genes related to metabolism of fatty acids, thyroid hormones, steroids and bile acids that were either significantly up- or down-regulated by the depletion of CD301b⁺ MNPs (Figure 6B). Consistently, serum cholesterol, free thyroid hormone T3

and corticosterone concentrations were significantly changed in mice depleted of CD301b⁺MNPs (Figure 6C). Collectively, these results indicate that depletion of CD301b⁺MNPs affects multiple metabolic pathways in the liver, while it mainly results in a reduction in the genes expressed by the CD301b⁺MNPs in the WAT.

Resistin-like molecule α (RELM α) is responsible for the weight loss and enhanced glucose clearance induced by CD301b⁺MNP depletion

Among the 42 genes commonly downregulated in both inguinal and epididymal WAT, the expression of *Retnla* gene was most dramatically reduced upon depletion of CD301b⁺MNPs (Figure 6A), which was further confirmed by real-time PCR analysis of the WAT (Figure 7A). The *Retnla* gene encodes RELM α protein (also known as FIZZ1), which was originally identified as a tissue-specific cytokine derived from WAT and lung epithelia but later found to be highly expressed in M2 macrophages (Holcomb et al., 2000; Raes et al., 2002; Stepan et al., 2001b). In WT mice, CD301b⁺ MNPs were the dominant cell type that expressed intracellular RELM α protein in both epididymal and inguinal WAT (Figure 7B). In addition, the depletion of CD301b⁺MNPs in *Mgl2*-DTR mice resulted in a severe reduction in the amount of circulating RELM α protein (Figure 7C). To directly address if the reduced RELM α concentration is responsible for the metabolic changes in CD301b⁺MNP-depleted mice, we supplemented the mice with recombinant RELM α intraperitoneally as previously described (Osborne et al., 2013) in parallel with the DT treatment. The reconstitution of RELM α fully restored the body weight as well the glucose concentrations in GTT in CD301b⁺MNP-depleted mice (Figure 7C–E). Collectively, these results revealed that CD301b⁺ MNPs are the major source of circulating RELM α , and place RELM α as the major effector mechanism by which CD301b⁺MNPs maintain the whole body metabolism.

Discussion

In the present study, we have demonstrated that CD301b molecule marks a distinct subset of MNPs present in various organs involved in systemic metabolism including the adipose tissue, skeletal muscle, colon and pancreas. A transient depletion of CD301b⁺ MNPs induced weight loss and enhances insulin sensitivity in both lean and obese animals. Depletion of CD301b⁺ MNPs led to reduced food intake, increased fatty acid oxidation, and reduced gluconeogenesis. These phenotypes were accompanied by increased concentrations of adiponectin and cholesterols, and decreased free thyroid hormone T3 and RELM α in circulation. Importantly, reconstituting RELM α normalizes the weight loss and hypoglycemia in CD301b⁺ MNP-depleted mice. Our results indicate a role for CD301b⁺ MNPs in maintenance of net energy balance even under lean conditions.

RELM α is a member of the resistin family of hormones that has been identified in the adipose tissue with roles in promoting insulin resistance and linked to obesity with insulin resistance (Stepan et al., 2001a). Although RELM α has not been identified in humans, human resistin expression pattern is more similar to mouse RELM α than mouse resistin, and is expressed in myeloid cells (Nair et al., 2006). In addition, RELM α has been shown to inhibit adipocyte differentiation (Blagoev et al., 2002), suppress type 2 inflammation (Nair

et al., 2009; Pesce et al., 2009), and promote T helper 17 cell-dependent colitis and skin wound healing (Knipper et al., 2015; Munitz et al., 2008; Osborne et al., 2013). *Retnla*^{-/-} mice show faster glucose clearance in GTT in mice with dextran sodium sulfate-induced colitis (Munitz et al., 2009). Furthermore, RELM α deficiency in *Ldlr*^{-/-} atherosclerotic mice results in hypercholesterolemia, while its overexpression in WT animals reduces serum cholesterol and directly induces bile acid synthases *Cyp7a1* and *Cyp8b1* in the liver (Lee et al., 2014). Our results of the elevated serum cholesterol and reduced hepatic *Cyp8b1* expression in CD301b⁺MNP-depleted mice are thus consistent with these studies. While the reason for the lack of metabolic phenotype reported in *Retnla*^{-/-} mice under steady-state conditions is unclear, these animals seem to have developed a compensatory mechanism to maintain normal metabolic homeostasis, as they maintain lower leptin concentrations in the sera despite their normal body weight and normal weight gain upon HFD feeding (Munitz et al., 2009). Thus, leptin sensitivity may be enhanced in *Retnla*^{-/-} mice. Following acute depletion of CD301b⁺MNPs, or in colitic or atherosclerotic animals, the drop in RELM α concentrations has a strong impact on glucose and cholesterol metabolism, perhaps because of the host's inability to engage compensatory mechanisms that are present in animals genetically deficient for RELM α . How RELM α secreted by CD301b⁺ MNP controls feeding behavior and energy balance remains to be determined.

Along with *Mgl2* (CD301b), *Mrc1* (CD206) and *Arg1*, *Retnla* (RELM α) mRNA expression is one of the most commonly used indicators of the M2 activation status. Indeed, all of these genes are highly inducible by IL-4 stimulation in peritoneal macrophages in an IL-4R-dependent manner (Raes et al., 2005; Raes et al., 2002). The expression of *Retnla* in MNPs *in vivo* seems to be dependent on IL-4R signaling cascade (Knipper et al., 2015; Pesce et al., 2009), and our data show that CD301b⁺MNPs are required to maintain systemic RELM α protein concentrations *in vivo*. However, the depletion of CD301b⁺MNPs results in a selective loss of certain M2 marker genes such as *Retnla* but not all (those that were not lost include *Mrc1* and *Arg1*), reflecting the heterogeneity within the so-called M2-like population.

Although the M1 and M2 paradigm has been helpful in our current understanding of the role of MNP subsets in metabolic homeostasis *in vivo* (Biswas and Mantovani, 2012; Odegaard and Chawla, 2011), CD301b⁺ MNP in the adipose tissue described here does not fit into either M1 or M2 classification. *In vivo* analyses of CD301b expression revealed that while CD301b expressing cells were uniformly CD11b⁺ and CD11c⁺ (typically considered M1 marker) in different organs, some but not all M2 markers were expressed by the same MNP subset. Consistently, phenotypic heterogeneity among M1- and M2-like populations have been previously described in both mice and humans (Li et al., 2010; Shaul et al., 2010; Wentworth et al., 2010), which collectively necessitated a major revision in the simplified M1–M2 view in describing *in vivo* MNP subsets (Mantovani, 2016; Martinez and Gordon, 2014; Murray et al., 2014). Nevertheless, the IL-4 receptor (IL-4R)-driven signaling cascade, a prototypical M2 differentiation driver (Stein et al., 1992), in MNPs seems to play crucial roles in regulating homeostasis under metabolic stress conditions such as over-nutrition (Kang et al., 2008; Odegaard et al., 2007; Odegaard et al., 2008) or cold exposure (Nguyen et al., 2011; Qiu et al., 2014). For instance, MNP-specific deletion of PPAR γ or PPAR δ , two major transcription factors downstream of the IL-4R-STAT6 axis, results in increased weight

gain and insulin resistance upon HFD feeding, along with a marked reduction in M2 marker expression including the *Mgl2* gene (Kang et al., 2008). In contrast, inactivating inflammatory signaling component such as IKK- β or JNK in MNPs suppresses expression of inflammatory M1 signature genes and ameliorates insulin resistance in HFD-fed mice without affecting body weight (Arkan et al., 2005; Han et al., 2013; Solinas et al., 2007). In addition, depletion of CD11c⁺ MNPs in bone marrow (BM) chimeric mice transplanted with *Itgax*-DTR BM cells also dampens the inflammatory cytokine expression and improves the insulin sensitivity in diet-induced obese mice but not in lean mice, without affecting the body weight (Patsouris et al., 2008).

Collectively, in light of our findings, these observations suggest the importance of the IL-4R signaling cascade within CD301b⁻ M2-like MNPs in maintaining insulin sensitivity and in controlling body weight under HFD conditions. Alternatively, since *Lyz2* promoter has been widely used in previous studies as an expression driver for Cre recombinase to generate MNP-specific gene-deficient mice, it is also possible that common MNP precursors that express *Lyz2* promoter are involved in promoting insulin sensitivity under HFD-induced stress. In contrast, our results reveal the role of CD301b⁺CD11c⁺ MNPs in maintaining the blood glucose and the body weight in both lean and obese animals. Taken together, this study contributes to the mounting evidence for MNPs in regulating whole body metabolic homeostasis, and reveals the role of CD301b⁺ MNP in maintaining positive energy balance through the secretion of RELM α .

Experimental Procedures

Mice and treatments

WT C57BL/6 (WT), *Mgl2*-DTR (*Mgl2*^{+/DTReGFP}) and homozygotic *Mgl2*^{DTReGFP/DTReGFP} mice were maintained in our specific pathogen-free facility at room temperature (20–22°C). *CD207*-DTR mice were a gift from Daniel Kaplan and maintained in our colony. YARG mice were a gift from Ruslan Medzhitov. *Adipoq*^{-/-} mice were purchased from the Jackson Laboratory and crossed with *Mgl2*-DTR mice. Unless otherwise stated, 3–6 month-old males were used for experiments. Where indicated, mice were fed with HFD (60% kcal% fat, D12492, Research Diets) for indicated period of time. For *in vivo* cell depletion, DT (0.5 μ g/mouse/dose, List Biological Laboratories) was given intraperitoneally every three days for four times, then mice were sacrificed on day 10. In HFD-fed animals, DT was given in the last 10 days of the indicated HFD feeding period. For chemical sympathectomy, WT mice were injected intraperitoneally twice with 3.5 mg/mouse/dose 6-hydroxydopamine (~100 μ g/g body weight, MP Biomedicals) dissolved in 0.07% (w/v) ascorbic acid (Sigma) in PBS on 1 and 3 days prior to the first DT treatment, as previously described (Riol-Blanco et al., 2014). For reconstitution of RELM α , 10 μ g of bacteria-expressed RELM α (Peprotech) was injected intraperitoneally every three days for four times as previously described (Osborne et al., 2013), at the same time with the DT treatments. In general, animals with different treatments were co-housed in the same cages to avoid cage-to-cage variation. For indirect calorimetry, mice were individually housed during the 10-day DT treatment period and their metabolic activity was monitored in metabolic cages in the last

five days. All animal protocols were approved by the Institutional Animal Care and Use Committee at Yale University.

Cell preparation and flow cytometry

Cells were isolated from tissues by digestion with collagenase D (Roche) and stained with monoclonal antibodies against CD11b (clone M1/70), CD11c (N418), F4/80 (BM8), MHCII (M5/114.15.2), CD206 (C068C2), CD301b (11A10-B7), Siglec F (E50–2440), CD3e (17A2), NK1.1 (PK136), CD25 (PC61), CD127 (A7R34), CD4 (GK1.5), CD8a (53–6.7), CD45 (30-F11), CD49b (DX5), and FcεRI (MAR-1). All antibodies were purchased from Biolegend except anti-SiglecF and anti-CD301b, which were purchased from BD Biosciences and prepared in-house, respectively (Kumamoto et al., 2013). For intracellular RELM α staining, cells pre-stained for MNP markers were fixed and permeabilized, then stained with biotinylated rabbit anti-mouse RELM α (Peprotech).

Gene expression analyses

Total RNA was isolated from tissues with Trizol (Invitrogen) and RNeasy Mini Kit (Qiagen) and analyzed with Affymetrix Mouse Gene 2.0ST Microarray (GEO accession number for the microarray data is GSE84809). For real-time PCR, cDNA was synthesized with iScript cDNA synthesis kit (Bio-Rad). Genes were amplified with iTaq Universal SYBR Green Supermix (Bio-Rad) with the primers listed in Supplementary Information and their relative amount to *Hprt* was calculated.

ELISA and metabolite measurements

Serum leptin (Sigma), adiponectin (Adipogen), free and total cholesterol (BioVision), free and total T3 (Alpha Diagnostic) and corticosterone (Abnova) were measured by commercial ELISA kits. For quantifying RELM α , ELISA plates were coated with rabbit anti-murine RELM α and serum RELM α was detected by biotinylated rabbit anti-murine RELM α (all reagents are from Peprotech).

Glucose tolerance tests and hyperinsulinemic euglycemic clamps

Experiments evaluating glucose homeostasis were performed according to recommendations by the NIH-funded Mouse Metabolic Phenotyping Center (MMPC) consortium (Ayala et al., 2010). GTTs were performed by intraperitoneally injecting 1 mg/g body weight dextrose. Glucose was measured in the tail blood using a YSI Glucose Analyzer or a Breeze2 Glucometer (Bayer) and insulin was measured by radioimmunoassay (Linco). Hyperinsulinemic euglycemic clamps were performed as previously described (Jurczak et al., 2012).

c-Fos staining in the brain

Under deep anesthesia, mice were perfused with a fixative containing 4% paraformaldehyde, 15% picric acid, 0.1% glutaraldehyde in 0.1 M phosphate buffer. Fifty micron sections were cut through the ARN and PBN of the brain, and every third section was used for immunostaining. Sections were stained with goat anti-c-Fos (Santa Cruz) and rabbit anti-POMC (Phoenix Pharmaceuticals).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Wang Guillin and Yong Kong for microarray analysis, and Daniel Okin and Ruslan Medzhitov for critical reading of the manuscript. This study was funded by support from HHMI, and grants from NIH AI054359, MMPC NIH DK059635 and Pilot and Feasibility Project Grants from Diabetes Research Center at Yale.

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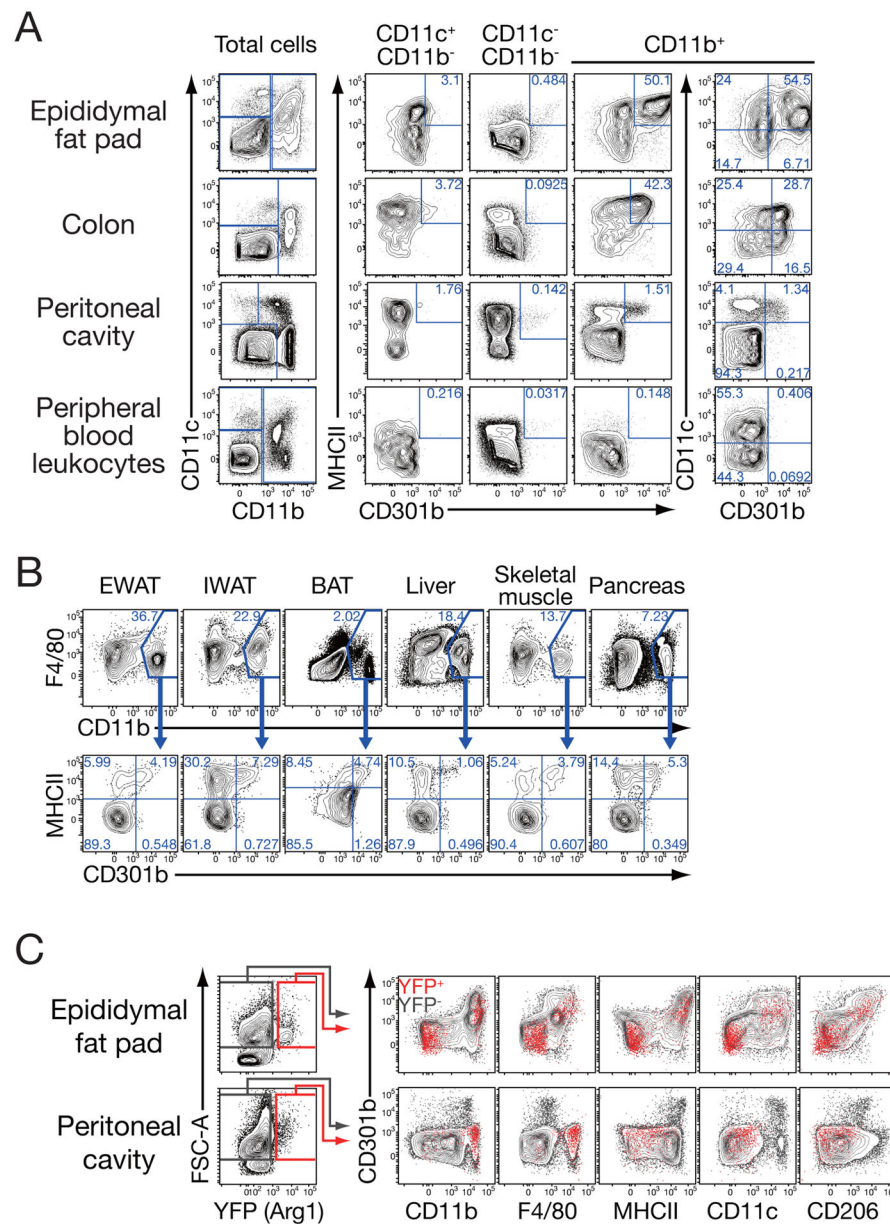


Figure 1. CD301b is expressed in a subset of MNPs

Expression of CD301b in MNPs isolated from indicated organs of regular chow-fed male WT (A, B) or YARG (C) mice. Cells were gated and overlaid as indicated. Representative data from more than five (A), three (B), and two (C) independent experiments are shown. EWAT: epididymal WAT, IWAT: inguinal WAT, BAT: interscapular brown adipose tissue.

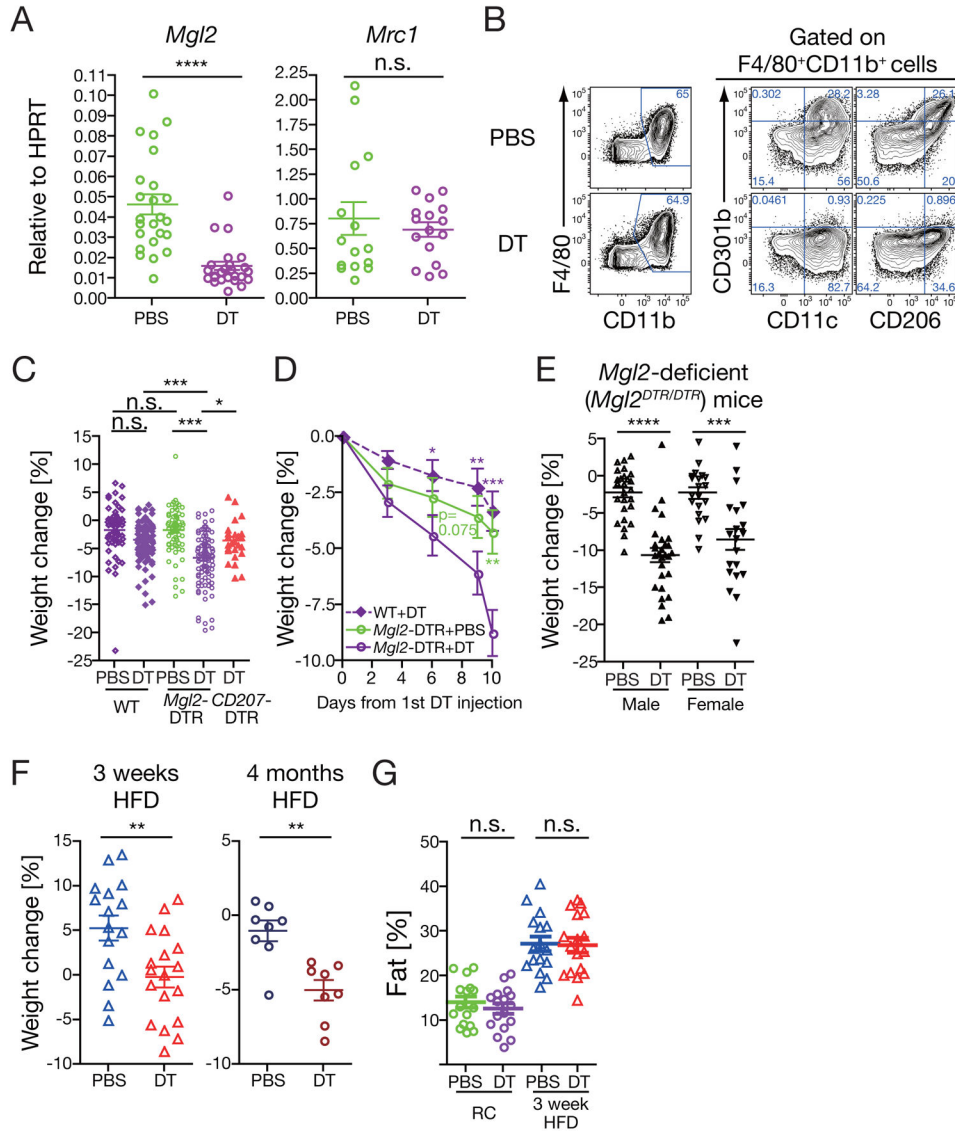


Figure 2. Depletion of CD301b⁺ MNPs results in weight loss

(A) Relative expression of *Mgl2* (CD301b) and *Mrc1* (CD206) in the epididymal WAT in DT-treated regular chow-fed *Mgl2*-DTR mice. *Mgl2*-DTR mice were injected intraperitoneally with 0.5 μg DT or PBS on days 0, 3, 6 and 9. On day 10, mice were sacrificed and mRNA expression was determined by real-time PCR. (B) Depletion of CD301b⁺ MNPs by DT treatment in *Mgl2*-DTR mice. Stromal vascular fraction was isolated from the epididymal WAT in *Mgl2*-DTR mice fed with HFD for 3 weeks and treated with DT or PBS for 10 days. Cells were stained and gated as indicated. Representative data from five independent experiments are shown. (C,D) Regular chow-fed mice of indicated strains were treated with DT as in (A) and weighed at day 10 (C) or at indicated time-points (D). Changes in body weight are indicated as percentage of the weight on day 0. Statistics in C was calculated by One-way ANOVA with Tukey’s post test. Statistics in D is calculated from 16 WT+DT, 19 *Mgl2*-DTR + PBS and 20 *Mgl2*-DTR +DT mice and indicates

comparison to DT-treated *Mgl2*-DTR mice with two-sided Student's t-test. **(E)** Male or female regular chow-fed homozygotic *Mgl2^{DTR/DTR}* mice were treated with DT for 10 days. Changes in body weight between day 0 and day 10 of DT treatment are shown. **(F)** *Mgl2*-DTR mice were fed with HFD for indicated period of time and treated with DT during the last 10 days of the HFD treatment as in **(A)**, and the changes in body weight were calculated as in **(C)**. **(G)** Adiposity was measured by nuclear magnetic resonance in *Mgl2*-DTR mice that were fed with regular chow or HFD for 3 weeks and treated with DT for 10 days. Data were pooled from two **(A)**, six **(B)**, five **(D)** and three **(E–G)** independent experiments. Each dot in graphs indicates an individual mouse and bars indicate mean \pm s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. n.s., not significant by two-sided Student's t-test unless otherwise indicated. Please see Figure S1.

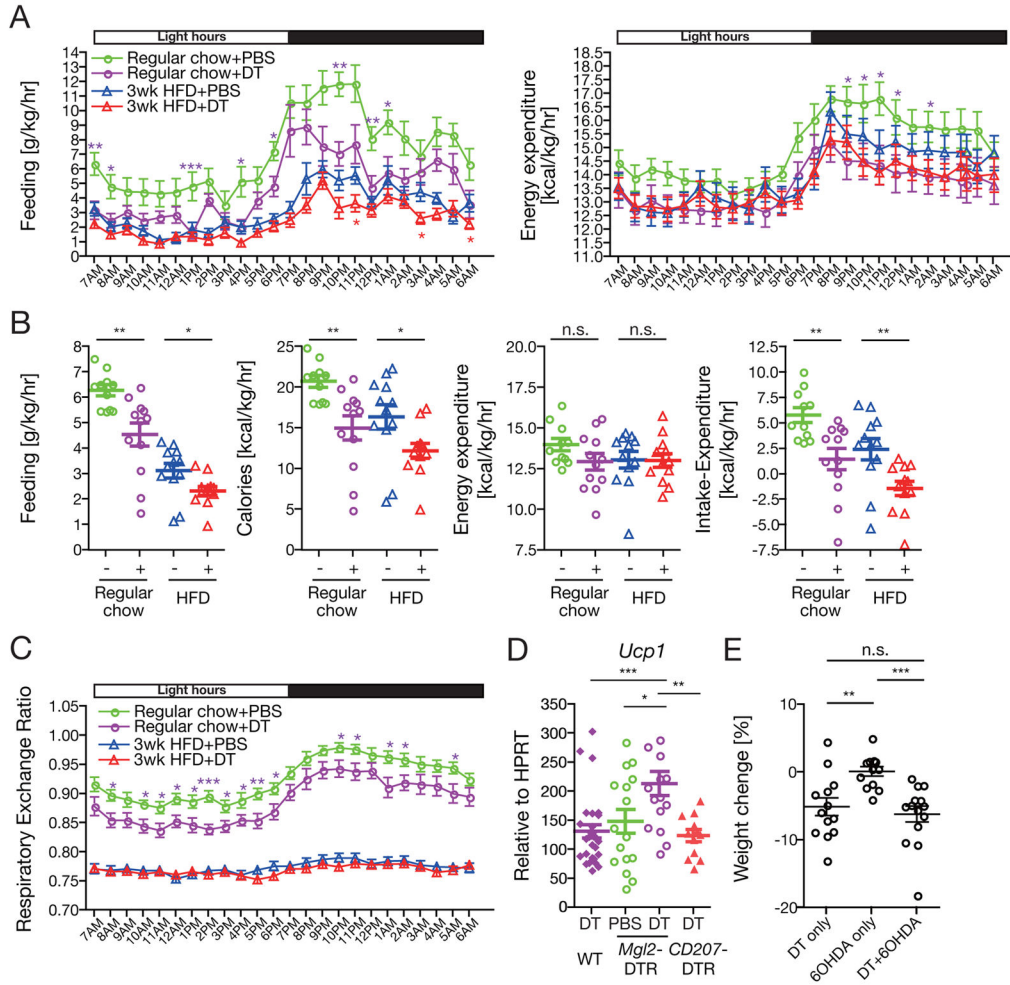


Figure 3. Depletion of CD301b⁺ MNPs results in a negative shift in the energy balance (A–C) *Mgl2*-DTR mice fed on regular chow or HFD for 3 weeks were individually housed and treated with DT for 10 days. Indirect calorimetry was performed in metabolic cages during the last 5 days of treatment. Hourly (A, C) and a whole-day daily (B) average data are shown. Data were pooled from two independent calorimetry experiments with total n=11–12 per group. (D) Regular chow-fed mice of indicated genotype were treated with DT for 10 days. Relative expression of *Ucp1* in the interscapular brown adipose tissue was determined by real-time PCR. (E) Regular chow-fed *Mgl2*-DTR mice were intraperitoneally injected with 6-hydroxydopamine (6OHDA) on days –3 and –1, then treated with DT on days 0, 3, 6 and 9. Changes in body weight between day 0 and day 10 were calculated. Bars indicate mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n.s., not significant by two-sided Student’s t-test. Please see Figure S2.

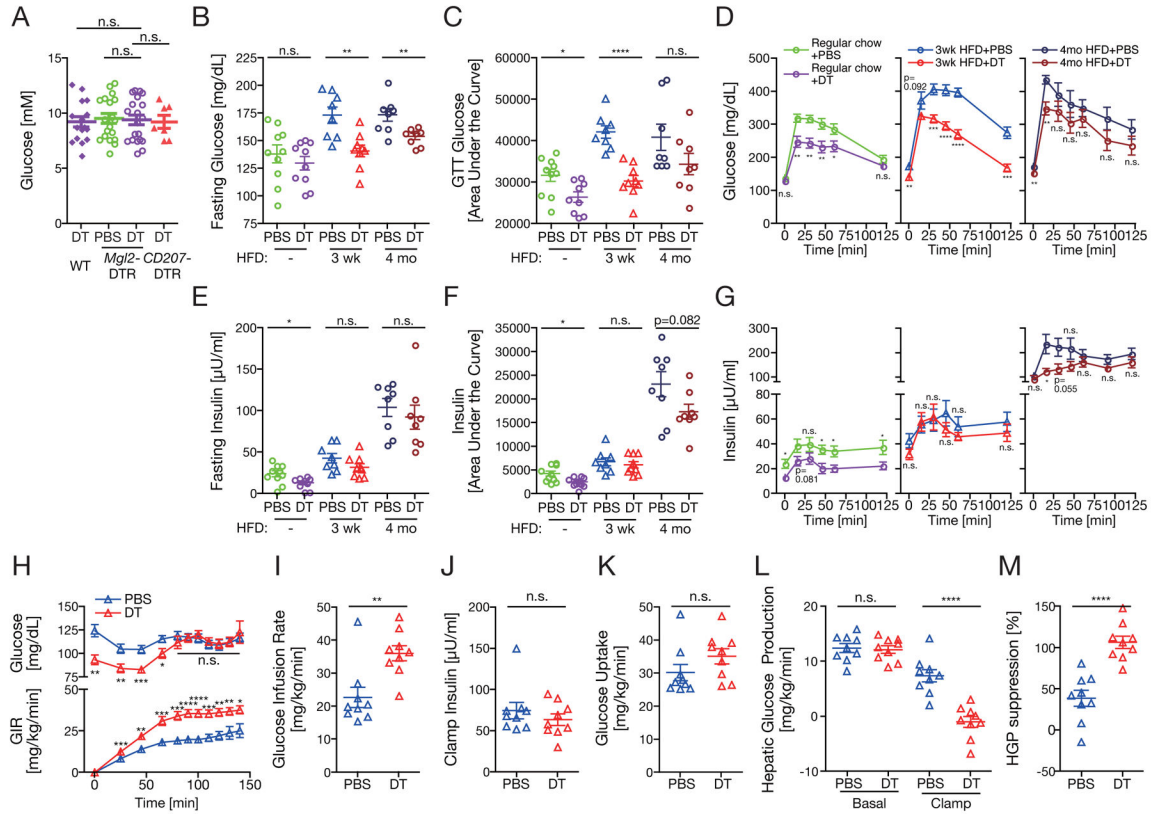


Figure 4. Depletion of CD301b⁺ MNPs results in hypoglycemia in both lean and obese mice (A) Serum glucose concentrations in DT-treated mice fed *ad libitum*. (B–G) GTTs were performed after an overnight fasting in *Mgl2*-DTR mice fed with regular chow, HFD for 3 weeks or HFD for 4 months and treated with DT for 10 days. Plasma glucose (B–D) and insulin (E–G) concentrations are shown. (H–M) Hyperinsulinaemic-euglycaemic clamp was performed on *Mgl2*-DTR mice fed with HFD for 3 weeks and treated with DT for 10 days. ³H-glucose tracer was included in the infusate so that rates of whole-body glucose uptake (K) and hepatic glucose production (L, M) could be determined during steady-state (final 40 min of study). Data were pooled from two independent experiments. Bars indicate mean \pm s.e.m. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001. n.s., not significant by two-sided Student’s t-test.

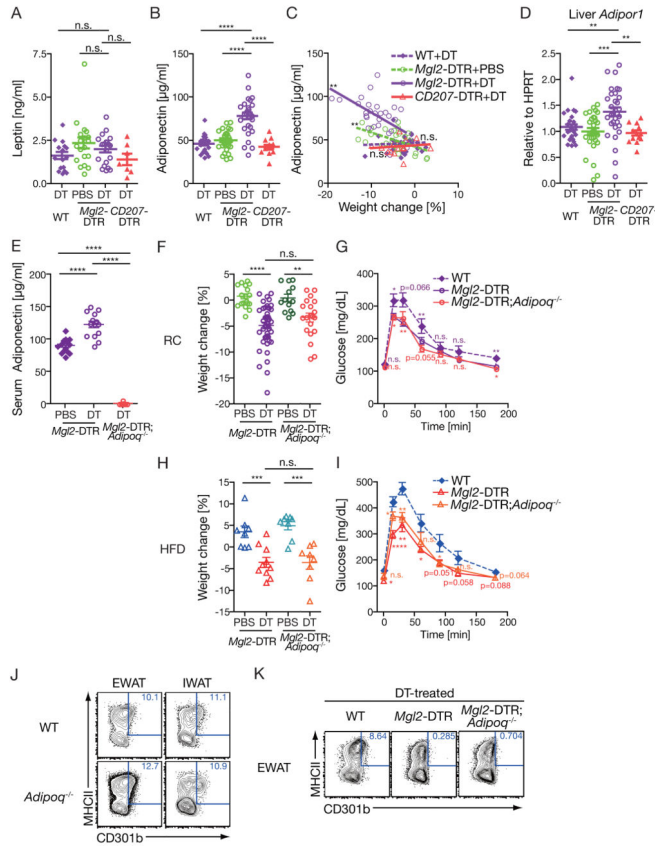


Figure 5. Depletion of CD301b⁺ MNPs results in elevated circulating adiponectin concentrations
 Regular chow-fed mice of indicated genotype were treated with DT for 10 days. (A,B,E) Circulating Leptin (A) and adiponectin (B,E) concentrations were determined by ELISA. (C) Correlation between weight loss and circulating adiponectin concentrations is shown. (D) Relative expression of *Adipor1* (Adiponectin Receptor 1) in the liver. (F–J) Weight change (F,H) and GTT (G,I) in *Mgl2*-DTR mice on *Adipoq*^{-/-} background fed with regular chow (F,G) or HFD for 3 weeks (H,I). (J, K) CD301b expression (J) and depletion (K) in mice on *Adipoq*^{-/-} background. Data are gated on F4/80⁺CD11b⁺ cells in epididymal (EWAT) or inguinal (IWAT) fat pads. Blood and tissue samples were collected in two (A–D), five (E–G) and two (H,I) independent experiments. Bars indicate mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n.s., not significant by two-sided Student’s t-test. Statistics in GTT curves indicates comparison to *Mgl2*-DTR mice.

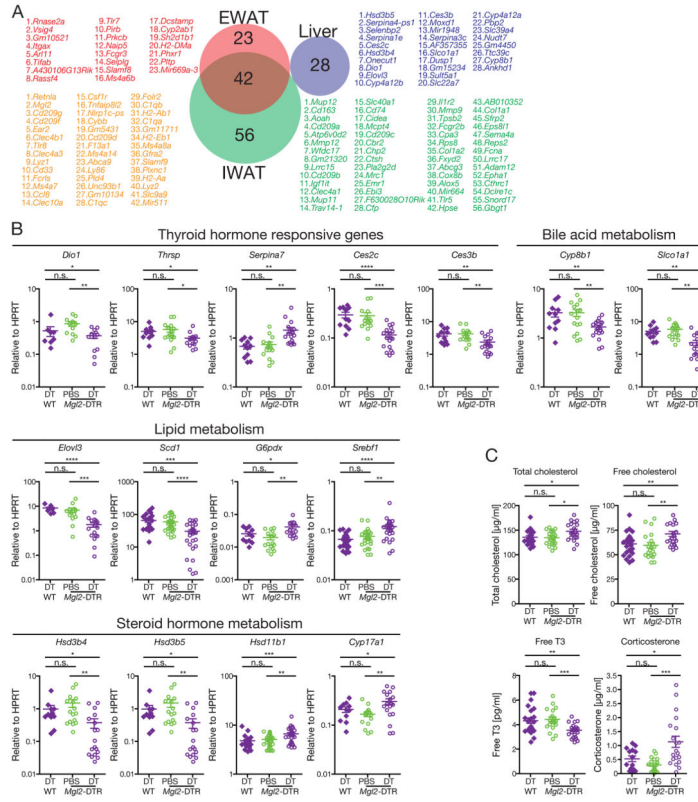


Figure 6. Depletion of CD301b⁺MNPs alters hepatic metabolism
 Regular chow-fed mice were treated with DT or PBS for 10 days. **(A)** Gene expression in indicated organs was compared between DT-treated WT and DT-treated *Mgl2-DTR* mice by microarray. Genes significantly downregulated more than 50% (two mice per group, $p < 0.01$) are shown in the order of magnitude of reduction. **(B)** Expression of indicated genes in the whole liver was examined by real-time PCR. **(C)** Cholesterol, T3 and corticosterone were measured in the sera. Each dot in graphs indicates independent mouse and bars indicate mean \pm s.e.m. Tissue samples were collected in four independent experiments **(B,C)**. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. n.s., not significant by two-sided Student's t-test. Please see Figure S3.

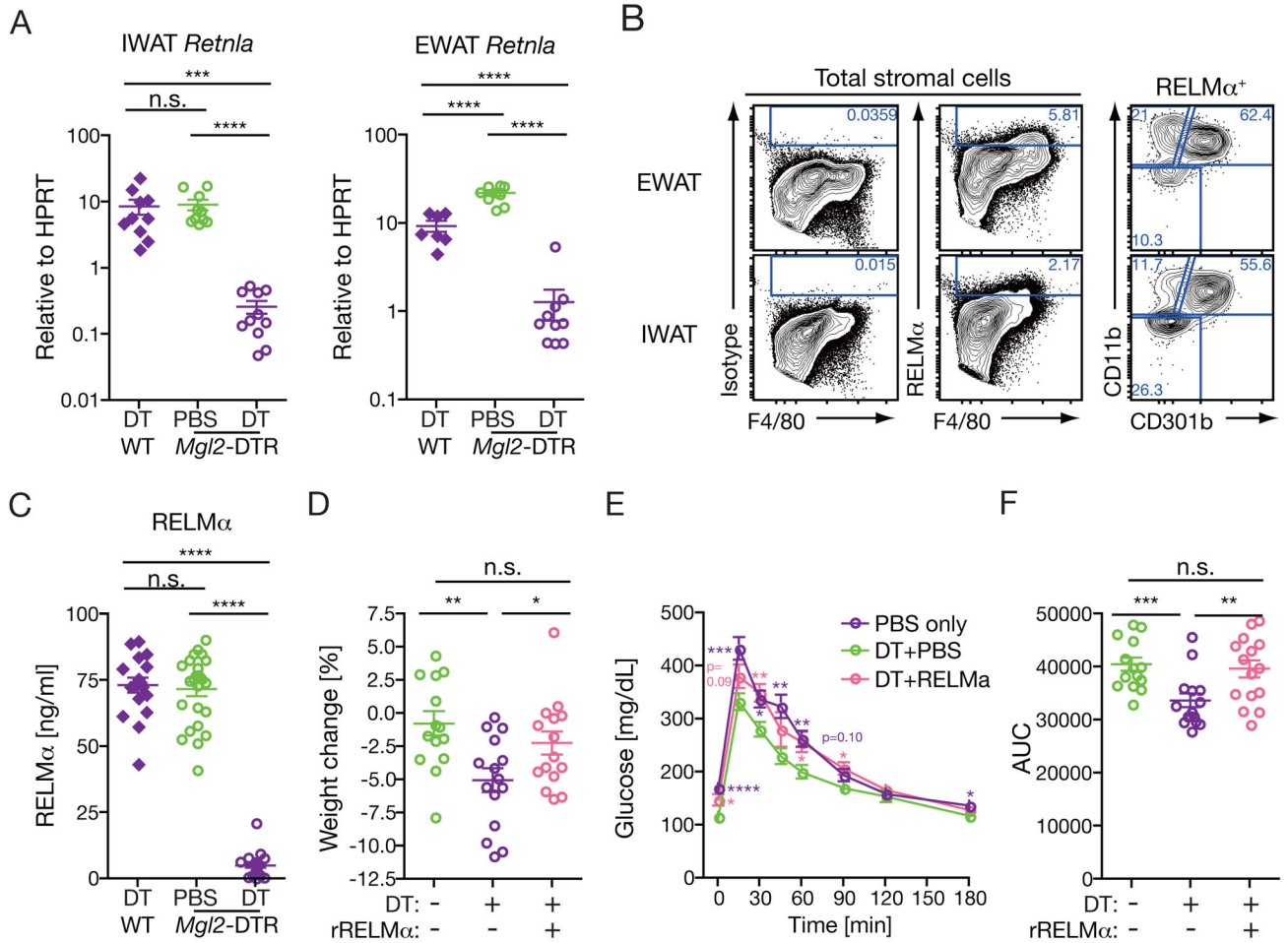


Figure 7. Reconstitution of RELM α restores body weight and normoglycemia in CD301b⁺MNP-depleted mice

(A,B) Regular chow-fed mice were treated with DT or PBS for 10 days. (A) RELM α mRNA expression was measured by real-time PCR in the whole IWAT and EWAT. (B) Intracellular RELM α expression in WAT MNPs. Representative data from two independent experiments are shown. (C) RELM α was quantitated in the sera by ELISA. (D–F) Regular chow-fed *Mgl2*-DTR mice were treated with DT with or without recombinant RELM α for 10 days. Weight change (D) and GTT (E,F) were examined as in Figure 4. Statistics in GTT curves indicates comparison to *Mgl2*-DTR mice with DT treatment alone. Bars indicate mean \pm s.e.m. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001. n.s., not significant by two-sided Student's *t*-test. Pooled data from three independent experiments are shown.