Macrophages are important for maintaining intestinal immune homeostasis. Here, we show that PPARβ/δ directly regulates CD300a in macrophages that express the immunoreceptor tyrosine based-inhibitory motif (ITIM)-containing receptor. In mice lacking CD300a, high-fat diet (HFD) causes chronic intestinal inflammation with low numbers of intestinal lymph capillaries and dramatically expanded mesenteric lymph nodes. As a result, these mice exhibit triglyceride malabsorption and reduced body weight gain on HFD. Peritoneal macrophages from Cd300a−/− mice on HFD are classically M1 activated. Activation of toll-like receptor 4 (TLR4)/MyD88 signaling by lipopolysaccharide (LPS) results in prolonged IL-6 secretion in Cd300a−/− macrophages. Bone marrow transplantation confirmed that the phenotype originates from CD300a deficiency in leucocytes. These results identify CD300a-mediated inhibitory signaling in macrophages as a critical regulator of intestinal immune homeostasis.
PPARβ/δ agonist represses inflammatory gene expression by releasing transcriptional co-repressor BCL-6 in macrophagesFig. 1b. In addition, PPARβ/δ has been supposed to attenuate chemokine receptor signaling by the induction of RGS proteins, which is involved in the termination of G protein signalFig. 1b. However, the mechanisms underpinning anti-inflammatory properties of PPARβ/δ have not been fully understood.

In the current study, we performed comprehensive analysis of PPARβ/δ regulated genes and genome-wide PPARβ/δ binding sites to facilitate our understanding of the function of PPARβ/δ in macrophages. We found that lack of CD300a, a novel PPARβ/δ target gene, expression in leucocytes relieves the TLR4/Myl88 signaling which leads to increase in proinflammatory cytokines in macrophages.

Results

PPARδ activates Cd300a in macrophages. To investigate the function of PPARβ/δ (NR1C2) in macrophages, we treated THP-1 macrophages with the high-affinity PPARβ/δ agonist GW501516 and performed a time course of global gene-expression analyses. These were combined with ChIP-seq analyses using newly-generated monoclonal antibodies against PPARβ/δ, as well as antibodies against its heterodimer partner, RXRαFig. 1a–c). We also generated genome-wide maps of modification sites for histone H3 lysine 4 mono- and tri-methylation (H3K4me1 and H3K4me3, respectively). Additionally, we identified binding sites for histone H3K4me1 modifications (Fig. 1c, Supplementary Fig. S2a). Because H3K4me1 is a marker for histone chromatin modification of enhancer sites (TGCCCT T TCACCT/C; PPRE) in this intron (Fig. 1d). This PPRE with this result, we identified a potential PPAR responsive element (PRE) in the promoter region of Cd300a (Fig. 1a, Supplementary Fig. S1a). This PPRE binds to PPARδ and RXRα (Fig. 1a). In fact, Cd300a was one of the most robustly induced genes.

Cd300a is an inhibitory immunoreceptor that fine tunes innate immune cell activity through an ITIM-mediated inhibitory signal (Fig. 1b)Fig. 1c, Supplementary Fig. S2a). Because H3K4me1 is a marker for histone chromatin modification of enhancer regions, these results indicate that Cd300a gene expression is regulated directly by the PPARδ/RXR heterodimer. In agreement with this result, we identified a potential PPAR responsive element (TGCCCT T TCACCT/C; PPRE) in this intron (Fig. 1d). This PPRE is conserved among mouse, rat, and human and was able to mediate PPARβ/δ dependent transactivation. Cotransfection of the luciferase reporter vector with a PPARβ/δ and RXRα expression vector increased luciferase activity, and this was further enhanced by the addition of GW501516 (Fig. 1e). Moreover, GW501516 treatment consistently increased the abundance of Cd300a mRNA in TIP-1 macrophages in a time- and dose-dependent manner (Fig. 1f,g). This induction was abrogated by siRNA-mediated knock down of PPARβ/δ (Supplementary Fig. S2b,c). GW501516-mediated Cd300a gene induction was observed in peritoneal macrophages from wild-type mice but not from PPARβ/δ-null mice (Fig. 1h). Another PPARβ/δ specific agonist, L-165041, also induced Cd300a mRNA (Supplementary Fig. S2d). Taken together these data identify Cd300a as a bona fide target of PPARβ/δ.

Cd300a deficiency causes gut inflammation under HFD feeding. To gain insights into the biological role of Cd300a, we generated Cd300a-null mice as described elsewhereFig. 1i. We fed these animals a diet rich in lard-based SFAs (HFD) (Supplementary Table S1a). Histological examination revealed enhanced immune staining of F4/88 (CD68) and F4/80 macrophages and Thy1.2 T cells in the intestine and colon of Cd300a−/− mice fed HFD (Fig. 2a,b, Supplementary Fig. S3). Oil red O (ORO) staining revealed marked lipid accumulation in the intestinal epithelium of these mice (Fig. 2c,d). Lymphatic capillaries were very narrow and hardly visible in the intestine from these Cd300a−/− mice fed HFD (Fig. 2e,f). Furthermore, the mesenteric lymph nodes (MLNs) were markedly expanded with accumulation of lipid-laden macrophages and foam cell formation indicating inflammation in MLNs (Fig. 2g–i). In addition, the length of small intestine and colon of Cd300a−/− mice are shortened by HFD feeding (Fig. 2m,n).

Transcriptome analyses of intestine showed up-regulation of the expression of chemokine and pro-inflammatory genes (FCγ1r, Fcγr3, Ccxl4, Ccxl3, and Il6), adhesion molecules (Vcam1, Esam1, and Pecam1) and the macrophage marker Cd68 in the same 18-week-old Cd300a−/− mice on HFD. These data suggest that macrophages are responsible for this chronic intestinal inflammation (Fig. 2o).

Cd300a−/− mice shows impaired lipid absorption under HFD feeding. Since dietary lipids are absorbed from the intestine by the lymph capillaries, we next examined the fat absorption of HFD fed Cd300a−/− mice. Acute fat-loading demonstrated that triglyceride (TG) levels in Cd300a−/− mice remained low in contrast to the Cd300a+/+ mice, and no peak in serum TG levels at 2 h occurred (Fig. 3a,b). Consistently, serum lipid content, including non-esterified free fatty acid (NEFA) and TG, is significantly reduced in the same Cd300a−/− mice on HFD (Fig. 3c–e). HPLC analysis of plasma lipoproteins showed a substantial reduction in chylomicron and very low-density lipoprotein (VLDL) TG levels in HFD-fed Cd300a−/− mice (Fig. 3f). Food intake, oxygen consumption, and rectal temperature showed no significant differences between Cd300a+/+ and Cd300a−/− mice (Fig. 3g–k). RQ value is significantly higher in HFD fed Cd300a−/− mice than that of Cd300a+/+ (P < 0.05) indicating lower utilization of lipid as an energy source (Fig. 3j). These observations indicated that the chronic intestinal inflammation associated with lymph capillary obstructions led to impaired intestinal lipid absorption of HFD fed Cd300a−/− mice. As a result of impaired lipid absorption, Cd300a−/− mice gained less body weight relative to Cd300a+/+ mice on HFD (Fig. 2p, Supplementary Fig. S4a–e). Feeding palm oil-based high saturated fat diets resulted in a similar phenotype to the lard-based HFD (Supplementary Table S1b, Supplementary Fig. S5a–c).

Hematopoietic deficiency of Cd300a augments TLR4 mediated IL6 production and exacerbates intestinal inflammation. To examine the influence of Cd300a deficiency on macrophages, we used oligonucleotide microarray and qPCR to examine classically-activated pro-inflammatory M1 and alternatively-activated anti-inflammatory M2 macrophage marker gene expressions in peritoneal macrophages. This analysis revealed that macrophages from Cd300a−/− mice on HFD resemble alternatively-activated anti-inflammatory M2 macrophages. They express a number of M2 markers including Cd163, Mrc1, Fobr2, Igf1, Clec4a3–22, whereas those from Cd300a−/− mice expressed classically-activated M1 markers including Il1a, Il6, Ccl3, Ccl4, Serpine1, Ptgs2, Tnfα20,21 (Fig. 4a,b, Supplementary Fig. S6). Lyve-1P, is considered another M2-macrophage related gene, whose product is pivotal in lymphatic vessel development. Intriguingly, Lyve-1 was highly induced upon HFD in Cd300a−/− mice and this was not observed in Cd300a+/− mice (Fig. 4a,b). These data suggested that Cd300a−/− mice may have a defect in preventing the
**Figure 1 | Cd300a is a direct target of PPARδ.** (a) Heat map: Color denotes the GW501516-induced changes in THP-1 macrophages. (b) Schematic diagram of CD300a. (c) Histogram of ChIP fragments. (d,e) PPRE present in intron 4 of Cd300a gene is conserved among species (d) and can mediate Cd300a induction by PPARδ (e). Error bars show s.e.m. **P < 0.01. (f,g) Time course of Cd300a expression in THP-1 cells exposed to GW501516/PMA (f) and dose response in THP-1 macrophages treated with GW501516 (g). (h) Cd300a induction by GW501516 is blunted in peritoneal macrophages from PPARδ-null mice (n = 3). Error bars show s.e.m. *P < 0.05; **P < 0.01 compared with DMSO treatment.
appropriate responses seen in chronic inflammatory and autoimmune diseases.

Because HFD induces activation of TLR4 signaling pathway and causes low-grade intestinal inflammation, we asked whether lack of CD300a expression relieves the inhibition of TLR4 signaling and in turn lead to an increase in pro-inflammatory cytokine production in macrophages. To examine this hypothesis, we treated peritoneal macrophages from Cd300a+/+ and −/− mice with LPS for 3 h to trigger the TLR4 signaling pathway and subsequently cultured them in LPS-minus media (Fig. 4c). LPS treatment led to a 60% reduction of Cd300 expression (Fig. 4d), consistent with results previously reported in monocytes and induced Il6 expression (Fig. 4e). Lack of Cd300a in macrophages resulted in 2.5-fold higher expression of Il6 upon LPS induction (Fig. 4e). Furthermore, while IL-6 secretion from Cd300a+/+ macrophages continued at similar levels during 24 h incubation after removal of LPS, IL-6 secretion from Cd300a−/− macrophages did not cease even after 24 h in the absence of LPS (Fig. 4f). These data together with our previous data indicate that Cd300a is pivotal in preventing or terminating TLR4-triggered Il6 expression and IL-6 secretion (Fig. 4g).

To specify whether HFD-induced intestinal inflammation in Cd300a−/− mice originates from malfunction of intestinal resident macrophages, we adoptively transferred CD300a-deficient bone marrow into lethally-irradiated wild-type mice (male C57BL/6 strain). After reconstitution for 4 weeks, age-matched cohorts were placed on HFD or NCD for 15 weeks (Fig. 4h). qPCR analysis confirmed >84% replacement of wild-type marrow by Cd300a−/− cells (Fig. 4i). Reconstitution of wild-type mice with Cd300a−/− bone marrow led to enhanced macrophage infiltration in the intestine of BMT-Cd300a−/− mice (Fig. 4j), resulting in resistance to body weight gain on HFD (Fig. 4h) with no apparent changes in food intake (Supplementary Fig. S7a). This is similar to results observed in Cd300a−/− mice on a BALB/c genetic background mice fed on HFD from 4 weeks old (n = 5–6 per group). Photograph shown is representative 18-week-old littermates. *P < 0.05; **P < 0.01 compared with Cd300a+/+ mice on HFD.

Discussion

The small intestine is the organ that digests and absorbs dietary nutrients such as lipids. It is also exposed to a host antigens from the diet and from commensal bacterial. Fatty acids are a constituent of lipid nutrients and also serve as signaling molecules that influence biological processes. Fatty acids are also part of the lipid moiety of LPS and play an important role in activation of TLR4. Fatty acids thereby induce NF-κB target genes such as cyclooxygenase 2 (Cox2) and Il6 in macrophages. By contrast, PPARβ/δ regulates expression of its target genes as a fatty acid sensor in macrophages.

In the current study, we show that activation of PPARβ/δ induced the Cd300a immunoreceptor. HFD feeding significantly induced intestinal inflammation in the Cd300a−/− and BMT-Cd300a−/− mice (Fig. 2A). PPARβ/δ target Cd300a was induced in HFD fed...
Figure 3 | Cd300a deficiency causes triglyceride malabsorption under HFD feeding. (a,b) Acute fat loading test (Cd300a+/+, n = 4; Cd300a−/−, n = 5). (c–e) Serum TG, NEFA, and cholesterol concentrations (n = 5–6 per group). (f) HPLC analysis of serum lipoproteins. Chylo, chylomicron; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein. (g) Food intake. (h) Oxygen consumption [VO₂], (i) CO₂ production rate [VCO₂], and (j) Respiratory quotient (RQ) (left) of Cd300a+/+ and Cd300a−/− mice (11–15 weeks old, n = 5 per group) fed on HFD used in Fig. 2. Note that RQ was significantly higher in Cd300a−/− mice during light cycle (i.e. Fasting phase) indicating fat utilization is lower in Cd300a−/− mice during fasting phase. (k) Rectal temperature at 18 weeks of age (n = 6 per group). Data represent the mean ± s.e.m. *P < 0.05; **P < 0.01.
Cd300a+/+ mice macrophages (Fig. 2m,4b). In parallel, expression of M2 macrophage marker genes was also increased. By contrast, in macrophages of Cd300a−/− mice fed HFD, pro-inflammatory Il6, ptgs2 (whose gene product is COX2), and Tnfa expression were increased (Figure 4a,b). These are all M1 macrophage markers. In cultured macrophages, LPS treatment led to the reduction of Cd300a expression (Fig. 4d). We also found that Il6 expression and secretion by LPS pretreatment were enhanced in the Cd300a−/− macrophages.
phages (Fig. 4e,4f). Based on the previous literature, we postulate that this is mediated by the TLR4/Myd88 signaling pathway.27,28 However, we do not exclude the possibility that other PPARβ/δ target molecules inhibit ITAM signaling (e.g. SH2B5 and DSCR1) or that an anti-inflammatory co-repressor BCL-6 suppresses inflammation, PPARβ/δ-Cd300a axis suppressed inflammatory cytokine IL6 production induced by TLR4/Myd88 signaling by dietary antigens. Based on these finding we propose that Cd300a, a novel PPARβ/δ target gene in macrophages, maintains intestinal immune response. In wild type mice, HFD-derivated fatty acids can activate the TLR4/Myd88 pathway, but the PPARβ/δ-Cd300a pathway inhibits TLR4/Myd88 pathway; therefore IL6 production is suppressed. In Cd300a−/− mice, HFD-derivated fatty acids activate only the TLR4/Myd88 pathway and IL6 production proceeds as illustrated in Figure 4g.

Dysregulation of the intestinal innate immune response is linked to metabolic disease29 and type 1 diabetes (T1D).30 Interestingly, Cd300a−/− mice exhibit higher blood glucose levels with reduced serum insulin levels in Cd300a−/− mice and BMT-Cd300a−/− mice compared to controls (Supplementary Fig. S9a, S7h,i, respectively) despite of impaired lipid absorption. Glucose tolerance tests further showed that glucose-induced increases in serum insulin levels were significantly reduced in Cd300a−/− mice on HFD (Supplementary Fig. S9b). It has been demonstrated that pancreatic islet-infiltrating lymphocytes express 4α/β7 integrin, which is a homing receptor to the gut mucosa31. In addition, it is well known that endocrine and exocrine cells of the pancreas are derived from a common set of epithelial cells from early gut endoderm. These observation suggested that pancreatic β-cells are affected by the homing lymphocytes during intestinal inflammation. Therefore, the PPARβ/δ-Cd300a axis may prevent food antigen-induced intestinal inflammation and metabolic diseases such as insulin resistance, atherosclerosis, and T1D.

These results suggest a novel mechanism through which PPARβ/δ activation leads to immuno-inhibitory receptor signaling to suppress chronic inflammation. The ability of PPARβ/δ to integrate metabolism and the innate immune system suggests that PPARβ/δ activation and subsequent CD300a induction could be a new therapeutic strategy to treat enteropathic diseases such as inflammatory-bowel-disease-like disease including Celiac disease32,33. In addition to our previous work which identified PPARβ/δ as a therapeutic target for the metabolic syndrome34, PPARβ/δ activation and its key role in intestinal immune modulation may also prove effective for the management of T1D.

Methods

The methods used in this study are described in detail in Supplementary Information.

Antibodies. Mouse monoclonal IgG-Y9705 against human PPARβ/δ GW501516 and fenofibric acid were synthesized as described previously35. lg-165041 was purchased from Sigma-Aldrich, rosiglitazone (BRL 49653) from Cayman Chemical, phenol 12-myristate 13-acetate (PMA) from Wako Pure Chemical Industries.

Chromatin immunoprecipitation (ChIP) was performed as described22 (Supplementary Information). Briefly, THP-1 cells after 24-h treatment with 10 nM PMA in the absence or presence of 100 nM GW501516 were treated with reagent samples (1% formaldehyde) for 10 min at room temperature and chromatin DNA was sheared by sonication. The resultant was immunoprecipitated with the indicated antibodies overnight at 4°C14. After washing and elution, the protein-DNA complexes were reverses by heating at 65°C overnight. Immunoprecipitated DNA was purified by using QIAquick spin columns (Qiagen). ChIP sequencing (ChIP-seq) was performed according to the manufacturer's instructions as described elsewhere (llumina)36. ChIP samples were also analyzed by gene-specific quantitative real time PCR.

Quantitative real-time PCR (qPCR). The qPCR method has been described22 (Supplementary Information). All primer sequences used in this paper are available on request.

Lentiviral shRNA knockdown. To deplete cellular PPARβ/δ, MISSION Lentiviral Packaging Mix and the lentiviral shRNA transfer vectors (Sigma-Aldrich) were co-transfected into 293FT cells using Lipofectamine 2000 (Invitrogen). Lentiviral particles carrying shRNAs targeting human PPARβ/δ were used to infect THP-1 cells.

 Luciferase reporter assay. Raw264.7 cells were transduced with the infected reporter together with expression plasmids (pCMV-hPPARα and pCMV-hRXRα) together with renilla luciferase plasmids (pGL-3 CMV) using Lipofectamine 2000 (Invitrogen) and treated with either with 100 nM GW501516 or vehicle (DMSO) 24 h after transfection. After overnight treatment, cells were lysed in lysis buffer (Promega) and analyzed using the Dual-Luciferase® Reporter Assay System (Promega). Firefly luciferase signal was normalized to renilla luciferase signal. All Luciferase assay data represent the mean ± s.e.m of triplicate samples.

Animal experiments. Cd300a-null (BALB/cA) (BALB/c back ground)37 and PPARβ/δ-null mice were generated as described elsewhere. All animals were housed in a temperature-controlled (24°C) facility with 12-h light/dark cycles (08:00 to 20:00 light) and allowed free access to water and NCD (CE-2; CLEA Japan) or HFD described in Supplementary Table 1. Food intake and body weights were monitored twice a week and core body temperature was measured using a rectal thermometer probe at 13:00. All mice were sacrificed at 13:00 and blood was taken from inferior vena cava. Serum TG, cholesterol, NEFA, and glucose levels were determined by Triglyceride E-Test Wako, Cholesterol E-Test Wako, NEFA C-Test Wako, Glucose
C-II Test Wako (Wako Pure Chemical Industries), respectively. Serum insulin, leptin and adiponectin levels were determined by ELISA using an immunonassay kit (Shibayagi, Gunma, Japan). For bone marrow transplantation, total bone marrow hematopoietic progenitor donor cells harvested from Cd300a−/− or wild-type mice on CBL/6J[c57BL/6]) background (backcrossed into C57BL/6J for 8 generations) were transplanted via orbital vein injection into lethally irradiated wild-type or Cd300a−/− mice, respectively, on C57BL/6J background (1100 rats; Cohal-6 source) with a minimum cell dose of 10⁶ mononuclear cells per mouse. Transplanted mice were housed in microisolator cages for 4 weeks prior to challenge with HFD. All data are presented as mean ± s.e.m. All mouse protocols were approved by the Animal Care and Use Committee of the University of Tokyo and Tsukuba.

Resting metabolic rate measurement. Seven to eleven weeks after HFD feeding was started, oxygen consumption was measured using open circuit indirect calorimetry (Model MK-5000, Muramachikika, Tokyo). The chamber volume was 720 ml, airflow to the chamber was 500 ml/min, samples were taken every 3 min and a standard gas reference was taken every 30 min. Mice were kept in the metabolic chamber and acclimated for 1 day.

Acute fat loading test. Acute fat loading test were performed in 12 week old HFD fed Cd300a+/+ and Cd300a−/− mice. Mice were orally administered 3 ml/kg BW of olive oil (Sigma, O1514) and blood samples were drawn from the tail vein at 0, 1, 2, 4, and 6 h after administration. Serum triglyceride and NEFA were measured.

HPLC analysis. An improved high resolution HPLC analysis of plasma lipoprotein was performed as described previously. 20 μl of plasma was mixed with 180 μl of saline and applied to four columns of TSK Gel Lipopack XL (Toho, Tokyo) connected in tandem. The detection of cholesterol and triglycerides in the post-column effluent was conducted by a simultaneous profiling system for lipoprotein cholesterol, triglyceride, and free glycerol in an on-line system.

Statistical analyses. All data are presented as mean ± s.e.m. The homogeneity in variance was evaluated by Bartlett test followed by parametric or non-parametric Dunnett’s multiple comparison test (one-side). The Student’s or Aspin-Welch t-test (one-side) was used to compare the data between the control and treated groups. *P < 0.05, **P < 0.01.

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How to cite this article: Tanaka, T. et al. PPARβ/δ activation of CD300a controls intestinal immunity. Sci. Rep. 4, 5412; DOI:10.1038/srep05412 (2014).

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