Free fatty acid receptor 2, a candidate target for type 1 diabetes, induces cell apoptosis through ERK signaling

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Abstract

Recent reports have highlighted the roles of free fatty acid receptor 2 (FFAR2) in the regulation of metabolic and inflammatory processes. However, the potential function of FFAR2 in type 1 diabetes (T1D) remains unexplored. Our results indicated that the mRNA level of *FFAR2* was upregulated in peripheral blood mononuclear cells of T1D patients. The human *FFAR2* promoter regions were cloned, and luciferase reporter assays revealed that NF- κ B activation induced *FFAR2* expression. Furthermore, we showed that *FFAR2* activation by overexpression induced cell apoptosis through ERK signaling. Finally, treatment with the *FFAR2* agonists acetate or phenylacetamide 1 attenuated the inflammatory response in multiple-low-dose streptozocin-induced diabetic mice, and improved the impaired glucose tolerance. These results indicate that *FFAR2* may play a protective role by inducing apoptosis of infiltrated macrophage in the pancreas through its feedback upregulation and activation, thus, in turn, improving glucose homeostasis in diabetic mice. These findings highlight *FFAR2* as a potential therapeutic target of T1D, representing a link between immune response and glucose homeostasis.

Key Words

- ▶ type 1 diabetes
- ▶ short-chain fatty acids
- ▶ free fatty acid receptor 2
- ▶ inflammatory
- ▶ peripheral blood mononuclear cells

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Introduction

Type 1 diabetes (T1D) is a multi-factorial, organ-specific autoimmune disease, characterized by selective and progressive loss of insulin-producing β -cells (Tisch & McDevitt 1996). T1D involves mononuclear cell infiltration

into the pancreatic islets of Langerhans, termed insulinitis, along with elevated levels of proinflammatory cytokines and chemokines, ultimately resulting in the selective destruction of pancreatic β -cells (Lehuen *et al.* 2010,

Baumann *et al.* 2012). Results from studies of T1D patients indicate that innate immune cells such as macrophages, dendritic cells, and natural killer cells are crucial components of the infiltrates as well as T cells (Lehuen *et al.* 2010, Coppieters *et al.* 2012). Therefore, numerous studies have investigated the therapeutic approaches for T1D prevention and interventions targeting the innate immune system. IL1 β (Mandrup-Poulsen *et al.* 2010), NF κ B (Lamhamedi-Cherradi *et al.* 2003), tumour necrosis factor alpha (Skyler *et al.* 2005), RAGE (Beyan *et al.* 2012), and HMG-CoA reductase (Strom *et al.* 2012) have all been demonstrated to be effective in treating T1D patients. However, more clinical studies are underway to evaluate their long-term efficiency (Baumann *et al.* 2012), and more details of T1D pathogenesis need to be explored in both human patients and animal models.

Short-chain fatty acids (SCFAs) are fatty acids with aliphatic tails of fewer than six carbons, mainly derived from the fermentation of dietary fibers and carbohydrates in the intestinal tract (Bindels *et al.* 2013). In addition to being minor nutrient sources in humans, SCFAs are also emerging as a class of signaling molecules in inflammation and metabolic processes (Layden *et al.* 2013). In 2003, two G-protein-coupled receptors (GPCRs), free fatty acid receptor 2 (FFAR2, also known as GPR43) and FFAR3 (also known as GPR41), were first identified as receptors for SCFAs (Brown *et al.* 2003, Le Poul *et al.* 2003, Nilsson *et al.* 2003). Functional studies have been carried out on FFAR2 and FFAR3 in different tissues and physiological conditions, but some of the results are controversial. Yanagisawa's group first demonstrated that FFAR3-stimulated leptin secretion in adipocytes and regulated gut hormones and motility (Xiong *et al.* 2004, Samuel *et al.* 2008). Then, several other groups argued that FFAR2 rather than FFAR3 inhibited lipolysis and triggered leptin secretion and adipogenesis (Hong *et al.* 2005, Ge *et al.* 2008, Zaibi *et al.* 2010). More recently, FFAR2 has also been reported to play a pivotal role in energy balance and GLP1 secretion induced by SCFAs (Kaji *et al.* 2011, Tolhurst *et al.* 2012, Kimura *et al.* 2013). However, these studies gave inconsistent results in mice fed normal-chow diets or high-fat diets due to different experimental models, methods of analysis and observation time points (Bjursell *et al.* 2011, Tolhurst *et al.* 2012, Kimura *et al.* 2013). In the immune system, FFAR2 has been shown to act as a chemotactic receptor for neutrophils and to affect inflammatory responses in models of colitis, arthritis, and asthma (Maslowski *et al.* 2009, Sina *et al.* 2009, Vinolo *et al.* 2011). Controversially, FFAR2 has been reported to induce exacerbated or persistent inflammation in different animal models (Maslowski *et al.* 2009, Sina *et al.* 2009).

Also, as inflammatory responses are involved in a range of metabolic diseases, FFAR2 may contribute to metabolic homeostasis through its role in the inflammatory response. Therefore, the function of FFAR2 is of importance in various ways, which need to be studied in more detail to uncover its role in metabolic and inflammatory regulation.

Our results indicate that FFAR2 expression is elevated in peripheral blood mononuclear cells (PBMCs) of recent-onset T1D patients, and we further explored its function both *in vitro* and *in vivo*. PBMCs are blood cells with round nuclei, including monocytes and lymphocytes, which are critical components of the immune system in both defense and autoimmune diseases. Our results indicate that the activation of FFAR2 improves glucose tolerance by inducing apoptosis of infiltrated immune cells. The transcriptional regulation of FFAR2 is also investigated in this study. Moreover, the synthetic agonist of FFAR2, phenylacetamide 1 (PA1), is shown to improve glucose tolerance and attenuate macrophage infiltration in pancreatic islets of diabetic mice. Taken together, these results indicate a novel mechanism of FFAR2 function in inflammatory responses in diabetes, and that therapeutics targeting FFAR2 might become a viable and effective therapy to T1D treatment.

Materials and methods

Subjects

Protocols in this study were approved by the Institutional Review Board of the Ruijin Hospital affiliated to Shanghai Jiaotong University School of Medicine. All procedures adhered to the tenets of the Declaration of Helsinki.

Patient recruitment, PBMC isolation, and other clinical and biochemical measurements were carried out as described previously (Zhang *et al.* 2012). The subjects were diagnosed as T1D within 6 months of onset, from January 2009 to October 2012 in our hospital, based on clinical findings of hyperglycemia and positive antibodies against glutamic acid decarboxylase (GAD-Ab). The ten recent-onset T1D patients in the Zhang *et al.* (2012) study were used as pilot subjects. In total, the study enrolled 33 patients with recent-onset T1D (16 men and 17 women, aged 13–31 years, mean $\pm$ S.E.M. 19.03 ± 4.61 years) and 34 healthy controls (24 men and ten women, aged 18–31 years, mean $\pm$ S.E.M. 22.88 ± 3.56).

Oral glucose tolerance tests were performed between 0700 and 0800 h after 10–12 h fasting. The blood samples were collected before administration of a standard dose of 75 g of glucose and at 30 min, 1, 2, and 3 h after the dose.

Animal models

Male C57BL/6 mice of 6–8 weeks of age were used in this study. The mice were kept under pathogen-free conditions and were given rodent diet and free access to water. For the multiple-low-dose streptozocin (MLDS) model, mice received an i.p. injection of 50 mg single-high-dose streptozocin (STZ)/kg body weight for 5 consecutive days. For the STZ model, mice received an i.p. injection of STZ at a dose of 200 mg/kg body weight. The mice received STZ that was freshly dissolved in 0.05 M citrate buffer (pH 4.5).

For treatment, mice received i.p. injections of sodium acetate at 500 mg/kg body weight, 10 or 30 mg/kg PA1 ((S)-2-(4-chlorophenyl)-3-methyl- N-(thiazol-2-yl)butanamide) (PA1), or vehicle for 3 consecutive days before and 2 h after STZ injection, and then daily injections for another 8 weeks.

Feeding blood glucose was measured with a glucose meter (OneTouch, LifeScan, CA, USA). The intraperitoneal glucose tolerance test was performed 4 weeks after STZ treatment with 2 g glucose/kg bodyweight. The insulin tolerance test (ITT) was performed 5 weeks after STZ treatment with 0.75 U insulin/kg bodyweight. The plasma insulin levels were measured with an ELISA (R&D Systems, Minneapolis, MN, USA) after 12 h of fasting.

Real-time quantitative PCR

RNA was extracted using TRIzol reagent (Invitrogen), followed by RT using the GoScript System (Promega). Real-time quantitative PCRs were carried out with QuantiFast probe assays (Qiagen) for human samples, and with LightCycler 480 SYBR Green I Master (Roche) for mouse samples on a LightCycler 480 Instrument II (Roche). All RNA expression levels were normalized to *Gapdh* expression. Primers used for mouse gene expression detection are listed in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article.

Cell transfection and treatments

Raw264.7 cells were cultured in RPMI 1640 (Invitrogen) with 10% heat inactivated fetal bovine serum (FBS) (Invitrogen). MCF7 and HEK293T cells were cultured in DMEM (11965 Invitrogen) with 10% FBS. The human FFAR2 expression plasmid (pcDNA/FRT/TO-FFAR2-eYFP) was kindly provided by Graeme Milligan (University of Glasgow). The cells were transfected with siRNA-targeting human FFAR2 (huFFAR2-145215, [Yonezawa et al. 2007](#))

using Lipofectamine 2000 (GenePharm, Shanghai, China) according to the manufacturer's instructions.

The Raw264.7 cells were treated with D-glucose (100 mM), lipopolysaccharide (LPS, 1 µg/ml), and methylglyoxal (MGO, 1 mM, Sigma-Aldrich). The NFκB inhibitor BAY11-7082 (10 µM), the p38 inhibitor SB203580 (10 µM), the JNK inhibitor SP600125 (20 µM), the ERK inhibitor U0126 (10 µM), and the protein kinase C (PKC) inhibitor GF109203X (5 µM) were all purchased from Merck. The cells were pretreated with inhibitors 1 h before LPS treatment.

The cells were starved with a medium containing 0.2% BSA without FBS for 8–12 h before treatment with compounds. Sodium acetate and sodium propionate were resolved in PBS (Sigma-Aldrich). Fluo-2 (Sigma-Aldrich) was used to calculate the relative Ca^{2+} concentration by measuring OD 340/380 nm under a patch clamp amplifier (EPC10; HEKA Elektronik, Lambrecht, Germany).

Luciferase reporter assay

The promoter sequence of human FFAR2 was cloned into the pGL4.15 luciferase reporter plasmid. The candidate transcription factor binding sites were analyzed with TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/>) and GeneCards (<http://www.genecards.org/>). The primers used for promoter cloning are listed in [Supplementary Table 1](#).

For the luciferase reporter assay, the cells were transfected with 500 ng plasmid/well in a 24-well plate (Corning Inc., Corning, NY, USA) with an SV40 internal control plasmid (250 ng for Raw264.7 and 20 ng for MCF7 cells). Luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega). For the serum-induced hFFAR2 reporter assay, sera from T1D patients or healthy controls were added to the cell culture medium (10% (v/v)) after reporter transfection. Serum from single-high-dose STZ or control treatment mice was added (20% (v/v)) for determination of mouse serum-induced hFFAR2 reporter activity.

Apoptosis detection

The number of living cells was quantified using the Cell Counting Kit 8 (CCK8; Dojindo, Kumamoto, Japan). Mitochondrial membrane potential and intracellular reactive oxygen species (ROS) were detected by using the JC-1 staining kit and the dichloro-dihydro-fluorescein diacetate (DCFH-DA) staining kit (Beyotime, Hangzhou, China) respectively with the BioTek Synergy Luminescence Reader (BioTek, Winooski, VT, USA). Flow cytometry analysis (FCM) was carried out using the anti-annexin V

and/or propidium iodide (PI) staining kits (R&D Systems) on a BD FACSCalibur Flow Cytometer. For microscopic observation, the cells were observed using a LEICA DMIRB light microscope with an Olympus DP71 Digital Camera (Olympus).

Western blotting and immunofluorescence

Western blotting was carried out as described previously (Jin *et al.* 2011). Polyclonal anti-poly-(ADP-ribose) polymerase (PARP) (full-length and cleaved form), anti-BCL2 and anti-caspase-3 (detecting both the full and cleaved forms) were purchased from Abcam (Cambridge, MA, USA). Polyclonal anti-phospho-p38 (Thr180/Tyr182), anti-phospho-ERK1/2 (Thr202/Tyr204), anti-phospho-SAPK/JNK(Thr183/Tyr185), anti-ERK, and anti- α -tubulin were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

For immunofluorescence analysis, the pancreases were fixed for 24 h in formalin, embedded in paraffin, and sectioned. Insulin and F4/80 antibodies were purchased from Abcam and Santa Cruz respectively. Secondary antibodies were purchased from Abcam and Jackson ImmunoResearch (West Grove, PA, USA). Nuclei were counterstained with Hoechst 33258 (Beyotime, Haimen, China). Macrophage infiltration was measured as the percentage of F4/80-positive islets and the F4/80-positive cell counts in islets based on measurements of all islets from three fixed-interval sections per mouse pancreas, with six animals analyzed for each group. The slides were visualized under a fluorescence microscope (Zeiss LM510) using Image Pro Plus 6.0 Software (Media Cybernetics, Rockville, MD, USA).

Statistical analyses

All the results are shown as the mean $\pm$ s.e.m. unless otherwise stated. Statistical comparisons between two groups were made with the Mann–Whitney *U* test, Wilcoxon signed-rank test, or the Student's *t*-test unless otherwise stated. The significance level was set at $\alpha = 0.05$. All tests were two-sided.

Results

The *FFAR2* mRNA level is elevated in PBMCs of patients with recent-onset T1D

The gene expression profiles in PBMCs of pilot subjects consisting of ten healthy controls and ten recent-onset T1D patients revealed that *FFAR2* mRNA expression was

Figure 1

FFAR2 expression is elevated in the peripheral blood mononuclear cells (PBMCs) of type 1 diabetes (T1D) patients. (A) Relative *FFAR2* mRNA expression in PBMCs of patients with recent-onset T1D ($n = 33$) and healthy controls ($n = 34$). *** $P < 0.001$. (B) HbA1c levels of healthy controls ($n = 9$), the low-*FFAR2* subgroup (normalized *FFAR2* mRNA expression less than twofold that of controls, $n = 18$), the high-*FFAR2* subgroup (normalized *FFAR2* mRNA expression greater than twofold that of controls, $n = 15$). * $P < 0.05$.

significantly elevated in T1D patients, whereas *FFAR1* and *FFAR3* expression were similar for patients and controls (Supplementary Figure 1A, see section on supplementary data given at the end of this article). When the subject group was expanded to 33 patients and 34 controls, the *FFAR2* mRNA expression of the patients was found to be 3.95 times higher than that of the controls ($P < 0.001$; Fig. 1A). However, when patients were subdivided according to *FFAR2* mRNA expression in PBMCs, the high-*FFAR2* subgroup exhibited a significantly lower HbA1c level than the low-*FFAR2* subgroup (Fig. 1B). Furthermore, a linear regression analysis among these T1D patients demonstrated a positive correlation between *FFAR2* mRNA expression and C-peptide level (Supplementary Figure 1B and C). These results indicate a potential correlation between *FFAR2* mRNA expression in the PBMCs of recent-onset T1D patients and their insulin secretion.

Sodium acetate ameliorates glucose tolerance in MLDS diabetic mice

Acetate is considered to be a more specific endogenous ligand of *FFAR2* than other SCFAs, which activate both *FFAR2* and *FFAR3* (Brown *et al.* 2003, Le Poul *et al.* 2003, Maslowski *et al.* 2009). Sodium acetate was used to activate *FFAR2* and to evaluate its role in diabetic mice. Treatment of MLDS mice with acetate led to a significant reduction in their feeding blood glucose compared with the vehicle group ($P < 0.01$; Fig. 2A). The glucose tolerance test (GTT) of acetate-treated MLDS mice showed significant improvement compared with that of the vehicle MLDS group (Fig. 2B), while the ITT was not significantly different

Figure 2

FFAR2 activation by sodium acetate ameliorates glucose tolerance in multiple-low-dose streptozotocin (MLDS) diabetic mice. Male C57BL/6 mice at 6–8 weeks of age were randomly assigned to vehicle or STZ and treated with sodium acetate or vehicle. (A) Random blood glucose levels of vehicle-treated control mice ($n=6$), acetate-treated control mice ($n=6$), vehicle-treated MLDS mice ($n=14$), and acetate-treated MLDS mice ($n=16$). $*P<0.05$, vehicle vs acetate-treated MLDS mice at the indicated time, the date indicated is after sodium acetate treatment; $**P<0.01$, paired Student's t -test, vehicle vs acetate-treated MLDS mice.

(Supplementary Figure 2A, see section on [supplementary data](#) given at the end of this article). Serum insulin analysis further showed a reduction in insulin secretion by 49.8% ($P<0.001$) in MLDS mice; whereas acetate treatment in those MLDS mice restored the insulin level by 37.5% ($P<0.01$; Fig. 2C). As has been reported, the immune response was shown to be activated in MLDS diabetic mice (Bellenger *et al.* 2011). To examine whether inflammatory pathways were involved in the protective role of acetate treatment, the expression of inflammatory genes in the spleen was analyzed. Splenic mRNA expression of IL6, one of the major inflammatory cytokines, increased by 95.5% ($P<0.01$) in MLDS mice, whereas acetate treatment reduced IL6 mRNA expression by 63.6% ($P<0.001$) (Fig. 2D). The expression levels of Vdac1 and Bid in the spleen were also upregulated by acetate treatment (Supplementary Figure 2B and C). Vdac1 and Bid are both dispensable for Bax activation and mitochondrial apoptosis (Baines *et al.* 2007, Kaufmann *et al.* 2007). These results indicate that FFAR2 activation by acetate can reduce the

(B) Intraperitoneal glucose tolerance test (IPGTT) of vehicle and acetate-treated MLDS mice at 4 weeks after STZ treatment with 2 g glucose/kg bodyweight. The right panel shows the statistical analysis of the area under the curve (AUC) of the IPGTT. $*P<0.05$. Mice were killed after 8 weeks of acetate treatment following STZ injection. The plasma insulin level (C) and the level of IL6 mRNA expression in the spleen (D) are reported. $**P<0.01$, $***P<0.001$, control vs MLDS mice; $##P<0.05$, $###P<0.05$, vehicle vs acetate-treated mice.

inflammatory response of immune cells, most probably by inducing their apoptosis, and protect pancreatic β -cells from destruction and increase insulin secretion.

Mutation of NF κ B-binding site in the FFAR2 promoter inhibits its reporter activity in Raw264.7 cells

To investigate the regulatory mechanism of FFAR2 expression, the 2.3-kb (–2266/+49) promoter of hFFAR2 was cloned and studied. Both LPS and phorbol 12-myristate 13-acetate (PMA), which are potent stimuli triggering immune responses, induced hFFAR2 transcriptional activity significantly in Raw264.7 cells, whereas only a slight effect was observed in MCF7 cells, breast cancer cells with a nonfunctional Toll-like receptor 4 (TLR4, LPS receptor; Supplementary Figure 3A, see section on [supplementary data](#) given at the end of this article). Reporter analysis on truncations of the 2.3-kb promoter further identified that the 0.5 kb promoter region (–500/+49 bp) exerted the highest transcriptional activity (Fig. 3C). These results

Figure 3

FFAR2 promoter activity in Raw264.7 cells. The luciferase activity of the *hFFAR2* 0.5 kb promoter (−500/+49 bp) is elevated in Raw264.7 cells cultured with serum from recent-onset T1D patients ($n=10$) (A) and serum from single-high-dose STZ model mice ($n=4$) (B). *** $P<0.001$, normal controls vs T1D patients; * $P<0.05$, control vs STZ mice. (C) Truncated *hFFAR2* promoter luciferase activities in the Raw264.7 cell line. Candidate transcription factor binding sites of the *hFFAR2* promoter (−500/+49 bp) are analyzed using online tools. *AP1*, *ELK1*, *FOXO1*, *NFκB*, and *SRF* are selected as candidate binding targets. (D) Transcriptional activities of *hFFAR2* promoters with mutations in the binding sites of their transcription factors treated by 1 μg/ml LPS for 16 h. * $P<0.05$, control vs LPS stimulated

WT promoter activity; # $P<0.05$, WT vs mutations with LPS treatment. Data are normalized to control levels for treatment with transcription factors. (E) Transcriptional activities of LPS-treated *hFFAR2* promoter pretreated with the *NFκB* inhibitor BAY11-7082 (10 μM), the p38 inhibitor SB203580 (10 μM), the JNK inhibitor SP600125 (20 μM), the MEK inhibitor U0126 (10 μM), and the PKC inhibitor GF109203X (5 μM). * $P<0.05$, ## $P<0.01$, DMSO vs inhibitors with LPS treatment; data are normalized to control levels for treatment with transcription factors. At least two independent experiments were carried out with four replicates for each assay.

indicate that the cloned 2.3-kb *hFFAR2* promoter exerts basic and inducible transcriptional activity with specificity, and that the 0.5-kb promoter region represents the core promoter. Elevated *FFAR2* expression in PBMCs was further reproduced *ex vivo* using sera from ten patients with recent-onset T1D or sera from four diabetic mice treated with a single high dose of STZ. The 0.5-kb *hFFAR2* promoter was significantly activated in Raw264.7 cells by the addition of sera from either T1D patients (175.4%, $P<0.01$; Fig. 3A) or STZ mice (199.8%, $P<0.05$; Fig. 3B).

Transcription factors such as *AP1* (*SYNRG*), *ELK1*, *FOXO1*, *NFκB*, and *SRF* were suggested as candidates after analysis of the core 0.5 kb promoter region with online tools described in the Materials and methods. Then, reporter plasmids carrying point mutations on the binding sites of the corresponding transcription factors were cloned and analyzed. Basic transcriptional activity tests showed that *AP1*, *ELK1*, *NFκB*, and *SRF*-binding site mutations significantly reduced *hFFAR2* transcriptional activity (Supplementary Figure 3B). When LPS was introduced to study the mutated binding sites of candidate transcription factors, *FOXO1*, *NFκB*, and *SRF* were shown to be involved in

LPS-induced *hFFAR2* transcriptional regulation (Fig. 3D). The inhibitor study further confirmed that the *NFκB* and JNK signaling pathways were involved in LPS-induced *hFFAR2* transcriptional activity in Raw264.7 cells (Fig. 3E). The *NFκB* pathway was further shown to be specifically involved in LPS-induced *hFFAR2* transcription, while not involved in glucose- or MGO-stimulated *hFFAR2* transcription (Supplementary Figure 3C).

FFAR2 activation reduces the number of living cells

Acetate and propionate, the endogenous ligands of *FFAR2*, both interact potently with *FFAR2* (Brown *et al.* 2003, Le Poul *et al.* 2003). A reduced number of living cells was observed after addition of acetate or propionate to Raw264.7 cell culture (Fig. 4A). Quantification by the CCK8 assay confirmed this effect of acetate and propionate (Fig. 4D). Furthermore, the overexpression of *hFFAR2* in HEK293T cells reduced cell density (Fig. 4B) and living cell number (Fig. 4E). Accordingly, knockdown of *hFFAR2* by siRNA (Yonezawa *et al.* 2007) in HEK293T cells increased cell density (Fig. 4C) and the number of living cells (Fig. 4F). Sera obtained from

Figure 4

Effect of FFAR2 activation on the number of living cells. (A) Microscopic observation of Raw264.7 cells treated with vehicle, 25 mM acetate, or propionate for 24 h. The statistical analysis of living cell number quantification by CCK 8 after treatment is shown in (D). (B) Microscopic observation of vector or hFFAR2 expression plasmid-transfected HEK293T cells. The statistical analysis of living cell number quantification by CCK8

24 h after transfection is shown in (E). (C) Microscopic observation of NC or hFFAR2 siRNA (huFFAR2-145215)-transfected HEK293T cells. The statistical analysis of living cell number quantification by CCK8 after transfection is shown in (F). Original magnification: 100-fold. At least two independent experiments were carried out with four replicates for each assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, control vs treatment.

STZ diabetic mice was also able to reduce the number of live Raw264.7 cells (Supplementary Figure 4A, see section on supplementary data given at the end of this article), which might be attributed to the induction of FFAR2 signaling (Fig. 3B). These results indicate that FFAR2 activation affects the number of living cells *in vitro*.

FFAR2 overexpression increases cell apoptosis, cytosolic Ca^{2+} concentration, and ROS level

FCM assays with annexin V and PI staining revealed a higher proportion of positive cells in hFFAR2-overexpressing HEK293T cells (Fig. 5A). The loss of the mitochondrial

membrane potential and the increase in the concentration of ROS are key mitochondrial events in apoptosis (Kroemer & Reed 2000). FFAR2-overexpressing HEK293T cells (Fig. 5B) and HeLa cells (Supplementary Figure 4C) exhibited a low mitochondrial membrane potential (green) and a reduced ratio of JC-1 polymers (red): monomers (green). Cytosolic ROS levels as measured by DCFH-DA staining were significantly higher in FFAR2-overexpressing HEK293T cells (Fig. 5C).

To exclude the possibility that transfected hFFAR2 was expressed as a nonfunctional protein, its function of releasing Ca^{2+} into the cytosol from the endoplasmic reticulum was tested after ligand stimulation (Tolhurst

Figure 5

Effect of hFFAR2 overexpression on cell apoptosis, cytosolic Ca^{2+} level, and ROS level. (A) HEK293T cells transfected with hFFAR2 or empty vector for 24 h were labeled with annexin V and PI and analyzed by flow cytometry (FCM). The right panel shows the quantification and statistical analysis of the FCM results shown in the left panel. (B) HEK293T cells transfected with hFFAR2 or empty vector were stained with JC-1. Red fluorescence indicates cells with normal mitochondrial membrane potentials, whereas green indicates an abnormal potential. The right panel shows the quantification and statistical analysis of the JC-1 ratio of polymer (red) to

monomer (green). (C) Quantification of cellular ROS level by DCFH-DA staining in HEK293T cells as indicated. (D) Cytosolic Ca^{2+} level of HEK293T cells stimulated by 10 mM acetate as indicated. The right panel shows the statistical analysis of the cytosolic Ca^{2+} level at the baseline as shown in the left panel. (E) Western blotting analysis of PARP, BCL2, and caspase-3 protein levels in HEK293T cells. At least two independent experiments were carried out in three replicates for each assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, empty vehicle vs hFFAR2.

et al. 2012). An instant Ca^{2+} release was detected after stimulation with 10 mM acetate (Fig. 5D). We also observed a higher baseline cytosolic Ca^{2+} concentration in hFFAR2-overexpressing cells than in the vector-transfected cells in the static state (Fig. 5D). As dysregulation of Ca^{2+} concentration is also a marker of apoptotic cells (Hajnoczky *et al.* 2006), these results further indicate that hFFAR2

activation by overexpression induces cell apoptosis. Then other markers of apoptotic cells were examined by western blotting analysis. The pro-apoptotic signals, cleaved PARP, and caspase-3 were increased, whereas the anti-apoptotic BCL2 signaling was decreased in hFFAR2-overexpressing cells (Fig. 5E). These results indicate that FFAR2 overexpression induces cell apoptosis *in vitro*.

Figure 6

FFAR2 activation induces cell apoptosis through ERK signaling. (A) Activity of ERK, p38, and JNK induced by hFFAR2 overexpression. (B) Quantification of mitochondrial membrane potential after treatment with MAPK inhibitors by JC-1 staining. (C) AP1 reporter assay after dose gradient transfection with the hFFAR2 plasmid in HEK293T cells. (D) Overexpression of hFFAR2 in the AP1 reporter assay following treatment by MAPK inhibitors, the p38 inhibitor SB203580 (10 μM), the JNK inhibitor SP600125

(20 μM), and the MEK inhibitor U0126 (10 μM). (E) FCM analyses of PI staining of hFFAR2 or empty vehicle transfected HEK293T cells treated by U0126 or DMSO. (F) Quantification of PI-positive HEK293T cells in (E). At least two independent experiments were carried out for each assay. ** $P < 0.01$, *** $P < 0.001$, empty vehicle vs hFFAR2. # $P < 0.05$, DMSO vs treatments.

FFAR2 activation induces cell apoptosis through ERK signaling

MAPK are recognized as being involved in apoptotic regulation (Wada & Penninger 2004). Analysis of the MAPK pathway in FFAR2-induced apoptosis revealed a clear activation of ERK signaling, as well as p38 and JNK activation (Fig. 6A). Quantification of JC-1 staining indicated that the inhibition of ERK signaling by U0126 reversed hFFAR2-overexpression-induced mitochondrial dysfunction, while JNK or p38 inhibitor showed slight effects (Fig. 6B). ERK activity was also analyzed by measuring AP1 transcriptional activity, which is downstream of ERK signaling. As shown in Fig. 6C, the AP1-LUC reporter assay showed that FFAR2 overexpression markedly activated AP1-driven luciferase activity in a dose-dependent manner. Inhibition of ERK by U0126 significantly reduced hFFAR2-induced AP1-LUC transcriptional activity, while other inhibitors had no significant effect (Fig. 6D). Furthermore, direct PI staining showed a significantly higher proportion of dead cells among hFFAR2-overexpressing cells than control cells, and

inhibition of ERK signaling by U0126 reversed this effect (Fig. 6E and F). These results indicate that hFFAR2 overexpression induces cell apoptosis through an ERK-dependent pathway.

FFAR2 activation by its agonist PA1 attenuates macrophage infiltration in MLDS diabetic mice

To further demonstrate the protective role of FFAR2 activation on T1D, the specific FFAR2 agonist PA1 (Lee *et al.* 2008) was i.p. injected into MLDS diabetic mice at doses of 10 or 30 mg/kg body weight daily along with the MLDS treatment. Mice were then killed for histological examination. Pancreatic islets showed that PA1 attenuates macrophage infiltration of MLDS mice (Fig. 7A), as indicated by the percentage of F4/80-positive islets (Fig. 7B) and the F4/80 positive cell count in islets (Fig. 7C). As macrophages play a crucial pathogenic role in both the initiation and destruction phases of T1D (Lehuen *et al.* 2010), this result further indicates a potential role of FFAR2 activation in the progress of diabetes involving

Figure 7

FFAR2 activation by the specific agonist phenylacetamide 1 (PA1) attenuates macrophage infiltration in MLDS diabetic mice. MLDS diabetic mice received i.p. injections of PA1 at 10 or 30 mg/kg body weight or of vehicle for 8 weeks after STZ treatment. Confocal images show F4/80 positive (red) macrophages and insulin positive (green) β-cells in islets. (A) Macrophage infiltration is measured as the percentage of F4/80 positive islets (B) and the F4/80 positive cell counts of islets (C), with measurements based on six pancreata per group and 59–107 islets per pancreas. * $P < 0.05$, control vs MLDS. # $P < 0.05$, ## $P < 0.01$, vehicle vs PA1. A schematic model of FFAR2-activation-induced protection of pancreatic islets of T1D (D). Insulitis

in T1D involves infiltration of lymphocytes and monocytes, resulting in the secretion of cytokines and chemokines. Next, NFκB, a master regulator of inflammatory responses, is elevated in T1D pathogenesis, contributing to the upregulation of FFAR2 in monocytes/macrophages. FFAR2 activation and upregulation induce cell apoptosis of monocytes/macrophages through ERK signaling. The attenuated inflammatory response improves the β-cell function and glucose tolerance of T1D patients. SCFAs and PA1 can play a positive role in the progression of T1D through the activation of FFAR2 signaling. The novel information on FFAR2 function revealed in this study is indicated by red arrows.

macrophage infiltration and viability. It is noteworthy that PA1 treatment at high doses also reduced mRNA expression of F4/80 and iNOS2 in liver, but not in adipose tissue (Supplementary Figure 5A and B, see section on supplementary data given at the end of this article) in the MLDS group. These results indicate that PA1 inhibits macrophage infiltration that applies to different tissues, although not all tissues.

Discussion

Pancreatic insulinitis frequently leads to T1D (Lehuen *et al.* 2010). Herein, we show that elevated FFAR2 expression is observed in recent-onset T1D patients and that FFAR2 expression can be activated by NFκB, a central regulator of inflammation (Baumann *et al.* 2012). Moreover, activation of FFAR2 can induce the apoptosis of macrophages

through ERK signaling, ameliorate macrophage infiltration, and improve glucose tolerance. These results indicate that inflammatory status in T1D patients may trigger a feedback protective mechanism involving the activation of FFAR2 in macrophages, leading to their apoptosis in pancreatic islets, and to the amelioration of insulinitis as well as the improvement of glucose tolerance (Fig. 7D).

PBMCs exhibit elevated expression of FFAR2 but not FFAR1 or FFAR3 (Fig. 1A and Supplementary Figure 1A), and this elevation is positively correlated with C-peptide levels in T1D patients (Supplementary Figure 1B and C). FFAR2, as a receptor for SCFAs, shares endogenous agonists with FFAR3 (Brown *et al.* 2003, Le Poul *et al.* 2003). Results from a recent study with reporter mice have highlighted that sensing of SCFAs in immune cells involved FFAR2, rather than FFAR3 (Nohr *et al.* 2013). Furthermore, acetate exhibited a much greater potency of interaction with FFAR2 than FFAR3, while propionate and butyrate have a similar potency of interaction with both receptors (Brown *et al.* 2003, Le Poul *et al.* 2003). As a major ingredient of vinegar, acetate has long been used as a food additive, and exhibits benefits for blood glucose control and diabetic management (Johnston & Gaas 2006). However, acetate supplementation does not result in any change in glucose tolerance in mice, and neither does *FFAR2* deficiency (Bjursell *et al.* 2011, Kimura *et al.* 2013). Bjursell *et al.* (2011) further showed that *FFAR2*-deficient mice exhibited an improved glucose tolerance and reduced body fat mass after a long duration (up to 20 weeks) of HFD feeding. In contrast, growing evidence shows that rather than *FFAR2* deficiency, both *FFAR2* transgenic expression and *FFAR2* activation by SCFAs improve glucose tolerance and insulin secretion (Tolhurst *et al.* 2012, Kimura *et al.* 2013). In this study, our results demonstrate that acetate improves glucose metabolism in MLDS diabetic mice, while acetate treatment alone shows no effect on control groups without MLDS (Fig. 2A and B).

Macrophage infiltration contributes to pancreatic insulinitis during the development of T1D (Denis *et al.* 2004, Cnop *et al.* 2005, Uno *et al.* 2007). Monocyte/macrophage *FFAR2* expression is among the highest of any human tissues (Brown *et al.* 2003, Le Poul *et al.* 2003). The macrophage cell line Raw264.7 used in this study reveals that *hFFAR2* transcription activity can be specifically induced by an immune stimulus (Supplementary Figure 3A), and NF κ B is involved in LPS-induced transcriptional activation (Fig. 3D and E). Involved in various steps of T1D pathogenesis (Baumann *et al.* 2012), NF κ B controls the expression of genes responsible for the activation and differentiation of macrophages, such as GM-CSF (Schreck

& Baeuerle 1990) and *iNOS* (Xie *et al.* 1994). The macrophages from NOD mice have increased NF κ B activity, supporting the secretion of high levels of inflammatory cytokines (Sen *et al.* 2003). In diabetic patients, NF κ B activation is inversely correlated with the quality of glycemic control (Baumann *et al.* 2012).

Overexpression of *FFAR2* sensitizes colon cancer cells to apoptosis when stimulated by propionate and butyrate (Tang *et al.* 2011a,b). Also, acetate treatment leads to the dose- and *FFAR2*-dependent apoptosis in bone marrow cells (Maslowski *et al.* 2009). Overexpression and siRNA knock-down of *hFFAR2* show that the activation of *FFAR2* is responsible for cell apoptosis not only in Raw264.7 cells but also in HEK293T and HeLa cells (Fig. 4 and Supplementary Figure 4). As indicated by the elevation of *hFFAR2* promoter activity induced in patients' serum (Fig. 3B), we deduced that diabetic status might trigger apoptosis in monocytes/macrophages through *FFAR2* activation, which was proven by *FFAR2* overexpression (Fig. 5). *FFAR2* has been reported to be a $G\alpha_{i/o}$ - and $G\alpha_q$ -coupled GPCR (Hong *et al.* 2005). $G\alpha_q$ -coupled *FFARs* trigger transient elevations in Ca^{2+} and MAPK activation (Hirasawa *et al.* 2005), whereas MAPKs are among the key pathways involved in immune and apoptotic regulation (Wada & Penninger 2004). MAPK signaling pathway analysis indicates that ERK signaling is necessary for *FFAR2*-overexpression-induced cell apoptosis (Fig. 6). As *FFAR2* has been shown to activate ERK1/2, which is required for chemotactic responses (Le Poul *et al.* 2003, Vinolo *et al.* 2011), the results of this study further emphasize the role of ERK1/2 activation in *FFAR2*-induced cell apoptosis.

In knockout mouse models of *FFAR2* and *FFAR3*, the compensatory expression of these proteins affects the results of functional studies of these receptors (Zaibi *et al.* 2010, Tolhurst *et al.* 2012). Therefore, PA1, a specific synthetic agonist of *FFAR2* (Lee *et al.* 2008, Maslowski *et al.* 2009, Vinolo *et al.* 2011, Kimura *et al.* 2013), has been used in this study to treat MLDS mice, resulting in attenuated macrophage infiltration (Fig. 7). Development of T1D arouses an inflammatory response, along with macrophage infiltration into pancreatic islets and an increase in pro-inflammatory cytokine secretion (Denis *et al.* 2004, Cnop *et al.* 2005, Uno *et al.* 2007). The controlled expansion and contraction of immune cells both during and after the immune response are imperative for the maintenance of a healthy, balanced immune system. Both extrinsic and intrinsic pathways of immune cell apoptosis are programmed to eliminate cells at the proper time to ensure immune homeostasis. A recent study with *FFAR2*-deficient and transgenic mice showed that *FFAR2*

activation reduced macrophage infiltration in adipose tissue (Kimura *et al.* 2013). Consistently with the results of this study, the change in inflammatory status represented by the IL6 level and macrophage infiltration in MLDS diabetic mice indicates that the activation of FFAR2 by acetate or PA1 limits the inflammatory response and improves glucose tolerance, most likely through feedback apoptosis of macrophages. Although we did not propose the difference in macrophage number in the circulation, as it is believed that activated macrophages with increased FFAR2 expression are more sensitive to PA1 treatment. Yet, it will be valuable to measure the difference in macrophage number in blood or spleen. Unexpectedly, the insulin-positive area of pancreatic islets showed no significant difference after PA1 treatment (Supplementary Figure 5C). However, the quantification of the plasma insulin levels in Fig. 2D showed that MLDS significantly reduced plasma insulin levels through induction of β -cell death, while acetate treatment significantly restored plasma insulin levels, possibly by inducing apoptosis of infiltrated/activated macrophages. Thus, the inconsistency of differences between the natural ligands and synthetic ligands provides the possibility for us to develop our study further to clarify the model.

The role of PA1 itself in the chemotaxis response of neutrophils (Vinolo *et al.* 2011) and $G\alpha_{i/o}$ -mediated intracellular signaling in adipocytes (Kimura *et al.* 2013) has been evaluated. PA2 (also termed (S)-2-(4-chlorophenyl)-N-(5-fluorothiazol-2-yl)-3-methylbutanamide (CFMB)) was found to elevate intracellular Ca^{2+} in intestinal L cells (Tolhurst *et al.* 2012). Another derivative of phenylacetamide, 4-chloro- α -(1-methyl ethyl)-N-2-thiazolyl-benzeneacetamide (CMTB), was shown to reduce the proliferation of leukemia cells (Bindels *et al.* 2012). The application of these synthetic agonists based on phenylacetamide facilitates studies of biological and pathological functions of FFAR2 in various processes, as they have significantly greater potency and specificity than any SCFAs (Lee *et al.* 2008).

In conclusion, the results of the current study indicate that FFAR2 is upregulated in PBMCs of individuals with T1D and subsequently triggers macrophage apoptosis through ERK signaling. FFAR2 can be regarded as a promising target linking the immune response with glucose metabolism.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0065>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Dr G N is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. G S, C S, M Y, X Z, and N Z analyzed the data. G N, G S, C S, and W G designed the study and wrote the manuscript. Y L, Z Z, P S, and Z Z helped to conduct the experiments and revised the manuscript.

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