

Inhibition of Islet Immunoreactivity by Adiponectin Is Attenuated in Human Type 1 Diabetes

Terence T. L. Pang, Myriam Chimen, Edward Goble, Natalie Dixon, Aled Benbow, Suzy E. Eldershaw, Dylan Thompson, Stephen C. L. Gough, and Parth Narendran

Centre of Endocrinology, Diabetes, and Metabolism (T.T.L.P., M.C., E.G., A.B., S.E.E., P.N.), School of Clinical and Experimental Medicine, College of Dental and Medical Sciences, University of Birmingham, Edgbaston B15 2TT, United Kingdom; Oxford Centre of Diabetes, Endocrinology, and Metabolism (S.C.L.G.), University of Oxford, Churchill Hospital, Headington OX3 7LJ, United Kingdom; and Department for Health (N.D., D.T.), University of Bath, Bath BA2 7AY, United Kingdom

Context: Adiponectin is an adipocyte-derived cytokine with insulin-sensitizing and antiinflammatory properties. These dual actions have not previously been examined in the context of human disease.

Objectives: Our objective was to examine the adiponectin axis in type 1 diabetes (T1D). T1D is an autoimmune inflammatory disease resulting from pancreatic β -cell destruction, in which insulin resistance associates with progression to disease.

Design, Patients, and Interventions: We measured circulating adiponectin and adiponectin receptor expression on blood-immune cells from 108 matched healthy, T1D, and type 2 diabetic subjects. We tested adiponectin effect on T cell proliferation to islet antigens and antigen-presenting function of monocyte-derived dendritic cells (mDCs). Lastly, we assessed the effect of a 3-week lifestyle intervention program on immune cell adiponectin receptor expression in 18 healthy subjects.

Results: Circulating concentrations of adiponectin were not affected by T1D. However, expression of adiponectin receptors on blood monocytes was markedly reduced and inversely associated with insulin resistance. Reduced adiponectin receptor expression resulted in increased T cell proliferation to islet-antigen presented by autologous mDCs. We demonstrated a critical role for adiponectin in down-regulating the costimulatory molecule CD86 on mDCs, and this function was impaired in T1D. We proceeded to show that lifestyle intervention increased adiponectin receptor but reduced CD86 expression on monocytes.

Conclusions: These data indicate that T cells are released from the antiinflammatory effects of adiponectin in T1D and suggest a mechanism linking insulin resistance and islet immunity. Furthermore, we suggest that interventions that reduce insulin resistance could modulate the inflammatory process in T1D. (*J Clin Endocrinol Metab* 98: E418–E428, 2013)

Adiponectin is the most abundant serum adipokine (1, 2). Adiponectin binds to at least 2 receptors, AdipoR1 and AdipoR2, which signal via ceramide and AMP kinase (3, 4). These receptors have been described on a variety of human tissues, including peripheral blood mononuclear cells (PBMCs) (5).

In contrast to most adipokines, adiponectin is distinguished by its insulin-sensitizing functions. Obesity and insulin resistance-related diseases such as type 2 diabetes (T2D) (6) and coronary artery disease (7) are associated with low levels of total adiponectin, and overexpression of adiponectin in animal models of these diseases confers protection (8, 9).

ISSN Print 0021-972X ISSN Online 1945-7197

Printed in U.S.A.

Copyright © 2013 by The Endocrine Society

doi: 10.1210/jc.2012-3516 Received October 2, 2012. Accepted December 10, 2012.

First Published Online February 5, 2013

Abbreviations: AdipoR, adiponectin receptor; APC, antigen-presenting cell; BMI, body mass index; CFSE, carboxyfluorescein-succinimidyl-ester; DC, dendritic cells; HbA1C, glycated hemoglobin; HC, healthy control; HLA, human leukocyte antigen; mDC, monocyte derived dendritic cells; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; SI, stimulation index; T1D, type 1 diabetes; T2D, type 2 diabetes; TT, tetanus toxoid; VO₂ max, maximal oxygen consumption.

Adiponectin is also distinguished by its antiinflammatory functions. Many of these functions have been demonstrated on cells of monocyte lineage. Chief among these are a change in the balance of proto-antiinflammatory cytokine production (10, 11) and a reduction in maturation, migration, and phagocytosis (12, 13). Recently adiponectin has also been demonstrated to affect the phenotype and function of murine monocyte-derived dendritic cells (DCs) (14). Here, adiponectin-treated DCs expressed lower levels of costimulatory molecules and showed less allostimulatory capacity when cocultured with T cells.

Although informative, these studies do not illustrate how the adiponectin pathway is modulated in human immunity, or how its antiinflammatory and unique insulin-sensitizing functions could interact in the context of human disease. We chose to examine these features of adiponectin in the context of type 1 diabetes (T1D). T1D is characterized by T cell-mediated destruction of insulin-producing pancreatic β -cells. It is a disease that requires professional antigen presentation by DCs, cells of myeloid lineage that are recognized to be adiponectin responsive (15, 16). It is also a disease in which insulin resistance associates with a more rapid onset of disease in genetically at-risk subjects (17–19).

We show that although circulating levels of adiponectin were unchanged in T1D, there was a striking reduction in adiponectin receptor expression by peripheral blood monocytes. We used serum-free conditions to demonstrate that reduced receptor expression resulted in protection from the antiinflammatory effects of adiponectin and a net proinflammatory effect on islet immunity. To dissect the mechanism of adiponectin effect, we examined the influence of adiponectin on antigen-presenting cell (APC) function and in particular on DCs. We demonstrated that adiponectin down-regulated expression of the costimulatory molecule CD86 by DCs, and this down-regulation was associated with suppression of antigen-specific T cell proliferation. We went on to show that an increase in adiponectin receptor expression by lifestyle intervention modulated CD86 expression on monocytes *in vivo*, thus illustrating translational application for our findings.

Materials and Methods

Subjects

Male Caucasian subjects with T1D and T2D were recruited from diabetic outpatient clinics at the University Hospitals Birmingham National Health Service Foundation Trust (United Kingdom) after informed consent. Matched healthy volunteers, with confirmed normal glucose tolerance and on no regular medications, were recruited on the same day. Full ethical approval was obtained for this study. Patients with T1D had been diagnosed according to guidelines outlined by the American Diabetes

Association. These included presentation with osmotic symptoms, weight loss, ketosis, and requirement for insulin within 3 months of diagnosis or presence of glutamic acid decarboxylase autoantibodies. Patients were presumed to have T2D if they did not have the above features, together with the absence of a family history of maturity-onset diabetes of the young, or a diagnosis of diabetes before the age of 40 years. They did not have associated endocrine autoimmune conditions and were all insulin treated. Specifically, insulin treatment was commenced more than 6 years after initial diagnosis, making the diagnosis of latent autoimmune diabetes of adulthood unlikely in our T2D cohort (20). Exclusion criteria include abnormal liver enzymes, microalbuminuria, poorly controlled diabetes [glycated hemoglobin (HbA1C) > 10%, 86 mmol/mol], acute illness and infection, heart failure, and use of thiazolidinediones [which independently increase AdipoR expression on monocytes (21)].

Measurement of adiponectin receptor expression of PBMCs by flow cytometry and quantitative PCR

The measurement of AdipoR1 and AdipoR2 has been described previously (5). Briefly, PBMCs were isolated from freshly drawn peripheral blood by density gradient centrifugation and preincubated with FcR blocker prior to staining with antibody. AdipoR1/2 antibodies (Phoenix Pharmaceuticals, Karlsruhe, Germany) were polyclonal, and their staining detected with a secondary goat antirabbit antibody (with preaddition of goat serum to avoid nonspecific binding). Specificity of AdipoR1/2 staining was confirmed by preincubating the antibodies with blocking peptides (Adipo R1/2; Phoenix Pharmaceuticals) before PBMC staining. Dead cells were excluded with propidium iodide staining. For each sample, at least 20 000 live cells were captured on flow cytometry (FACSCalibur; BD Biosciences, Abingdon, United Kingdom). The net mean fluorescence intensity (MFI) for AdipoR staining is the geometric mean fluorescence of the gated population, subtracted from fluorescence of the cells from the same subject stained with the secondary antibody only.

Quantitative RT-PCR was used to measure AdipoR1 and AdipoR2 gene expressions. Total RNA was extracted and reverse transcribed before analysis using previously published sequences of primers and probes for AdipoR1 and AdipoR2 (Eurogentec, Southampton, United Kingdom), standardized against 18S RNA endogenous control.

T cadherin was measured by RT-PCR using the intron-spanning primers (forward, CTGGACCGAGAGACTCTGGAA AAT, reverse, TGTGGCTTGAACTCTTTCTTGGT) using 35 cycles of PCR with 4 nM MgCl₂, 2 nM deoxynucleotide triphosphate, and Taq polymerase.

Specimens from all subjects were processed on the same day as phlebotomy.

Serum adiponectin by ELISA

Serum was stored at -80°C until analysis. Serum adiponectin levels were measured using a quantikine human total adiponectin immunoassay (R&D Systems, Abingdon, United Kingdom).

Antigens and reagents

Recombinant full-length adiponectin, produced from human embryonic kidney-293 cells, was purchased from Enzo (Exeter, United Kingdom). Tetanus toxoid was purchased from Calbiochem (San Diego, California). Cytokines were from Peptrotech

(Hamberg, Germany). Dr Hilary Murray (The Islet Research Laboratory, Worcestershire Acute Hospitals National Health Service Trust, Worcestershire, United Kingdom) prepared human islets from cadaveric specimens. Four preparations, each containing at least 20 000 islet equivalents IEQ, were sonicated and mixed. The OKT3 antibody was a kind gift from Dr Dave Sansom (University of Birmingham, Birmingham, United Kingdom).

Serum-free PBMC proliferative assay

The 2×10^5 carboxyfluorescein-succinimidyl-ester (CFSE)-labeled ($1.25 \mu\text{M}$) PBMCs in $100 \mu\text{L}$ of serum-free Cellgro SCGM media (Cellgenix, Freiberg, Germany), supplemented with penicillin 100 U/mL^{-1} , streptomycin 100 U/mL^{-1} , and glutamine 2 mM , were cultured in round bottomed 96-well plates. Incubation conditions were controlled at 37°C and 5% humidified O_2 . Each condition was tested in triplicate. The antigens and recombinant adiponectin were added at the beginning of the culture. IL-2 at $10^{-4} \mu\text{g/mL}^{-1}$ was added to cultures stimulated by islet lysates. The cells were fed complete media on day 4 and read on day 8. Stimulation index (SI) was calculated as the ratio of percentage of live CFSE low cells in a stimulated condition over that of the unstimulated control (22).

Serum-free DC generation and culture

CD14^+ monocytes were positively selected from whole PBMCs by magnetic sorting (Miltenyi, Church Lane Bisley, United Kingdom). The 5×10^5 cells were cultured in $500 \mu\text{L}$ of serum-free media Cellgro DC (Cellgenix), supplemented with $1 \mu\text{g}$ of granulocyte macrophage colony-stimulating factor and IL-4 and refed on day 2. Adiponectin at $10 \mu\text{g/mL}^{-1}$ was added at the beginning of culture. The DCs were harvested on day 6 and phenotyped with anti-CD80, CD86, human leukocyte antigen (HLA)-DR, dendritic cell-specific intercellular adhesion mole-

cule-3-grabbing non-integrin, CD1a, CD11c, and CD14 antibodies.

In experiments assessing the role of AMP kinase and p38 MAPK, compound C (Sigma, Dorset, United Kingdom), SB 203580 (Merck, Nottingham, United Kingdom), or dimethyl-sulfoxide was added to CD14^+ cells at 0.01 nM and then washed after an hour-long incubation.

To test the T cell stimulatory capacity, DCs were added at a ratio of 1:10 to 2×10^5 CFSE-labeled $\text{CD4}^+\text{CD25}^-$ cells in a final volume of $200 \mu\text{L}$ Cellgro SCGM complete media (Cellgenix). In experiments in which OKT3 antibodies were used ($0.5 \mu\text{g mL}^{-1}$), the effectors were obtained by sorting allogeneic whole PBMCs, first by CD4^+ negative selection and then CD25^+ depletion. With tetanus toxoid, fresh allogeneic PBMCs were used for sorting. Proliferation was assessed on days 4–6 by flow cytometry.

Exercise study in healthy individuals

Study participants were 45–64 years old, were nonsmokers, had a body mass index (BMI) of $25\text{--}35 \text{ kg m}^{-2}$, and were not on medication. Female subjects were all premenopausal. Prior to the study, they performed no regular structured exercise (defined as 30 minutes of moderate intensity activity for more than 5 days a week). All subjects followed a hypocaloric diet (5000 kcal/week) and had 5 supervised treadmill sessions per week for 3 weeks with 600 kcal of energy expended per session. Subjects were asked to perform at moderate intensity [50% maximal oxygen consumption ($\text{VO}_2 \text{ max}$), estimated based on target heart rate] for approximately 75 minutes per session. Venous peripheral blood samples were collected with full informed consent, in the fasting state, on the morning of day 1 (start) and day 22 (completion).

Table 1. Clinical Characteristics of Study Group

| | Healthy Control (n = 22) | T1D (n = 29) | T2D (n = 25) |
|--------------------------------|-----------------------------|-----------------|-------------------|
| Age, y | 32.6 ± 5.7 | 35.2 ± 10.7 | 54.8 ± 11.5^a |
| BMI, kg/m^{-2} | 25.2 ± 2.8 | 25.1 ± 4.1 | 35.3 ± 7.3^a |
| WHR | 0.90 ± 0.06 | 0.91 ± 0.09 | 1.00 ± 0.08^b |
| Systolic BP, mm Hg | 112 ± 11 | 137 ± 17 | 141 ± 15 |
| Diastolic BP | 75 ± 12 | 85 ± 10 | 85 ± 9 |
| Duration of diabetes, y | n/a | 15 ± 10 | 10 ± 7 |
| HbA1C, % | n/a | 9.0 ± 1.4 | 8.8 ± 1.9 |
| Total cholesterol, mmol | n/a | 4.4 ± 0.7 | 4.1 ± 0.9 |
| Triglyceride, mmol | n/a | 1.6 ± 1.0 | 2.3 ± 1.4 |
| Insulin dose, total daily U/kg | n/a | 0.85 ± 0.24 | 1.08 ± 0.15 |
| Statin use, % | n/a | 31 | 100 ^c |
| ACEI or ARB use, % | n/a | 52 | 80 ^c |
| Fibrate use, % | n/a | 0 | 4 |
| Diabetic retinopathy, % | n/a | 52 | 50 |

Abbreviations: ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BP, blood pressure; WHR, waist to hip ratio. All subjects were Caucasian males. Mean \pm SD values are represented for continuous variables. By *t* test, there were no significant differences in age, BMI, and WHR between T1D and HC groups. HbA1C, duration of diabetes, lipids, and rates of vascular complications were similar between T1D and T2D groups. The T2D subjects were older and had greater BMI and WHR compared with both HC and T1D subjects. By χ^2 test, ACEI/ARB and statin use was significantly higher ($P < .05$) in the T2D group, compared with the T1D subjects ($P < .0001$ and $P < .01$, respectively).

^a $P < .0001$.

^b $P < .01$.

^c $P < .05$.

Statistical analysis

Data are expressed as mean ± SD. The Mann-Whitney *U* test was used for nonparametric data and the χ^2 test for categorical data. For comparisons between more than 2 groups, ANOVA and Bonferroni adjustment for multiple testing was used. We used the paired *t* test for mean SI, of the triplicate from the same subject, between serum-free and adiponectin supplementation conditions. Two-tailed Pearson correlation analysis was used to analyze possible relationship between 2 set variables. Statistical analyses were performed on SPSS version 15.0 (SPSS Inc, Chicago, Illinois). Graphical plots were done using GraphPad Prism version 5.03 (San Diego, California) and Flowjo version 8.87 (Ashland, Oregon). Contour plots were done at the 5% probability level with the outliers shown. Dot plots depict a minimum of 8000 gated events.

Results

Total serum adiponectin concentrations are not affected by T1D

To examine the adiponectin axis in T1D, we first measured circulating adiponectin in subjects with this condition. Subjects with T1D were compared with age and healthy BMI-matched male Caucasian subjects (Table 1). We recruited a second comparator group of male Cauca-

sian subjects with insulin-treated T2D, matched for duration of diabetes, HbA1C, and exogenous insulin dose. Total serum adiponectin concentrations were not significantly different between T1D subjects and matched healthy controls (HCs) (T1D: $9.6 \pm 6.3 \mu\text{g/mL}^{-1}$; HC: $7.6 \pm 2.3 \mu\text{g/mL}^{-1}$, $P = .17$) (Figure 1A). In contrast, circulating adiponectin levels in T2D were significantly reduced (T2D: $4.2 \pm 2.8 \mu\text{g/mL}^{-1}$, $P = .0003$ vs HC). Within patients with T1D, there was a significant inverse correlation between serum adiponectin and BMI ($r = -0.397$, $P = .033$). Other than having BMI in the lowest quintile, the 3 T1D subjects with the highest serum adiponectin levels had no other distinguishing clinical characteristics.

Expression of AdipoR1 and AdipoR2 by peripheral blood monocytes is reduced in T1D

To further explore the effect of T1D on the adiponectin axis, we measured the expression of adiponectin receptors by PBMCs in subjects with this condition. We have previously described the presence of AdipoR1 and AdipoR2 on PBMCs, in particular on monocytes (5). We reproduced these findings (Supplemental Figure 1, published on The Endocrine Soci-

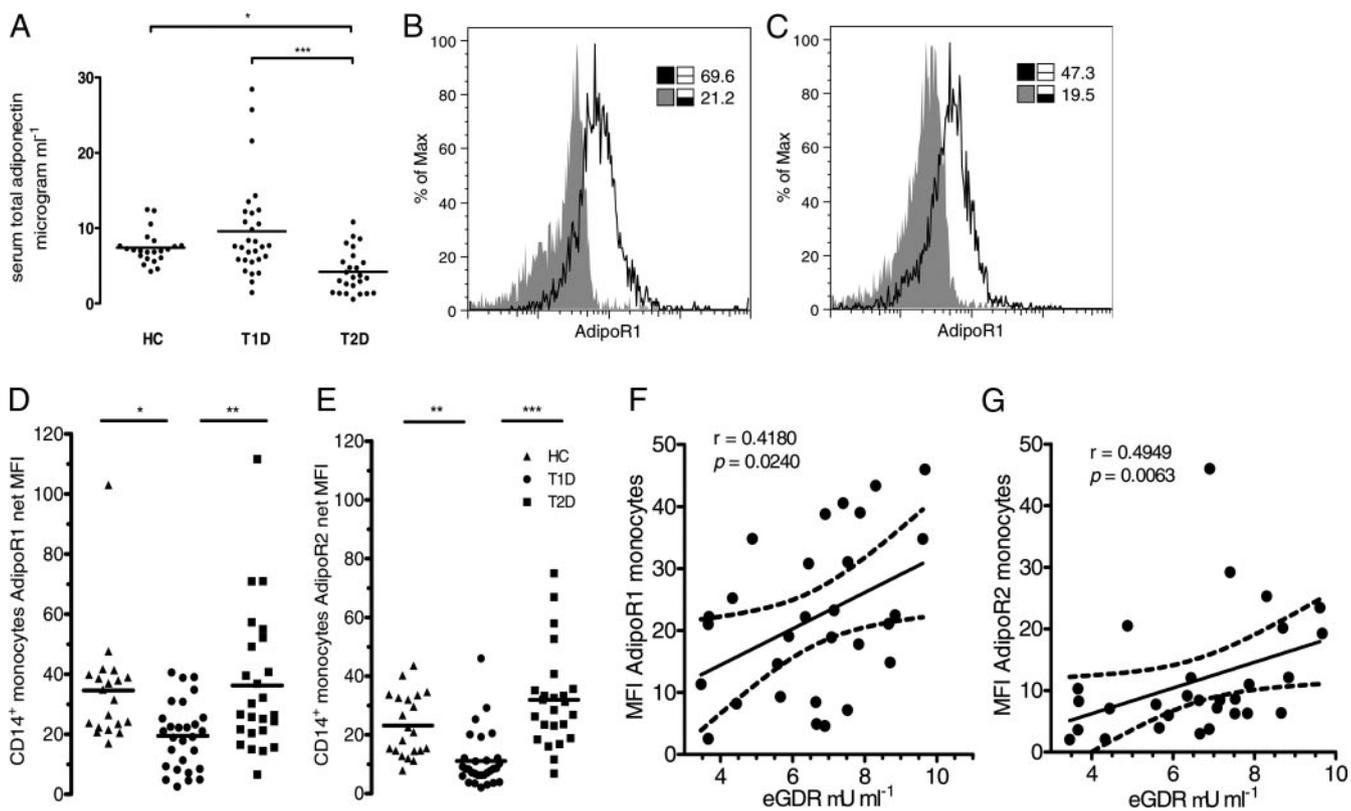


Figure 1. Reduced adiponectin receptor expression on monocytes in T1D. A, Serum adiponectin in healthy and diabetic subjects. B and C, AdipoR1 staining (open histograms) of CD14+ cells for healthy controls (B) and subjects with T1D (C), overlaid against isotype staining of cells from the same subject (filled histograms). In this example, net MFI for the healthy controls is 48.4 (B) and 27.8 in T1D subjects (C). AdipoR1 (D) and AdipoR2 (E) expression as measured by flow cytometry on CD14+ monocytes from HCs (triangles) and T1D (circles) and T2D (squares) subjects. Lines indicate the median value for each group. F and G, Pearson correlation of monocytic AdipoR1 and AdipoR2 MFI vs estimated glucose disposition ratio, a measure of insulin sensitivity in T1D. * $P < .05$, ** $P < .01$, *** $P < .005$ by a Bonferroni posttest.

ety's Journals Online web site at <http://jcem.endojournals.org>, including demonstrating a close correlation between expression at the level of protein and RNA (Supplemental Figure 2, A and C–E). T-cadherin, although reported to act as an adiponectin receptor on endothelial cells (23), was not detected on PBMCs (Supplemental Figure 2B).

Because monocytes expressed the highest levels of adiponectin receptors, we compared receptor expression on these cells between the T1D, healthy, and T2D subjects (Table 1). Expression of both AdipoR1 and AdipoR2 was decreased significantly in T1D, by approximately 40% when compared with matched healthy controls (Figure 1, B–E) (MFI AdipoR1, HC = 34.58 ± 18.47 , T1D = 19.46 ± 11.16 , $P < .01$; AdipoR2, HC = 23.14 ± 10.63 , T1D = 11.12 ± 9.36 , $P < .01$). The reduction in adiponectin receptor expression was also seen in all PBMCs (MFI AdipoR1, HC = 4.0 ± 1.6 , T1D = 2.3 ± 1.8 , $P < .01$; MFI AdipoR2, HC = 3.5 ± 1.7 , T1D = 1.3 ± 1.1 , $P < .01$, Supplemental Figure 2F), confirmed at the RNA level

(T1D AdipoR1 relative gene expression to HC = 0.27, $P < .01$; AdipoR2 = 0.55, $P < .05$) (Supplemental Figure 2G).

Within patients with T1D, monocytic expression of AdipoR correlated with estimated glucose disposition ratio, a clinical surrogate of insulin sensitivity in T1D (24) (Figure 1, F and G, AdipoR1, $r = 0.4180$, $P = .0240$; AdipoR2, $r = 0.4949$, $P = .0063$), and with BMI in patients with T2D (Supplemental Figure 2H). There was no significant correlation between AdipoR expression with HbA1C, insulin dose per kilogram, duration of disease, presence of vascular complications, or medication use in subjects with T1D.

Adiponectin inhibits antigen-driven CD4+ T cell proliferation, and this effect is reduced in T1D

We wanted to determine the functional significance of a reduced AdipoR expression in T1D. To do so, we first developed a standardized serum (adiponectin)-free assay of CD4+ T cell proliferation in which different PBMCs

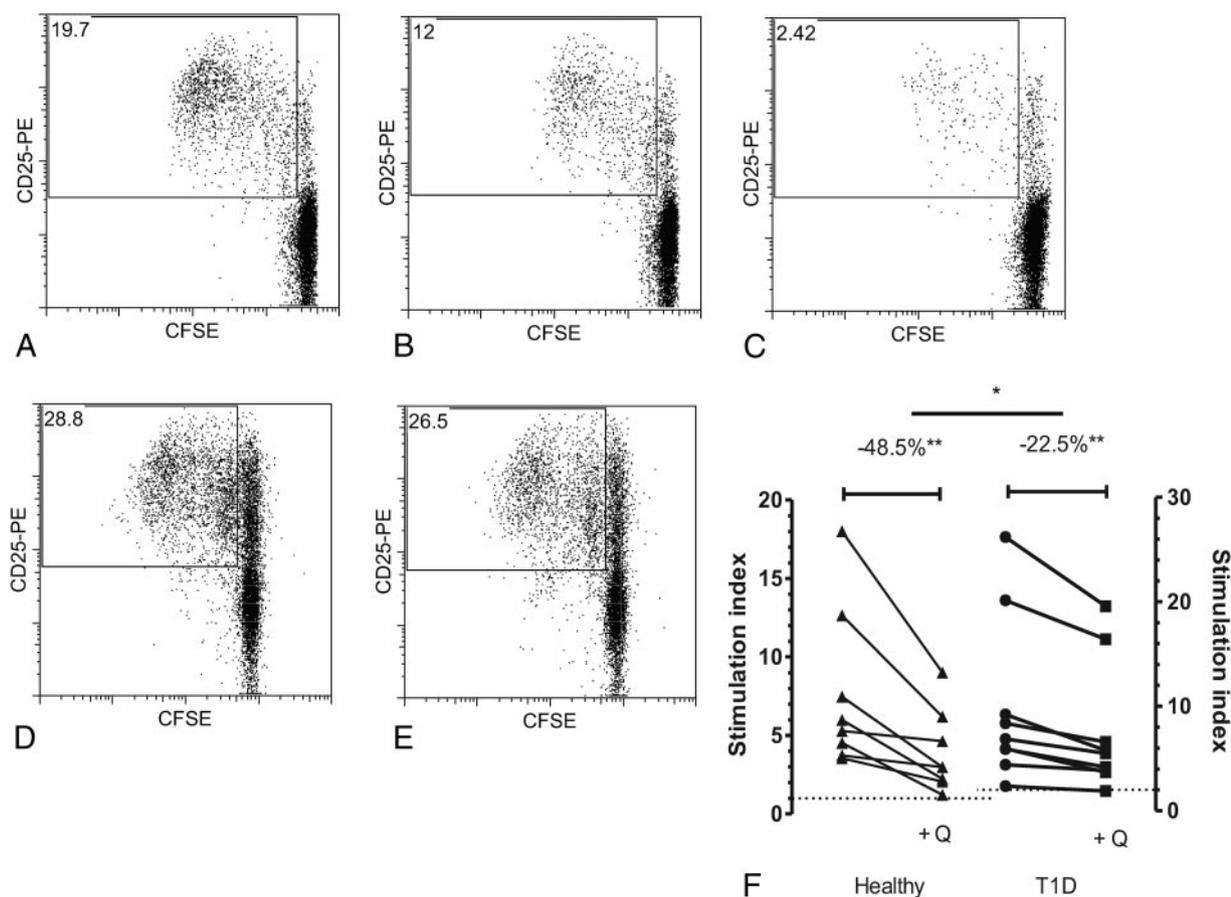


Figure 2. Adiponectin (Q) suppresses TT-stimulated CD4+ T cell proliferation in healthy individuals more than in people with T1D. Enumeration of proliferating CD4+ T cells in serum-free, whole-PBMC cultures stimulated by TT at $3 \mu\text{g}/\text{ml}^{-1}$ from a healthy subject (A) and after adiponectin supplementation at $10 \mu\text{g}/\text{ml}^{-1}$ (B) is shown. C, Background proliferation of CD4+ T cells in serum-free media in the absence of antigen and adiponectin. In this example, 2.42% of CD4+ T cells were gated in the no-antigen control. Thus, the SI to TT is $19.7 / 2.42 = 8.1$, and 5.0 after adiponectin supplementation. TT stimulation in serum free (D) and with adiponectin supplementation in a T1D subject (E) is shown. F, The SI of CD4+ T cells in serum-free culture and with adiponectin supplementation plotted as a joint pair (healthy, $n = 7$; T1D, $n = 8$). * $P < .05$, ** $P < .001$, paired t test.

could be cultured. Division of CD4+ T cells within whole PBMC cultures was tracked by CFSE dilution and concomitant up-regulation of IL-2 receptor (CD25) expression. In healthy subjects (n = 8), we demonstrated a consistent reduction in tetanus toxoid (TT)-stimulated CD4+ T cell proliferation when adiponectin was supplemented at a physiological concentration of 10 μg/mL⁻¹ (Figure 2, A and B, TT: serum free SI = 7.6 ± 5.1, adiponectin supplementation SI = 3.9 ± 2.6, P = .0085). This inhibition was also observed in response to human islet lysate (Figure 3, A and B) (serum free SI = 3.0 ± 0.6, adiponectin supplementation SI = 1.8 ± 0.3, P = .0004).

To determine the functional significance of reduced adiponectin receptor expression in T1D, we compared the effect of adiponectin on TT and islet antigen stimulated T cell proliferation in these patients. An adiponectin concentration of 10 μg mL⁻¹ was used to model the similar serum levels seen in vivo in health and T1D. Adiponectin inhibited T cell proliferative responses to TT and islet lysate in T1D, but this inhibitory effect was significantly lower than in HC (Figures 2 and 3, 3% reduction in SI after adiponectin treatment: TT, HC = 48.5 ± 7.4%, T1D = 22.5 ± 2.9%, P = .0124; islet lysate, HC = 38.7 ± 2.9%, T1D = 27.0 ± 5.6%, P = .0430). Similar distributions of CD14+ monocytes were observed in both groups, again matched in age, sex, ethnicity, and BMI.

Adiponectin-treated mDCs have reduced CD86 expression and CD4+ T cell stimulatory capacity

Resting T cells do not express adiponectin receptors (5). To explore the mechanism of adiponectin effect on T cell

proliferation, we therefore examined the effect of adiponectin on antigen presenting function. We chose to study DCs because they are professional APCs critical to the development of T1D. Also, they can be generated from blood monocytes that express high levels of adiponectin receptors.

Monocyte-derived DCs (mDCs) were expanded ex vivo in serum-free conditions and on day 6 had the phenotype of immature DC generated in 10% fetal calf serum, the standard approach to generating mDCs (Figure 4, A, C, and E; and Supplemental Figure 3). They were CD1a^{high}, CD14^{low}, and expressed HLA class II and the costimulatory molecules CD80 and CD86. The addition of physiological concentrations of adiponectin at the beginning of culture significantly reduced the expression of CD86 but not CD80 or HLA-DR (Figure 4, A–F).

The inhibition of CD86 was adiponectin dose dependent (Supplemental Figure 4, C and D) but not observed if adiponectin was added after the first 18 hours of the 6-day culture (Supplemental Figure 4B). Adiponectin is an established activator of AMP kinase and p38 MAPK in immune cells (10, 25). Inhibition of AMP kinase, but not p38 MAPK, ameliorated adiponectin effect on CD86 expression by CD1a+mDC (Supplemental Figure 4, E and F).

CD86 provides a costimulatory signal for T cell activation. Consistent with reduced CD86 expression, adiponectin-treated mDCs had reduced CD4+T cell stimulatory capacity (Figure 4, G–I). The proliferation of CFSE+CD4+CD25– effector cells was significantly re-

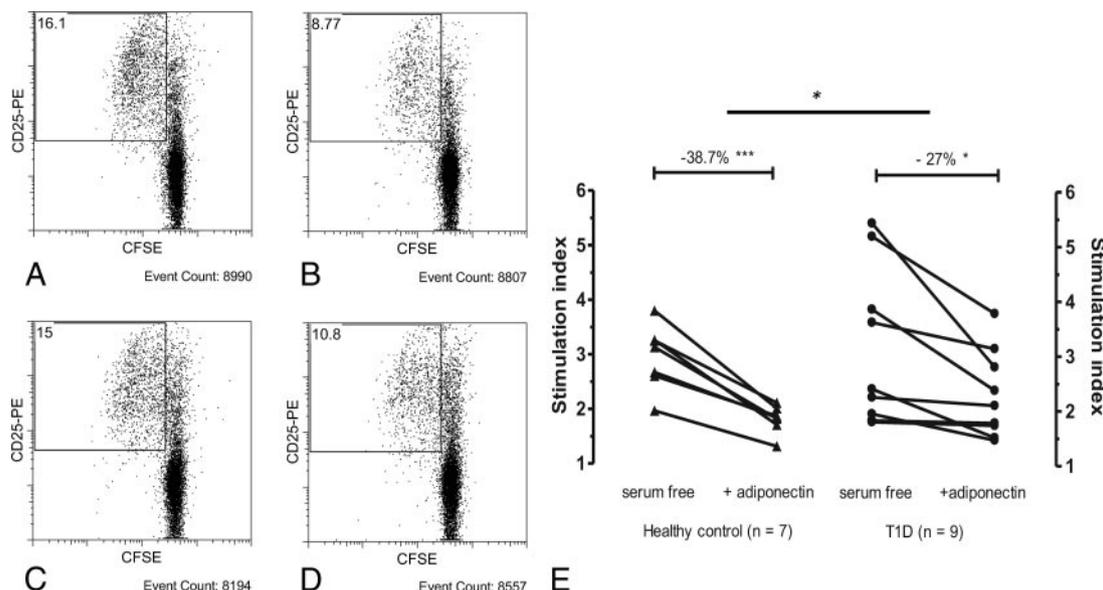


Figure 3. Adiponectin suppresses islet antigen-stimulated CD4+ T cell proliferation in healthy individuals more than in people with T1D. Enumeration of proliferating CD4+ T cells in serum-free, whole-PBMC cultures stimulated by human islet lysates (at 5 μg/ml⁻¹) and IL-2 (10⁻⁴ μg/ml⁻¹) from a healthy subject (A) and (B) after adiponectin supplementation. Islet lysate stimulation in serum-free culture (C) and with adiponectin supplementation in a T1D subject (D). E, SI of CD4+ T cells in serum-free culture and with adiponectin supplementation plotted as a joint pair. * P < .05, *** P < .001, paired t test.

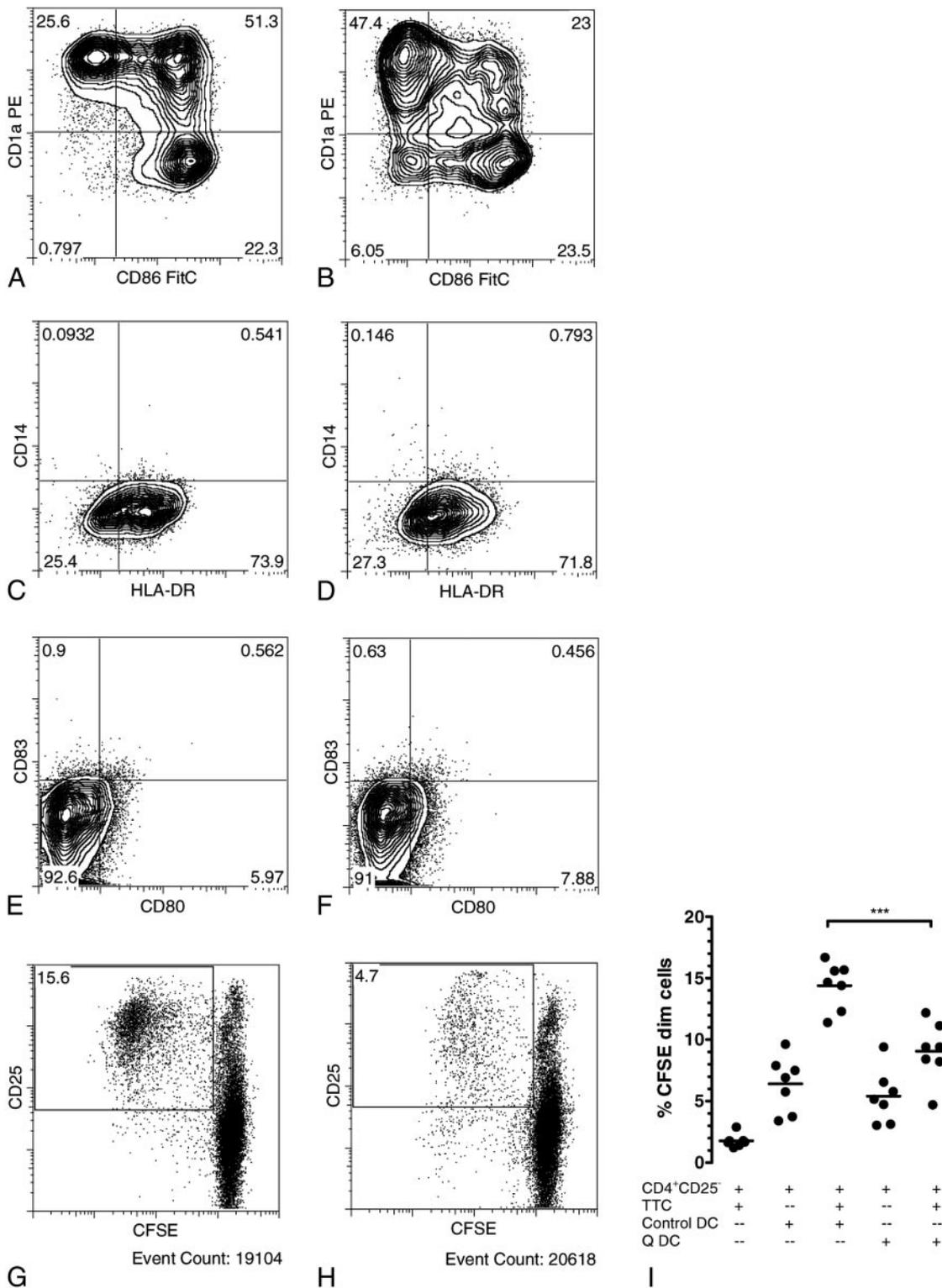


Figure 4. Adiponectin inhibits CD86 expression on CD11c+ mDCs and their T cell stimulatory capacity. A, CD14+ monocytes were transformed to mDCs expressing typically high levels of CD1a and CD86. B, Adiponectin supplementation 10 $\mu\text{g}/\text{mL}^{-1}$ at the commencement of culture significantly reduced CD86 expression. C and D, HLA-DR expression on mDCs generated in the absence (C) and presence (D) of adiponectin remains unaltered. E and F, CD80 expression on mDCs generated in the absence (E) and presence (F) of adiponectin remains unaltered. CD4+CD25⁻ CFSE+ effector cells from a healthy subject cocultured with TT and control mDCs (G) or with adiponectin-treated DCs from the same donor (H). I, Proliferation of effector cells from 7 donors, each point representing average of triplicate. Legend denotes culture conditions. TTC, tetanus toxoid; Q DC, adiponectin-treated DCs. *** $P < .001$, Bonferroni posttest.

duced after both antigenic (Figure 4, G–I) as well as mitogenic (Supplemental Figure 5) challenge.

Reduced adiponectin receptor expression on T1D monocytes is associated with a proinflammatory response

We then asked whether the reduction in AdipoR expression in T1D would affect mDC phenotype and function. We recruited a new cohort of T1D and matched HC subjects (n = 30) and again confirmed reduction in monocyte expression of AdipoR1 and AdipoR2 (data not shown). We demonstrated that adiponectin did not modulate CD86 expression as much in T1D as it did in HC (ratio CD86 MFI adiponectin-treated DC: serum free con-

trol DC, HC = 0.45 ± 0.13, T1D = 0.59 ± 0.17, P = .0207), whereas expression of CD80 and HLA-DR remained unchanged (Figure 5, A–G). The yield of CD1a mDC was not altered by diabetes or adiponectin supplementation in media.

The T cell stimulatory capacity of mDCs from adiponectin-supplemented conditions was compared in subjects with and without T1D (n = 17). Consistent with the effect of adiponectin on costimulatory molecule expression in T1D mDCs, there was reduced inhibition of T cell proliferation with adiponectin-treated mDC from T1D compared with healthy control subjects (Figure 5H, median reduction HC = 23.6%, T1D = 6.1%, P = .0079).

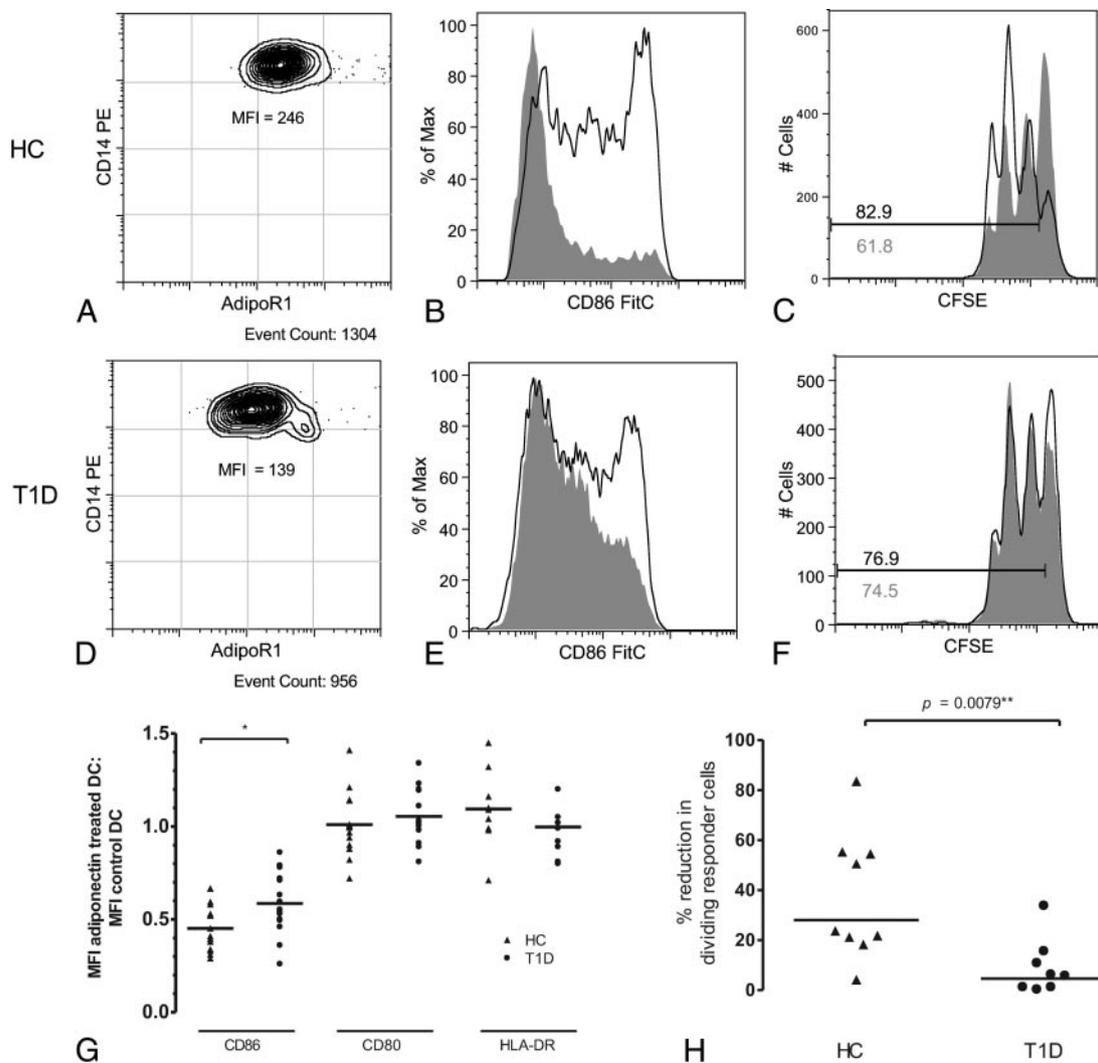


Figure 5. Reduced inhibition of CD86 expression on mDCs by adiponectin in T1D is associated with the lower AdipoR1 expression of monocyte precursors. A–F, Representative comparison from a pair of matched HC (A–C) and T1D (D–F) subjects, from the same experimental run. A and D, AdipoR1 labeling on CD14⁺ precursors. B and E, CD86 expression on CD1a⁺ mDC. Control DC (open histogram); adiponectin-treated mDC (gray-filled histogram). C and F, OKT3 stimulated proliferation of CFSE⁺CD4⁺CD25⁻ effector cells in cocultures with control DC (black line), and adiponectin-treated DC (gray filled). Bar indicates the percentage of dividing effector cells. G, CD86, CD80, and HLA-DR expression on adiponectin-treated mDC, standardized against untreated control DC, in healthy control and T1D subjects. H, The 2 mDCs were cocultured with effector cells and stimulated by OKT3. Each point represents triplicate experiments for a single subject. The line represents the median. The difference in the proportion of dividing effector cells with the 2 DCs was expressed as a percentage change, such that in the example (C), this is (82.9–61.8) of 82.9 = 25.5%. * Mann-Whitney U test.

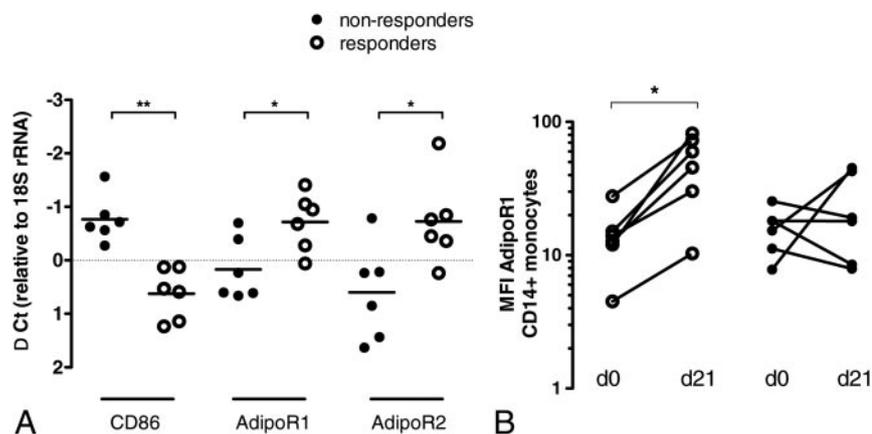


Figure 6. Exercise up-regulates adiponectin receptors and decreases CD86 expression in healthy nondiabetic subjects. A, Fold change in gene expression on day 21 (after the intervention) relative to baseline (day 0). Responders are defined by a change in VO_2 max greater than 10% or weight loss greater than 5%, at the end of the study. ** $P < .01$; * $P < .05$ t test. B, Change in MFI of AdipoR1 staining on CD14+ monocytes. * $P < .05$ paired t test.

Up-regulation of adiponectin receptors associates with down-regulation of CD86 on PBMCs

To explore the translational application for our findings, we examined whether a clinical intervention that increased AdipoR expression by immune cells had the potential to modulate their proinflammatory phenotype. In humans, an aerobic exercise programs that lead to weight loss or increase in VO_2 max, up-regulate adiponectin receptor expression on skeletal muscle (26, 27). We tested the association between adiponectin signaling and CD86 expression on PBMCs observed in vitro through an exercise trial with healthy nondiabetic subjects. We compared the effect in responders who had achieved an increase in VO_2 max greater than 10% or weight loss greater than 5% with those who did not (Supplemental Table 1). At baseline, clinical characteristics between the 2 groups were similar. After intervention, responders achieved increases in AdipoR gene expression on PBMCs (95% confidence interval fold change, AdipoR1 1.13–2.39; AdipoR2 0.94–2.91) (Figure 6A). These changes were accompanied by significant reduction in waist circumference and fasting insulin, in addition to the changes in VO_2 max ($+3.0 \pm 0.83$ ml/kg⁻¹·min⁻¹) and weight (-2.9 ± 0.6 kg). The increase in AdipoR was seen on monocytes at a protein level (Figure 6B). The increased AdipoR expression was associated with a decrease in CD86 expression on whole PBMCs (fold change 0.47–0.90) (Figure 6A). Serum adiponectin levels were not significantly altered at the end of the study in both groups.

Discussion

We demonstrate that adiponectin down-regulates APC expression of CD86, and this associates with a reduction

in antigen-specific T cell stimulation. Furthermore, we show that the reduced expression of adiponectin receptors in T1D is associated with release from inhibition by adiponectin, thus promoting an inflammatory T cell response to islet antigen. Lastly, we confirmed, in vivo, an increase adiponectin receptor expression on PBMCs through lifestyle interventions is coupled with a concomitant decrease in CD86.

We recognize mechanisms other than CD86 on myeloid APC may also play a role in the dampening of T cell antigen reactivity. For example, adiponectin can induce monocytes to secrete antiinflammatory cytokines IL-10 and IL-1RA (10) and also suppress the chemokine CCL2 (28). However, we have focused on CD86 because of its importance in autoimmune diabetes. CD86 expression on APCs has a critical role in priming of diabetogenic CD4+ T cells, and its knockout in the NOD mouse model of T1D prevents disease (29). Recent clinical trial data also confirm that blocking T cell costimulation preserves β -cell function in human T1D (30). Our results therefore demonstrate the potential for adiponectin signaling to modulate islet autoimmunity.

Our data are in agreement with recently published work in a murine system in which adiponectin was reported to alter the phenotype of bone marrow-derived DCs (14). A global reduction in costimulatory molecule expression was reported, in contrast to our findings of a decrease confined to CD86. We suggest that the potential for adiponectin effect in culture serum (in comparison with our serum free system) may explain the differences in results. Adiponectin is known to bind avidly to endotoxin (31), which if present at low levels can be tolerogenic and could confound our in vitro findings (32). However, we believe this to be not the case. The phenotypic change on APCs after endotoxin priming would involve other surface molecules including major histocompatibility complex class II expression (32), which was unchanged with recombinant human adiponectin. In addition, endotoxin contamination in our adiponectin preparation was undetectable by the LAL test.

In contrast to other reports (33, 34), we find that the serum total adiponectin levels is not affected by T1D. However, our T1D subjects were carefully matched for age, sex, ethnicity, and BMI as well as renal function and lipid profile. These factors have been reported to affect adiponectin levels in T1D (33, 34) but have not been ac-

counted for in other comparisons between T1D and health populations. In agreement with current knowledge, however, we found an inverse correlation of serum adiponectin with BMI within patients with T1D (35).

The mechanism leading to reduced adiponectin receptor expression by peripheral blood myeloid cells in T1D is unclear. From twins studies, heritability for AdipoR1 and AdipoR2 gene expression is estimated at 39%–78% (36). However, genes coding for AdipoR1/AdipoR2 are not located within regions associated with T1D risk (<http://t1dbase.org>). In our hands and in contrast to animal systems (37, 38), insulin, glucose, and adiponectin did not affect AdipoR expression in vitro (data not shown). Although adiponectin receptor expression is down-regulated in transgenic mice in which adiponectin is overexpressed (38), we do not believe this mechanism explains our findings because circulating adiponectin was not increased in our subjects, nor did adiponectin correlate inversely with receptor expression in our diabetic subjects (data not shown). Although we show that insulin resistance associates with low adiponectin receptor expression within patients with T1D, insulin resistance alone is unlikely to explain this disease specific decrease because receptor expression is higher in T2D (and these patients are likely to be more insulin resistant). It is likely that other mechanisms contribute, and we speculate that the broad activation of innate immunity associated with T1D (39) underlies the reduced expression described in PBMCs. As an example, inflammatory cytokines inhibit expression of AdipoR1 and AdipoR2 through the endoplasmic reticular stress-inducible transcriptional factor *ATF3* (40).

As with other studies involving human subjects, a limitation of our study is the correlative nature of the presented data. We cannot therefore state that the reduced adiponectin receptor expression leads directly to islet autoimmunity in T1D. Our findings do, however, describe a mechanism whereby an imbalance in adipokines such as adiponectin could contribute to the association between insulin resistance, inflammation, and the development of T1D in those at high genetic risk. Thus, we see confirmation of the mediatory role of AdipoR on APC costimulation and islet immunity in animal models to be an important focus for future studies. Also, our finding that adiponectin receptors are up-regulated by exercise suggests a physiological approach to modulating islet immunity in T1D, and this we believe requires further clinical investigation.

Acknowledgments

We are grateful to Dr Lucy Walker and Professors Dave Sansom and Peter Lane (University of Birmingham, Birmingham, UK);

Professor Susan Wong (University of Cardiff, Cardiff, UK); and Professor Len Harrison (Walter and Eliza Hall, Institute of Medical Research, Melbourne, Victoria, Australia) for the critical reading of this manuscript. We thank Dr Hilary Murray (Worcestershire Hospitals National Health Service Trust, United Kingdom) for access to cadaveric islet cells.

Address all correspondence and requests for reprints to: Dr Parth Narendran, Institute of Biomedical Research, Room 229, University of Birmingham, Birmingham B15 2TT, United Kingdom. E-mail: p.narendran@bham.ac.uk.

This work was supported by Novo Nordisk United Kingdom and the University of Birmingham.

Disclosure Summary: The authors have nothing to declare.

References

1. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem*. 1995;270(45):26746–26749.
2. Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr Rev*. 2005;26(3):439–451.
3. Yamauchi T, Nio Y, Maki T, et al. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. *Nat Med*. 2007;13(3):332–339.
4. Holland WL, Miller RA, Wang ZV, et al. Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin. *Nat Med*. 2011;17(1):55–63.
5. Pang TT, Narendran P. The distribution of adiponectin receptors on human peripheral blood mononuclear cells. *Ann NY Acad Sci*. 2008;1150:143–145.
6. Li S, Shin HJ, Ding EL, van Dam RM. Adiponectin levels and risk of type 2 diabetes: a systematic review and meta-analysis. *JAMA*. 2009;302(2):179–188.
7. Sattar N, Wannamethee G, Sarwar N, et al. Adiponectin and coronary heart disease: a prospective study and meta-analysis. *Circulation*. 2006;114(7):623–629.
8. Okamoto Y, Kihara S, Ouchi N, et al. Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 2002;106(22):2767–2770.
9. Yamauchi T, Kamon J, Waki H, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipodystrophy and obesity. *Nat Med*. 2001;7(8):941–946.
10. Wolf AM, Wolf D, Rumpold H, Enrich B, Tilg H. Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes. *Biochem Biophys Res Commun*. 2004;323(2):630–635.
11. Mandal P, Pratt BT, Barnes M, McMullen MR, Nagy LE. Molecular mechanism for adiponectin-dependent M2 macrophage polarization: Link between the metabolic and innate immune activity of full-length adiponectin. *J Biol Chem*. 2011;286(15):13460–13469.
12. Yokota T, Oritani K, Takahashi I, et al. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood*. 2000;96:1723–1732.
13. Sun Y, Lodish HF. Adiponectin deficiency promotes tumor growth in mice by reducing macrophage infiltration. *PLoS One* 2010;5(8):e11987.
14. Tsang JYS, Li D, Ho D, et al. Novel immunomodulatory effects of adiponectin on dendritic cell functions. *Int Immunopharmacol*. 2011;11(5):604–609.
15. Ludewig B, Odermatt B, Landmann S, Hengartner H, Zinkernagel RM. Dendritic cells induce autoimmune diabetes and maintain dis-

- ease via de novo formation of local lymphoid tissue. *J Exp Med*. 1998;188(8):1493–1501.
16. Green EA, Eynon EE, Flavell RA. Local expression of TNF α in neonatal NOD mice promotes diabetes by enhancing presentation of islet antigens. *Immunity*. 1998;9(5):733–743.
 17. Furlanos S, Narendran P, Byrnes GB, Colman PG, Harrison LC. Insulin resistance is a risk factor for progression to type 1 diabetes. *Diabetologia*. 2004;47(10):1661–1667.
 18. Xu P, Cuthbertson D, Greenbaum C, Palmer JP, Krischer JP, for the Diabetes Prevention Trial Type 1 Study Group. Role of insulin resistance in predicting progression to type 1 diabetes. *Diabetes Care*. 2007;30(9):2314–2320.
 19. Bingley PJ, Mahon JL, Gale EAM, for the European Nicotinamide Diabetes Intervention Trial Group. Insulin resistance and progression to type 1 diabetes in the European nicotinamide diabetes intervention trial (ENDIT). *Diabetes Care*. 2008;31(1):146–150.
 20. Furlanos S, Dotta F, Greenbaum C, et al. Latent autoimmune diabetes in adults (LADA) should be less latent. *Diabetologia*. 2005;48(11):2206–2212.
 21. Chinetti G, Zawadzki C, Fruchart JC, Staels B. Expression of adiponectin receptors in human macrophages and regulation by agonists of the nuclear receptors PPAR α , PPAR γ , and LXR. *Biochem Biophys Res Commun*. 2004;314(1):151–158.
 22. Mannering SI, Morris JS, Jensen KP, et al. A sensitive method for detecting proliferation of rare autoantigen-specific human T cells. *J Immunol Methods*. 2003;283(1–2):173–183.
 23. Hug C, Wang J, Ahmad NS, Bogan JS, Tsao T, Lodish HF. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. *Proc Natl Acad Sci USA*. 2004;101(28):10308–10313.
 24. Williams KV, Erbey JR, Becker D, Arslanian S, Orchard TJ. Can clinical factors estimate insulin resistance in type 1 diabetes? *Diabetes*. 2000;49(4):626–632.
 25. Crawford LJA, Peake R, Price S, Morris TCM, Irvine AE. Adiponectin is produced by lymphocytes and is a negative regulator of granulopoiesis. *J Leukoc Biol*. 2010;88(4):807–811.
 26. Bluher M, Williams CJ, Kloting N, et al. Gene expression of adiponectin receptors in human visceral and subcutaneous adipose tissue is related to insulin resistance and metabolic parameters and is altered in response to physical training. *Diabetes Care*. 2007;30(12):3110–3115.
 27. Christiansen T, Paulsen SK, Bruun JM, Ploug T, Pedersen SB, Richelsen B. Diet-induced weight loss and exercise alone and in combination enhance the expression of adiponectin receptors in adipose tissue and skeletal muscle, but only diet-induced weight loss enhanced circulating adiponectin. *J Clin Endocrinol Metab*. 2010;95(2):911–919.
 28. Medoff BD, Okamoto Y, Leyton P, et al. Adiponectin deficiency increases allergic airway inflammation and pulmonary vascular remodeling. *Am J Respir Cell Mol Biol*. 2009;41(4):397–406.
 29. Yadav D, Judkowski V, Flodstrom-Tullberg M, et al. B7–2 (CD86) controls the priming of autoreactive CD4 T cell response against pancreatic islets. *J Immunol*. 2004;173(6):3631–3639.
 30. Orban T, Bundy B, Becker DJ, et al. Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial. *Lancet*. 2011;378(9789):412–419.
 31. Peake PW, Shen Y, Campbell LV, Charlesworth JA. Human adiponectin binds to bacterial lipopolysaccharide. *Biochem Biophys Res Commun*. 2006;341(1):108–115.
 32. Wolk K, Döcke W, von Bachr V, Volk H, Sabat R. Impaired antigen presentation by human monocytes during endotoxin tolerance. *Blood*. 2000;96(1):218–223.
 33. Maahs DM, Ogden LG, Snell-Bergeon JK, et al. Determinants of serum adiponectin in persons with and without type 1 diabetes. *Am J Epidemiol*. 2007;166(6):731–740.
 34. Schalkwijk CG, Chaturvedi N, Schram MT, Fuller JH, Stehouwer CDA the EURODIAB Prospective Complications Study Group. Adiponectin is inversely associated with renal function in type 1 diabetic patients. *J Clin Endocrinol Metab*. 2006;91(1):129–135.
 35. Barnes MM, Curran-Everett D, Hamman RF, et al. Determinants of adiponectin levels in young people with type 1 diabetes. *Diabet Med*. 2008;25(3):365–369.
 36. Storgaard H, Poulsen P, Ling C, Groop L, Vaag AA. Relationships of plasma adiponectin level and adiponectin receptors 1 and 2 gene expression to insulin sensitivity and glucose and fat metabolism in monozygotic and dizygotic twins. *J Clin Endocrinol Metab*. 2007;92(7):2835–2839.
 37. Tsuchida A, Yamauchi T, Ito Y, et al. Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity. *J Biol Chem*. 2004;279(29):30817–30822.
 38. Bauche IB, Ait El Mkaem S, et al. Adiponectin downregulates its own production and the expression of its AdipoR2 receptor in transgenic mice. *Biochem Biophys Res Commun*. 2006;345(4):1414–1424.
 39. Schaumberg DA, Glynn RJ, Jenkins AJ, et al. Effect of intensive glycemic control on levels of markers of inflammation in type 1 diabetes mellitus in the diabetes control and complications trial. *Circulation*. 2005;111(19):2446–2453.
 40. Park HJ, Kang YM, Kim CH, Jung MH. ATF3 negatively regulates adiponectin receptor 1 expression. *Biochem Biophys Res Commun*. 2010;400(1):72–77.