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Insulin Inhibits IL-10–Mediated Regulatory T Cell Function: Implications for Obesity

Jonathan M. Han, Scott J. Patterson, Madeleine Speck, Jan A. Ehses, and Megan K. Levings

Chronic inflammation is known to promote metabolic dysregulation in obesity and type 2 diabetes. Although the precise origin of the unchecked inflammatory response in obesity is unclear, it is known that overproduction of proinflammatory cytokines by innate immune cells affects metabolism. For example, TNF-α contributes to the inability of cells to respond to insulin and to the increase in levels of insulin. Whether this hyperinsulinemia itself is part of a feedback loop that affects the progression of chronic adipose inflammation is unknown. In this article, we show that regulatory T cells (Tregs) express the insulin receptor, and that high levels of insulin impair the ability of Tregs to suppress inflammatory responses via effects on the AKT/mTOR signaling pathway. Insulin activated AKT signaling in Tregs, leading to inhibition of both IL-10 production and the ability of Tregs to suppress the production of TNF-α by macrophages in a contact-independent manner. The effect of insulin on Treg suppression was limited to IL-10 production and it did not alter the expression of other proteins associated with Treg function, including CTLA-4, CD39, and TGF-β. In a model of diet-induced obesity, Tregs from the visceral adipose tissue of hyperinsulinemic obese mice showed a similar specific decrease in IL-10 production, as well as a parallel increase in production of IFN-γ. These data suggest that hyperinsulinemia may contribute to the development of obesity-associated inflammation via a previously unknown effect of insulin on the IL-10–mediated function of Tregs. The Journal of Immunology, 2014, 192: 000–000.

Obesity-associated chronic inflammation contributes to metabolic impairment and the risk for development of type 2 diabetes (1, 2). In comparison with lean visceral adipose tissues (VAT), inflamed obese VAT accumulates proinflammatory immune cells such as T cells and classically activated macrophages, resulting in chronic inflammation and overproduction of proinflammatory cytokines such as TNF-α. These proinflammatory cytokines can block insulin receptor signaling in multiple cell types, including adipocytes and hepatocytes (3–6), leading to insulin resistance. In parallel with an increase in proinflammatory immune cells, inflamed obese VAT has a reduction in the proportion of regulatory T cells (Tregs) (7–9). Evidence that therapeutic manipulation of VAT can reduce inflammation and improve metabolic parameters of obese mice suggests that a deficit in Tregs contributes to chronic VAT inflammation (6, 9, 10).

One of the hallmarks of obesity is a marked increase in insulin (11), with recent evidence suggesting that hyperinsulinemia may even be required for obesity (12). The canonical role of insulin is to promote glucose uptake in cells such as adipocytes, hepatocytes, and myocytes, a function that is primarily mediated via insulin receptor–mediated stimulation of the AKT pathway (3, 13). Notably, the AKT signaling pathway also has a major role in regulating the development and function of Tregs, and hence immune tolerance and inflammation. For example, relatively low activity of the AKT pathway is essential for the normal development and function of Tregs (14). In addition, fully developed Tregs normally have diminished AKT activity in response to TCR or IL-2 stimulation (15, 16), and forced AKT activation inhibits their function (14, 15, 17). Downstream of AKT, mammalian target of rapamycin (mTOR) activation is also linked to reduced Treg differentiation and function (18–20), providing a molecular mechanism for why the mTOR inhibitor rapamycin promotes tolerance (21, 22).

Because obesity-associated hyperinsulinemia is correlated with a reduction in Treg proportion (9, 10), we speculated that insulin may have biological effects on Tregs via the AKT/mTOR pathway. In this article, we show that in contrast with TCR stimulation, insulin strongly activates AKT signaling in Tregs. Activation of AKT results in specific inhibition of IL-10 production and reverses the ability of Tregs to suppress TNF-α production from macrophages. Similarly, Tregs isolated from the VAT of obese, hyperinsulinemic mice were specifically impaired in IL-10 production and produced significantly higher amounts of IFN-γ. Together, these data reveal a previously unknown link between metabolism and immunity, and suggest that insulin-stimulated activation of the AKT pathway in Tregs is important for regulation of IL-10 production and may contribute to the perpetuation of chronic inflammation in obesity.

Materials and Methods

Mice
Mice were C57BL/6 (B6), B6 FOXP3-EGFP (23), or B6 FOXP3-mRFP (24) × IL-10–EGFP (25) mice (6–12 wk old; The Jackson Laboratory or
Glucose tolerance tests and determination of homeostatic model assessment of insulin resistance

For glucose tolerance testing, 8-h fasted mice were orally gavaged with 2 g/kg body weight glucose. Glycemia was measured using an OneTouch Ultra 2 glucometer, and plasma insulin was determined using luminex technology (Millipore). Homeostatic model assessment of insulin resistance was calculated as fasting blood glucose (mmol/l) × fasting insulin (mU/ml)/22.5 as described previously (26).

Cell isolation

Spleens and lymph nodes were mashed. Epididymal fat pads (VAT) were minced and digested with collagenase type II (Worthington) for 1 h at 37°C with shaking. The stromal-vascular fraction was obtained after centrifugation and passed through a cell strainer. CD4+ T cells were isolated with EasySep CD4+ selection kit (Stemcell Technologies), and CD25+ selection was performed with CD25 microbeads (Miltenyi Biotec). For gene expression analysis, CD4+ T cells were sorted into CD4+FOXP3-EGFP+ Tregs, CD4+FOXP3+EGFP+ Tconv cells, and CD4+FOXP3-EGFP+ conventional T cells (Tconvs) to a >98% purity on a FACSaria.

Cell culture and signaling

RPMI 1640 was supplemented with 5 or 10% FBS, HEPEs, and penicillin G and streptomycin. FBS contains a relatively small amount of endogenous insulin (0.002 ng/ml) (4), whereas serum insulin concentrations in obese hypertensive mice can increase to as high as 38 ng/ml upon glucose challenge (27). To mimic these conditions, where indicated, we cultured T cells with 0, 10, or 100 ng/ml insulin (Sigma-Aldrich) in the presence of 25 U/ml IL-2 for 24 h, then stimulated them with plate-bound anti-CD3 (10 μg/ml), soluble anti-CD28 (2 μg/ml), and 100 U/ml IL-2 in continuous insulin (0, 10, or 100 ng/ml). After 4 d, cell-free conditioned media was collected. In some cases, 25 nM rapamycin, 2 μM Akt1/2, or 2 μM U0126 (Sigma-Aldrich) was present throughout the culture. For suppression assays, irradiated APCs and soluble anti-CD3 (0.7 μg/ml) were used to stimulate CFSE-stained or cell proliferation dye eFluor670 (eBioscience)-stained Tconvs that were cocultured without or with Tregs (4:1 ratio) for 3 d. For cell signaling, CD4+ T cells were rested in serum-free RPMI 1640 for 4 h, or overnight in RPMI 1640 1% FBS and then serum-free RPMI 1640 for 4 h for VAT-derived cells, then stimulated with 10 μg/ml insulin, or 10 μg/ml anti-CD3 and 4 μg/ml anti-CD28 for the indicated time periods. Activation was arrested by fixation in fix/perm buffer (eBioscience), and methanol in the case of pS6; then phosphorylation was detected with anti-pAKT (Ser473) and anti-pS6 (Ser235/236) Abs (Cell Signaling Technology) using flow cytometry by measuring the geometric mean fluorescence intensity (MFI).

Flow cytometry and ELISA

All flow cytometry and ELISA Abs were commercially obtained from BD Pharmingen or eBioscience. For insulin receptor staining, cells were fixed and permeabilized with a Transcription Factor Staining Buffer Set (eBioscience), stained with anti-FOXP3 mAbs (eBioscience) and an unconjugated rabbit mAb against an intracellular epitope of the insulin receptor β (clone 4B8; Cell Signaling Technology), followed by staining with an Alexa Fluor 647–labeled anti-rabbit secondary Ab (Life Technologies). The isotype control was rabbit (DA1E) mAb IgG (eBioscience) or a mouse IgG1 isotype control (BD Pharmingen or eBioscience). Flow cytometry was performed on BD FACSCanto or LSR II and analysis was performed using FlowJo 8.7.

Bone marrow–derived macrophages

Bone marrow–derived macrophages (BMDMs) were differentiated with GM-CSF for 6 d and then incubated with 10 ng/ml IL-10, or media containing 12.5% of medium conditioned by Tregs or Tconvs (prepared as described earlier with or without insulin) for 4 h; then 10 ng/ml LPS was added for 20 h.

RT-PCR analysis

Gene expression was measured on Applied Biosystems 7500 Fast Real-Time PCR System. Primer sequences were: 18s, 5′-CAAGAAGCCAGCA-GAGCGAAA-3′ and 5′-GGCGGGGCATGCGGAAAC-3′; IL-10, 5′-TTGCAACGCTTATCCGAAA–3′ and 5′-TGTCTCCACTGCCTGCTT-T-3′. Data were normalized to 18s.

Statistical analysis

Student t tests or Mann–Whitney U tests were used to analyze significance. The p values are indicated as follows: *p < 0.05, **p < 0.01.

Results

Insulin activates the AKT pathway in Tregs

Insulin is arguably the best-characterized activator of AKT signaling. Because Tregs have diminished activation of the AKT pathway in response to TCR (14, 17) or IL-2 stimulation (16), we wondered whether Tregs would be similarly hyporesponsive to insulin. To test this possibility, CD4+ T cells were purified from B6 mice, stimulated with insulin for various times, then analyzed by flow cytometry to measure the amounts of AKT Ser473 phosphorylation in FOXP3+ Tregs versus FOXP3+ Tconv cells. As shown in Fig. 1A, surprisingly, insulin-induced AKT phosphorylation was significantly higher in Tregs than in Tconvs, which is the opposite of how these cells respond to TCR stimulation (Supplemental Fig. 1A). To test whether signaling downstream of AKT was also higher in Tregs, we measured insulin-induced phosphorylation of ribosomal protein S6, which is dependent on the activation of mTORC1 (28) (Fig. 1B). Similar to AKT, insulin-stimulated S6 phosphorylation was calculated as fasting blood glucose (mmol/l) × fasting insulin (mU/ml)/22.5 as described previously (26).

FIGURE 1. Insulin activates AKT in Tregs. (A and B) CD4+ T cells were purified from spleens and stimulated with 10 μg/ml insulin for the indicated times. Phosphorylation of (A) AKT Ser473 (n = 8) or (B) S6 Ser235/236 (n = 12) relative to unstimulated controls was measured by flow cytometry in Tregs and Tconvs gated as CD4+FOXP3+ or CD4+FOXP3- cells, respectively. MFIs relative to time 0 and representative histograms (with absolute MFIs indicated in brackets) are shown. Amounts of phosphorylation in unstimulated (t = 0) Tregs and Tconvs were equivalent. (C) Insulin receptor expression was measured by flow cytometry on FOXP3+ Tconvs and FOXP3+ Tregs ex vivo, or after 72 h of stimulation with anti-CD3 and anti-CD28 (n = 3–5). The MFI of the isotype control is shown. Error bars represent SD. **p < 0.01.
Ser235/236 phosphorylation was significantly higher in Tregs compared with Tconvs. This greater response to insulin in Tregs compared with Tconvs was not related to differences in expression of the insulin receptor, because Tregs and Tconvs express equivalent levels of the receptor ex vivo or after 72 h of TCR activation (Fig. 1C). These data indicate that despite the poor ability of TCR or IL-2 to stimulate the AKT pathway in Tregs (16, 17), they are fully competent to activate this pathway upon exposure to insulin.

**Insulin selectively inhibits IL-10 production by Tregs via AKT/mTOR activation**

Tregs suppress immune cells via a number of different mechanisms including production of inhibitory cytokines (29). Because high AKT activity is associated with loss of Treg function (14, 15, 17), we asked whether exposure to insulin might affect one or more of these suppressive mechanisms. Tregs and Tconvs were isolated, cultured overnight in 0, 10, or 100 ng/ml insulin to stimulate the AKT pathway, and then stimulated via the TCR. Neither cell proliferation nor survival was affected by insulin (Supplemental Fig. 1B, 1C), and consistent with our previous studies of forced AKT activation in Tregs (17), insulin-induced AKT activation did not reduce FOXP3 expression in Tregs (Fig. 2A). We then measured expression of surface proteins associated with contact-dependent Treg suppression, including CTLA-4, CD39, CD25, and latency-associated peptide (LAP), but did not find any significant effect of insulin (Fig. 2B). To test whether insulin might affect cytokine-mediated suppression, we stimulated Tregs and Tconvs for 4 d, and the amounts of secreted IL-10 and TGF-β were measured by ELISA (Fig. 2C). In contrast with the lack of effect on TGF-β production, 10 and 100 ng/ml insulin significantly inhibited the production of IL-10 by 40.9 ± 22.0 and 39.9 ± 13.2%, respectively.

To determine whether the specific effect of insulin on IL-10 production was dependent on insulin-induced activation of AKT signaling, we used inhibitors of AKT (Akti1/2), mTOR (rapamycin), or the AKT pathway–unrelated kinase MEK1/2 (UO126), which is also activated by insulin (30). Tregs or Tconvs were cultured overnight in 0, 10, or 100 ng/ml insulin in the presence or absence of the indicated inhibitors, then stimulated via the TCR for 4 d. As shown in Fig. 3A and 3B, inhibitors of the AKT pathway (Akti1/2 and rapamycin), but not the MEK1/2 pathway (UO126), completely reversed the inhibitory effect of insulin on IL-10 production. Fig. 3A displays a representative experiment, and the cumulative data in Fig. 3B were normalized to account for the fact that, as expected, Akti1/2 and rapamycin reduced cell proliferation and caused an overall reduction in IL-10. Thus, insulin-mediated suppression of IL-10 production was dependent on AKT and mTOR, but not the MEK/ERK signaling pathway.

**Insulin impairs the ability of Tregs to exert cytokine-mediated suppression**

Because IL-10 is one of the major mechanism used by Tregs to suppress target cells (29, 31, 32), we next tested whether insulin alters Treg suppressive function. Tregs were cultured with or without insulin for 24 h, then tested for their ability to suppress the proliferation of Tconvs (Fig. 4A) in an assay that is known to be cytokine independent (33). Consistent with our finding that insulin did not affect molecules associated with contact-dependent mechanisms of suppression, insulin had no effect on Treg-mediated suppression of Tconv proliferation. Similar results were obtained with coculture suppression assays in the presence or absence of insulin using Tregs that had not been pretreated with insulin (data not shown). Moreover, insulin did not affect the proliferation of Tconvs in this assay (Supplemental Fig. 1C).

To test whether insulin affects contact-independent Treg suppression, we tested the effects of insulin on the ability of Tregs to reduce TNF-α production by macrophages (Fig. 4B, 4C). BMDMs were incubated for 4 h in media that were conditioned by Tregs activated in the absence or presence of insulin, then stimulated with LPS. After 20 h, TNF-α production was measured. As expected, addition of IL-10 effectively inhibited TNF-α production. Similarly, Treg conditioned media suppressed TNF-α production by an average of 70.5 ± 7.5%. Addition of neutralizing anti–IL-10 mAbs blocked the suppression of TNF-α production by the Treg-conditioned media and further potentiated its production, demonstrating that Treg-mediated suppression of TNF-α is IL-10 dependent. In contrast with the effects of Tregs, media conditioned by Tconvs significantly enhanced TNF-α production by an average of 464 ± 112%. Tconvs alone produced an average of 2.3 ± 0.3 ng/ml TNF-α (n = 3, data not shown). Because only 12.5% of the total BMDM media was conditioned by Tconvs, T cell–derived TNF-α did not substantially contribute to the synergistic effect.

Next, we asked how exposure to insulin affected Treg-mediated suppression of macrophages. BMDMs were stimulated with LPS with or without supernatants from Tregs that had been cultured in the absence or presence of insulin. These supernatants had reduced concentrations of IL-10 because of the effect of insulin on Tregs as shown in Fig. 2C. Exposure of Tregs to 10 and 100 ng/ml insulin reduced their ability to suppress TNF-α by 46.8 ± 23.7 and 58.1 ± 12.1%, respectively. Addition of insulin itself did not
FIGURE 3. Insulin inhibits IL-10 production by Tregs via the AKT/mTOR pathway. (A and B) CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ Tconvs were cultured with 0, 10, or 100 ng/ml insulin for 1 d, then stimulated with anti-CD3, anti-CD28 mAbs, and IL-2 for 4 d in the continual presence of 0, 10, or 100 ng/ml insulin, in the presence or absence of 2 μM Akti1/2, 25 nM rapamycin, or 2 μM UO126. Supernatants were collected and IL-10 was measured via ELISA. In (A), the representative absolute amounts of IL-10 and, in (B), the average fold change in IL-10 compared to the without insulin condition are shown (n = 5–12). Error bars represent SD. **p < 0.01.

FIGURE 4. Insulin inhibits the ability of Tregs to suppress TNF-α production by macrophages via soluble factors. (A) CD4⁺CD25⁺ Tregs were cultured with 0, 10, or 100 ng/ml insulin for 24 h, then added in increasing numbers to CFSE-labeled CD4⁺CD25⁻ Tconvs. The cocultures were stimulated with anti-CD3 mAbs and irradiated APCs for 3 d in the absence of insulin. Tconv proliferation was measured by CFSE dilution and division index (DI). (A) Representative data and average percent suppression from multiple experiments at a 4:1 ratio of Tconvs/Tregs are shown (n = 2–5). (B and C) BMDMs were incubated with media containing 12.5% of medium conditioned by CD4⁺CD25⁺ Tregs that had been stimulated with anti-CD3, anti-CD28, and IL-2 with 0, 10, or 100 ng/ml insulin (n = 3) for 4 h. BMDMs were then stimulated with 10 ng/ml LPS and cultured for an additional 20 h, and TNF-α production was measured. The percentage suppression of TNF-α release compared with cultures without Treg conditioned medium is shown. Error bars represent SD. *p < 0.05, **p < 0.01.

Tregs from obese, hyperinsulinemic mice have reduced IL-10 and increased IFN-γ expression

We next examined the phenotype of Tregs residing in the VAT in a hyperinsulinemic environment in vivo. As expected in a model of diet-induced obesity, serum insulin levels were substantially increased to 9.48 ± 3.43 ng/ml in B6 FOXP-EGFP mice fed a high-fat/sucrose diet (HFD) for 16 wk compared with 3.17 ± 1.41 ng/ml in mice fed an NCD (Fig. 5A). Furthermore, mice fed an HFD had increased body weight (Supplemental Fig. 2A) and impaired glucose tolerance and insulin sensitivity, as measured by an oral glucose tolerance test and calculation of the homeostatic model assessment of insulin resistance (Supplemental Fig. 2B, 2C). These data are consistent with a previous report using the same diet (34) and confirm the suitability of the model to ask how changes in insulin sensitivity affect immune cells.

The proportion of Tregs among total CD4⁺ T cells was normal in the spleen of HFD mice but was reduced from 32.8 ± 3.9% in NCD mice to 16.4 ± 1.1% in the VAT of HFD mice (Fig. 5B). However, NCD and HFD mice had equivalent numbers of Tregs per gram of VAT tissue (Fig. 5C) (8–10) because of the increase in immune cell infiltration in this setting. Because diet-induced obesity causes insulin resistance in adipocytes residing in VAT (35), we examined the ability of VAT Tregs from HFD mice to respond to insulin and found that they remained sensitive to insulin as judged by induction of AKT phosphorylation (Supplemental Fig. 3A). Furthermore, Tregs remained sensitive to insulin in the presence of TNF-α (Supplemental Fig. 3B), which has been shown to promote a loss of insulin signaling in adipocytes (36).

Whether Tregs in the VAT of HFD mice have an altered suppressive function has never been examined. To investigate whether Tregs in this environment are dysfunctional, we measured expression of proteins associated with Treg suppressive capacity. Consistent with our in vitro data, in a setting of hyperinsulinemia in vivo, Tregs from the spleen or VAT of HFD mice had no significant change in expression of CTLA-4, CD39, CD25, or LAP in comparison with cells from NCD mice (Fig. 5D).

We next examined IL-10 production in Tregs from NCD and HFD mice. Tregs were sorted as GFP⁺ cells from the spleen and VAT of FOXP3-GFP-reporter mice fed an NCD or HFD, and expression of IL-10 mRNA was measured. We found that the Tregs from both the spleen and the VAT of HFD mice had a significant reduction in the amount of IL-10 mRNA (Fig. 6A). To confirm these data at the protein level, we fed FOXP3-RFP × IL-10–EGFP mice an NCD or HFD, then analyzed spleen and VAT Tregs by flow cytometry (gating shown in Fig. 5B). Both the MFI of IL-10 and percentage of IL-10⁺ cells indicate that VAT Tregs produce markedly greater levels of IL-10 compared with splenic Tregs (Fig. 6B, Supplemental Fig. 3C). In VAT Tregs from HFD mice,
however, production of IL-10 was significantly reduced compared with Tregs from NCD mice.

Treg dysfunction is associated with induction of expression of effector cytokines such as IFN-γ and IL-17 (37). Therefore, in parallel to IL-10, we measured production of IFN-γ in HFD versus NCD mice. As expected, HFD mice had an enhanced Th1 response in the VAT (10), as evidenced by an increased proportion of IFN-γ+ Tconv cells. VAT, but not splenic, Tregs in HFD mice

FIGURE 5. Tregs in the VAT of obese hyperinsulinemic mice are present in reduced proportions but express normal levels of proteins that mediate contact-dependent suppression. (A) B6 FOXP3-EGFP mice were fed an HFD for 16 wk and their serum insulin levels were measured (n = 8). (B) The percentage of FOXP3+ Tregs of CD4+ cells was measured in spleen and VAT of NCD and HFD mice (n = 5). Representative plots of VAT CD4+ cells and their FOXP3+ Treg gates are shown. (C) The absolute numbers of CD4+FOXP3+ Tconv cells and CD4+FOXP3+ Tregs per grams of tissue were measured (n = 3–4). (D) Expression of CTLA-4, CD39, CD25, and LAP on Tregs and Tconvs from spleen and VAT of HFD and NCD mice were measured by flow cytometry (n = 2–4). Error bars represent SD. **p < 0.01.

FIGURE 6. Tregs in hyperinsulinemic obese mice have reduced expression of IL-10 and increased expression of IFN-γ. (A) CD4+FOXP3– Tconvs and CD4+FOXP3+ Tregs were FACS sorted from spleen or VAT of FOXP3-GFP mice fed an NCD or HFD, and expression of IL-10 mRNA was measured by quantitative RT-PCR (n = 2–3). (B) B6 FOXP3-RFP × IL-10–EGFP mice were fed an HFD or NCD; then IL-10–EGFP expression was measured in FOXP3-RFP+ Tregs from the spleen and VAT. The average MFI and percentage of IL-10hi cells is shown (n = 8–9). (C) IFN-γ production of Tregs and Tconvs in spleen and VAT of NCD and HFD mice was measured via flow cytometry. Representative and average data are shown (n = 3). Error bars represent SD. *p < 0.05, **p < 0.01.

Treg dysfunction is associated with induction of expression of effector cytokines such as IFN-γ and IL-17 (37). Therefore, in parallel to IL-10, we measured production of IFN-γ in HFD versus NCD mice. As expected, HFD mice had an enhanced Th1 response in the VAT (10), as evidenced by an increased proportion of IFN-γ+ Tconv cells. VAT, but not splenic, Tregs in HFD mice
also had a significant increase in IFN-γ expression: 15.1 ± 0.4% of Tregs from HFD mice expressed IFN-γ compared with only 6.9 ± 1.8% in NCD mice (Fig. 6C). Expression of IL-17 was not detectable under any conditions (data not shown). These data suggest that in HFD mice, VAT Tregs may lose their lineage stability and take on a Th1-like phenotype.

Discussion

Insulin has long been known as a metabolic hormone that regulates glucose homeostasis, and its physiological effects on myocytes, hepatocytes, and adipocytes in health and disease are well established. Little is known, however, about the effects of insulin on immune cells, and its effects on Tregs have never been investigated. In this article, we show that Tregs express the insulin receptor, and we reveal a novel physiological role for insulin: regulation of IL-10 production and the suppressive function of Tregs. Tregs resident in the VAT of hyperinsulinemic mice produced significantly less IL-10 than lean mice fed a normal diet, suggesting that changes in metabolism may specifically affect this mechanism of Treg suppression.

Tregs normally have dampened TCR- or IL-2–induced AKT phosphorylation at Ser^473, at least partly because of high expression of the protein phosphatase PHLP (15, 16). This low AKT signaling is functionally relevant for Tregs, because forced activation of the pathway by overexpression of active forms of AKT or deletion of inhibitory phosphatases blocks their normal development and function (14, 15, 17). We show in this study, however, that unlike TCR or IL-2 stimulation, insulin-induced activation of AKT was significantly higher in Tregs compared with Tconvs, suggesting that Tregs may be uniquely responsive to this hormone. Insulin-induced AKT activation also resulted in the activation of mTORC1, as judged by phosphorylation of ribosomal protein S6, a downstream target of mTORC1 (28). This finding provides the first evidence, to our knowledge, that Tregs are not universally hyporesponsive to stimulation of the AKT pathway.

The effect of insulin on Tregs was remarkably specific: inhibition of IL-10 production without any effect on other proteins associated with Treg function, including CTLA-4, CD39, LAP, or TGF-β. To the best of our knowledge, such a specific cytokine/growth factor–stimulated effect on IL-10 production by Tregs has not been previously reported. The insulin-mediated suppression of IL-10 production was dependent on AKT and mTOR, but not the MEK/ERK signaling pathway, which is also activated by insulin (30). Of the few studies that have investigated the relationship between AKT signaling and IL-10 production in Tregs, some have reported an inverse association between AKT activation and IL-10 production in Tregs (38, 39) much like our data, whereas other studies suggest the opposite (40–42). Thus, the role of AKT signaling in IL-10 production by Tregs may be context dependent. Our data suggest that the major known mechanisms of Treg action, IL-10 production may be particularly sensitive to modulation of the AKT signaling pathway, and that, in the context of insulin stimulation, AKT activation negatively regulates this aspect of Treg function.

A well-established feature of obesity associated with hyperinsulinemia is chronic inflammation, characterized by a prominent infiltration of macrophages into adipose tissue, and an overproduction of TNF-α (43–45). We found that insulin significantly inhibited the ability of Tregs to exert IL-10–mediated suppression of TNF-α production by macrophages. Tregs in HFD mice also produced lower amounts of IL-10, in accordance with a previous report that IL-10 expression in the total stromal-vascular fraction of VAT from HFD mice is reduced (46). Because IL-10 inhibits TNF-α, which is overproduced by macrophages in obese VAT (43, 44), and can directly alleviate TNF-α–induced insulin resistance in adipocytes (46), reduced IL-10 production by Tregs may directly contribute to both inflammation by macrophages and insulin resistance in nonimmune cells.

As a mouse model of obesity, HFD mice are known to be hyperinsulinemic and have a reduced proportion of Tregs specifically in VAT, which is a key site for insulin resistance and chronic inflammation (47, 48). However, whether these Tregs are functionally altered is unknown. Consistent with a previous study (8, 10), we found that, on a per gram of tissue basis, the absolute number of adipose Tregs was unchanged in HFD mice, indicating that a simple reduction in Treg numbers likely does not contribute to obesity-associated inflammation. To our knowledge, our data are the first to suggest that there is major functional impairment of Tregs in HFD mice, specifically their cytokine-dependent function. Furthermore, the proportion of Tregs making IFN-γ in HFD mice is significantly increased, possibly indicating a loss of Treg lineage stability in the HFD environment.

There is striking resemblance between insulin-treated Tregs in vitro and Tregs from the VAT of hyperinsulinemic obese mice. In both settings, Tregs encountered high levels of insulin, and in both, Tregs acquired a specific defect in IL-10 production, whereas retaining normal expression of other proteins associated with Treg function. Thus, our data suggest a possible pathological effect of insulin on Tregs in the setting of obesity, similar to an observation previously made in myeloid cells (49), where insulin receptor expression has been shown to contribute to the development of obesity-associated inflammation. In conclusion, Tregs become phenotypically altered in conditions of hyperinsulinemia, and the loss of IL-10 production and gain of IFN-γ could contribute to the immune dysregulation that ensues in VAT during obesity. Our data establish a new molecular and cellular paradigm for how hyperinsulinemia might affect inflammation in obesity.

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Disclosures

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