Reduced Adipose Tissue Macrophage Content Is Associated With Improved Insulin Sensitivity in Thiazolidinedione-Treated Diabetic Humans

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Obesity is associated with increased adipose tissue macrophage (ATM) infiltration, and rodent studies suggest that inflammatory factors produced by ATMs contribute to insulin resistance and type 2 diabetes. However, a relationship between ATM content and insulin resistance has not been clearly established in humans. Since thiazolidinediones attenuate adipose tissue inflammation and improve insulin sensitivity, we examined the temporal relationship of the effects of pioglitazone on these two parameters. The effect of 10 and 21 days of pioglitazone treatment on insulin sensitivity in 26 diabetic subjects was assessed by hyperinsulinemic-euglycemic clamp studies. Because chemoattractant factors, cytokines, and immune cells have been implicated in regulating the recruitment of ATMs, we studied their temporal relationship to changes in ATM content. Improved hepatic and peripheral insulin sensitivity was seen after 21 days of pioglitazone. We found early reductions in macrophage chemoattractant factors after only 10 days of pioglitazone, followed by a 69% reduction in ATM content at 21 days and reduced ATM activation at both time points. Although markers for dendritic cells and neutrophils were reduced at both time points, there were no significant changes in regulatory T cells. These results are consistent with an association between adipose macrophage content and systemic insulin resistance in humans. ***Diabetes*** **62:1843–1854, 2013**

Obesity is an important causal factor in the global diabetes epidemic (1,2). Adipose tissue generates substantial amounts of proinflammatory molecules believed to contribute to insulin resistance (3). Obesity is associated with increased adipose tissue macrophage (ATM) infiltration in rodents and humans (4–6). Inflammatory cells, including macrophages, appear to be the main source of various fat-derived inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1b (7,8), and many rodent models suggest that increased ATM content is associated with insulin resistance (9–12). However, human studies have not universally shown a relationship between ATM content and insulin resistance, raising questions about the role of ATMs in the metabolic consequences of obesity in humans (13,14).

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Increased local production of macrophage chemoattractant protein-1 (MCP-1) appears to recruit circulating monocytes/macrophages through interaction with the MCP-1 receptor chemokine (C-C motif) receptor 2 (CCR2) (9–11), and MCP-1 expression is increased in human adipose tissue from obese subjects (6). Adipocytes in obesity also appear to express increased amounts of hyaluronan and its receptor CD44, thereby recruiting more monocytes into adipose tissue (15–18). In addition, a number of studies have suggested that regulatory T cells (Tregs) oppose recruitment of proinflammatory macrophages, thereby improving insulin sensitivity (19–21), although a recent study showed that Treg markers were paradoxically upregulated and correlated with inflammation in adipose tissue of obese human subjects (22). Furthermore, rodent studies suggest a role for additional adipose inflammatory cells, including neutrophils and dendritic cells, in macrophage recruitment and insulin resistance (23,24).

Therefore, many unanswered questions remain regarding the relationships among ATMs, chemoattractant factors, and insulin action in humans. Because thiazolidinediones have been shown to reduce insulin resistance and inflammatory factors in subjects with type 2 diabetes (25,26) and to reduce ATM content in subjects with impaired glucose tolerance (27), we used pioglitazone to prospectively study the temporal sequence of its effects on hepatic and peripheral insulin sensitivity, subcutaneous ATM content, chemoattractant factors, and immune cell populations. We conducted hyperinsulinemic-euglycemic pancreatic clamp studies along with adipose tissue biopsies after 10 and 21 days of pioglitazone treatment in individuals with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Human subjects. All studies and procedures were approved by the Albert Einstein College of Medicine Institutional Review Board. Before their enrollment in the study, informed, written consents were obtained from all subjects. A total of 26 adult volunteers with type 2 diabetes were enrolled in the 10-day and/or the 21-day protocols. Three subjects participated in both the 21- and 10-day studies, with a wash-out period of at least 6 months between studies. Subjects with a history of medical conditions other than type 2 diabetes and well-controlled hypertension were excluded. Women who were of child-bearing age were allowed to participate provided that they had negative pregnancy test results within a week of the studies.

Each subject was instructed to follow his or her usual diet and activity while participating in the studies. The subjects discontinued sulfonylureas and metformin for 3 days (the approximate half-life of a typical sulfonylurea, glyburide, is 10 h; the half-life of metformin is 18 h in blood) and long-acting insulin for 24 h before each clamp study. Subjects fasted overnight, but took their capsules on the morning of the study. Blood samples were collected before each study for lipid profile, plasma glucose, insulin, glycerol, and free fatty acid (FFA) levels.

The 21-day pioglitazone studies. A total of 16 subjects participated in a pair of clamp studies after 45 mg/day of pioglitazone (Pio21d) and placebo (Plc21d) treatment for 21 days each. Each participant received the experimental agents

in random order, in a double-blind fashion. There was a washout period of at least 3 weeks before they began taking the other experimental agent. Some clamp data from eight of these subjects were reported previously. Adipose tissue biopsies were performed during the final 30 min of the clamp studies under local anesthesia, as described below.

To examine the effects of 21 days of pioglitazone on hepatic and peripheral insulin action, we used 6-h "stepped" hyperinsulinemic clamp studies (Fig. 1*A*). The subjects were admitted the night before the study. After establishing intravenous access, insulin infusions (Novolin Regular) were begun at 3:00 A.M. and were adjusted according to hourly plasma glucose measurements to gradually attain euglycemia. At 7:30 A.M. the next day, an additional intravenous cannula was inserted in the opposite arm for blood sampling, and "stepped" hyperinsulinemic clamps were performed, as previously described (26).

Briefly, a primed-continuous infusion of high-performance liquid chromatography-purified [3-^{3}H]glucose (bolus 21.6 μCi for 5 min; continuous infusion of 0.15 μCi/min) was initiated at t = 0 min to quantify glucose turnover. To reduce interstudy variability, individualized basal insulin replacement rates were established from 0 to 120 min by means of variable rates of insulin infusion to keep plasma glucose levels at ~90 mg/dL without the need for glucose infusion. Insulin infusion rates were then increased by 20 mU/m^2/min above these basal rates to reproduce physiologic hyperinsulinemia ("low insulin" step of the clamp) from 120 to 240 min. Infusion rates were then further increased by 150 mU/m^2/min above the basal rate for the final 2 h of the studies ("high insulin" step of the clamp). All experiments consisted of 360-min somatostatin (250 μg/h) infusions with replacement of glucoregulatory hormones (glucagon 1 ng/kg/min; growth hormone 3 ng/kg/min) to maintain fixed levels of these hormones throughout.

Plasma glucose was measured every 5 to 10 min and maintained at euglycemia (~90 mg/dL) by a variable infusion of [3-^{3}H]glucose-enriched 20% dextrose. Blood samples were collected every 15 to 60 min to measure plasma insulin, C-peptide, FFA, glycerol, and [3-^{3}H]glucose. All infusions were stopped at t = 360 min. The subjects were then given a standard meal, and plasma glucose concentrations were monitored for an additional 60 min. Dextrose infusion was continued for ~30 min after completion of the clamp to avoid hypoglycemia.

The 10-day pioglitazone studies. A total of 13 subjects underwent adipose tissue biopsies and/or clamp studies at baseline (B10d) and after 45 mg/day (Pio10d) pioglitazone treatment for 10 days. Six of these 13 subjects participated in 3-h hyperinsulinemic clamp studies before and after pioglitazone treatment, with adipose tissue biopsies performed during the last 30 min of the clamp study. These shorter studies were conducted to reduce inconvenience to the subjects because the 21-day studies had already demonstrated that pioglitazone improved hepatic and peripheral insulin action. These 3-h clamp studies used insulin infusion rates previously used to examine insulin sensitivity in subjects with type 2 diabetes (28).

After an overnight fast, all subjects received insulin infusions to gradually lower glucose levels to euglycemia. Insulin infusions at 50 mU/m^2/min were initiated at 8:00 A.M. (t = 0) and continued throughout the study duration. A primed continuous infusion of [3-^{3}H]glucose was initiated at t = 0 min as above. In the two most recent subjects, primed continuous infusions of [6,6-^{2}H$_2$]glucose were used to quantify glucose turnover (29). Plasma glucose levels were measured every 5 to 10 min and glucose levels were maintained at euglycemia (~90 mg/dL) by a variable infusion of tracer-enriched 20% dextrose for the entire study. Samples were collected every 15 to 60 min to measure plasma insulin, C-peptide, FFA, glycerol, and labeled glucose. All infusions were stopped at

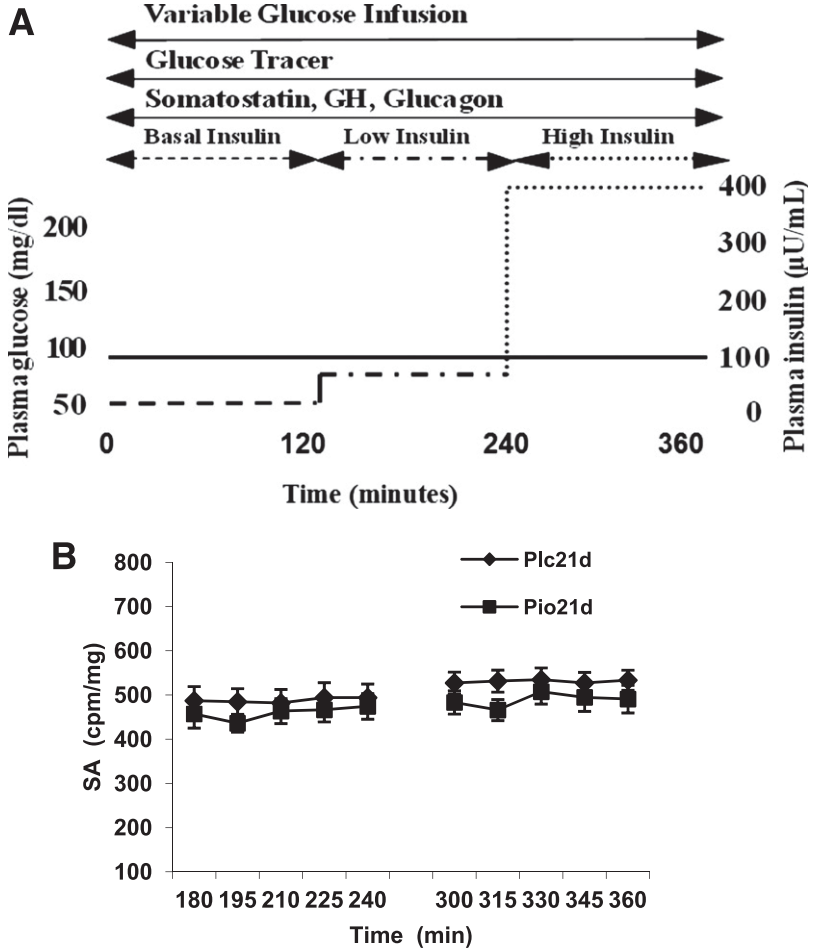


FIG. 1. *A*: Schematic depiction of "stepped clamp" protocol. Plasma glucose values were clamped at 90 mg/dL for the duration of each study. Glucose fluxes were measured by infusing tritiated glucose. Somatostatin was infused for the entirety of the clamp to inhibit pancreatic hormone secretion, with concomitant replacement of glucagon and growth hormone (GH). Basal insulin infusion rates (- - -) were established for the first 2 h, and rates were then increased by 20 mU/m^2/min to reproduce 2 h of physiologic hyperinsulinemia, the "low insulin" step of the clamp (• ▬ •). During the final 2 h of the clamp, the insulin infusion rate was increased by 150 mU/m^2/min above basal, the "high insulin" step of the clamp (·····). *B*: Glucose-specific activity (SA) (cpm/mg) during the steady state of "stepped clamp" shown after Plc21d vs. Pio21d.

t = 180 min. The subjects were then given a standard meal, and plasma glucose concentrations were monitored for an additional 60 min.

Adipose tissue biopsies. A small 0.25-cm cutaneous incision in the periumbilical region was performed under local anesthesia (Lidocaine, 1%) and 1–2 g of subcutaneous adipose tissue was obtained by aspiration (30). The biopsy specimens were immediately homogenized in TRIzol (Invitrogen) to inhibit any RNase activity and stored at −80°C.

Adipose tissue separation. Adipose tissue samples from all subjects were immediately washed at least three times with saline to remove contaminating blood. The cells were then digested with collagenase type 1 (0.05 g per 30 mL of Hanks Balanced Salt solution with 4% BSA; Worthington Biochemical) for 30 min at 37°C with intermittent shaking, followed by extensive washing with Dulbecco's phosphate buffered solution depleted of magnesium and calcium (Mediatech). The adipocytes were separated from the stromal-vascular fraction (SVF) by centrifugation at 3,000 rpm for 10 min. Macrophages were separated from the SVF by CD14+ antibody-coated magnetic Dynabeads (Dynal Biotech) by the manufacturer's recommended method. The separated adipocytes and macrophages were washed with PBS and stored in TRIzol and analyzed by real time RT-PCR.

Analytical procedures. Plasma glucose levels were measured using a Beckman glucose analyzer (Fullerton, CA; glucose oxidase method). Plasma insulin was measured by radioimmunoassay, and plasma FFA levels were measured by an acyl-CoA oxidase-based colorimetric kit (Wako, Osaka, Japan). Glycerol was measured by colorimetric enzymatic methods and plasma lactate by fluorometric enzyme techniques, as previously described (31).

Fluorescence-activated cell sorter analysis. After digestion with collagenase, the pellet consisting of stromal cells was treated with erythrocyte lysing buffer for 5 min, followed by incubation with saturating amounts of fluorescein isothiocyanate–labeled human CD14+ antibody (BD Pharmingen, San Diego, CA), and phycoerythrin-labeled human CCR2+ antibody (R&D Systems, Minneapolis, MN) in staining buffer (PBS containing 1 mg/mL BSA and 12 mmol/L NaN3, pH 7.2) on ice for 20 min, washed, and analyzed immediately by using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). Fluorescence-activated cell sorter (FACS) data were analyzed using FlowJo (V9.0.1) with the assistance of the Flow Cytometry Core Facility at Albert Einstein College of Medicine.

Quantitative real-time RT-PCR. From the adipose tissue samples, total RNA was extracted with TRIzol. cDNA was synthesized using Superscript First Strand Synthesis System for RT-PCR (Invitrogen Technologies). Gene expression was studied by quantitative, real-time RT-PCR using the specific protocol for the LightCycler (Roche Diagnostics, Indianapolis, IN), as previously described (26). Primer sequences are listed in Supplementary Table 3.

Reaction conditions were as follows: 40 cycles, denaturation at 95°C for 0 s, annealing at 59°C for 5 s, and elongation at 74°C for 12 s. All reactions were performed at least three times. Results are expressed as fold-change by determining the ratio of copy number of the gene of interest in a given individual after pioglitazone versus placebo treatment or baseline, corrected for the geometric mean of housekeeping genes in the same pair of samples.

Immunohistochemistry. Adipose tissue samples were fixed for 12 to 16 h at room temperature in neutral phosphate-buffered formalin and embedded in paraffin. Five-micrometer sections, cut at 50-μm intervals, were mounted on charged glass slides and deparaffinized in xylene and rehydrated. Epitope demasking was performed using Target Retrieval Solution pH 9 (Dako, Los Angeles, CA), as recommended by the manufacturer. Thereafter, endogenous peroxidase was inhibited with 3% H_2O_2 in immunohistochemistry (IHC) buffer (0.1% BSA, 0.1% Triton-X in PBS) followed by blocking nonspecific binding with IHC buffer containing 10% normal donkey serum. Subsequently, slides were incubated overnight at 4°C, with anti-human forkhead box P3 (Foxp3; 1:10; eBioscience clone: PCH101, Los Angeles, CA). Foxp3 immunopositive (+) cells were visualized with a peroxidase-based detection system using 3,3-diaminobenzidine as the chromogen (Vector Laboratories, Burlingame, CA). Slides were counterstained with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO), dehydrated, and mounted. Sections were analyzed using a light microscope with a ×40 objective. Foxp3+ cells were enumerated by counting 12 to 18 randomly selected fields per case. Data were normalized as Foxp3+ cells, percentage of total nuclei per field.

Statistical analysis. Statistical analysis of the data over time was performed using SPSS 11.5 software (SPSS Inc., Chicago, IL). For averaged data, paired Student t tests were used for comparisons of pioglitazone versus placebo studies. All data are presented as mean ± SE.

RESULTS

Subject characteristics. The study included 26 overweight or obese subjects (15 men) with type 2 diabetes with the following characteristics: mean age 47.0 ± 1.5 years; BMI 33.3 ± 1.0 kg/m^2; and HbA_{1c} 9.65 ± 0.45. Demographic characteristics did not differ significantly between subjects who participated in the 10-day or 21-day pioglitazone studies (Table 1). Pioglitazone treatment did not affect liver function tests or lipid profiles (Supplementary Table 2). Of note, there were no significant differences in results when analyzed by sex; results are presented as combined data from all participants.

Effects of pioglitazone on metabolic parameters and insulin action.

Twenty-one–day studies

Fasting conditions: Plasma glucose levels did not differ between the two experimental conditions before the insulin infusion was started at 3:00 A.M. (Plc21d = 188.09 ± 14.44 mg/dL vs. Pio21d = 186.67 ± 18.35 mg/dL; P = 0.56; Table 2). The overnight insulin infusion rate required to maintain plasma glucose in the target range of 90–120 mg/dL was significantly lower after 21 days of pioglitazone treatment compared with that of the baseline 21-day study (Plc21d = 1.90 ± 0.28 units/h vs. Pio21d = 1.52 ± 0.23 units/h; P = 0.03). At t = 0, after an overnight fast and insulin infusion for about 4 h, plasma insulin concentrations were significantly lower in the pioglitazone studies compared with placebo (P = 0.01), but plasma glucose levels did not differ and averaged 125.64 ± 4.06 mg/dL for Plc21d and 129.71 ± 5.77 mg/dL for Pio21d (P = 0.38).

Clamp conditions: Average plasma glucose levels were similar for both study types during the physiologic hyperinsulinemic step (low insulin; t = 120–240, P = 0.14) and the pharmacologic hyperinsulinemic step (high insulin; t = 300–360, P = 0.63; Table 2). C-peptide levels were initially moderately suppressed secondary to insulin infusion before the clamp and were further suppressed by somatostatin infusion for the duration of the clamp studies. FFA and glycerol levels did not differ significantly between the study types at the beginning of the clamp study or for the entire duration of the clamp studies. Glucose-specific activity was constant after tracer equilibration during the last hour of the low insulin (t = 180–240 min) and the high

TABLE 1
Subject characteristics

	10-day studies	21-day studies
	n = 13	n = 16
Age (years)	45.85 ± 2.18	47.88 ± 2.13
Sex		
Male	8	10
Female	5	6
BMI (kg/m^2)	34.12 ± 1.41	32.89 ± 1.29
Weight (kg)		
B or Plc	97.75 ± 4.72	93.44 ± 3.27
Pio	96.29 ± 4.89	93.75 ± 3.28
Medication group		
S	1	1
M	4	4
I	3	3
M + S	4	7
I + M	1	1
HbA_{1c} (%)	8.97 ± 0.68	9.87 ± 0.44

Data are means ± SE. Weight: B, weight at baseline study before 10-day pioglitazone treatment; Plc: weight after 21 days of placebo treatment; Pio, weight after 10 or 21 days of pioglitazone treatment. Medication group: S, sulfonylurea alone; M, metformin alone; I, insulin alone; M + S, metformin plus sulfonylurea; I + M, insulin plus metformin.

TABLE 2
Plasma hormones and substrate levels after 21 days of placebo or pioglitazone therapy

	Placebo			Pioglitazone		
	Preclamp	180–240	300–360	Preclamp	180–240	300–360
Glucose (mg/dL)	188.09 ± 14.44	96.43 ± 1.92	89.99 ± 1.06	186.67 ± 18.35	94.08 ± 1.37	90.77 ± 0.97
Insulin (μU/mL)	53.73 ± 9.83	83.13 ± 8.26	433 ± 30.37	34.91 ± 7.61*	74.34 ± 8.88	400.40 ± 25.11
FFA (μmol/L)	198.46 ± 29.91	93.40 ± 16.11	31.09 ± 3.92	148.24 ± 29.72	70.23 ± 19.17	33.10 ± 8.88
Glycerol (μmol/L)	47.80 ± 7.97	64.06 ± 10.46	50.46 ± 6.86	42.14 ± 5.77	59.96 ± 14.68	41.81 ± 7.36
C-peptide (nmol/mL)	0.54 ± 0.08	0.16 ± 0.02	0.08 ± 0.01	0.50 ± 0.06	0.14 ± 0.01	0.09 ± 0.01

Data are means ± SE. *$P < 0.05$ placebo vs. pioglitazone.

insulin (t = 300–360 min) steps, during which time glucose fluxes were measured. After an initial 2-h equilibration, specific activity remained stable throughout both the Plc21d and Pio21d studies (Fig. 1*B*). Pioglitazone administration for 21 days markedly increased the ability of insulin to suppress EGP during the low insulin step (t = 180–240 min; P = 0.0003; Fig. 2*A*), but there was no significant difference in the rate of disappearance (Rd) of glucose (P = 0.28, Fig. 2*B*). However, during the high insulin step of the clamp (t = 300–360min), EGP was nearly completely suppressed in the pioglitazone and placebo groups (Fig. 2*A*), and there was a significant increase in Rd in the pioglitazone group (P = 0.03, Fig. 2*B*). Neither EGP nor Rd was significantly affected after 10 days of pioglitazone treatment, compared with baseline studies (Fig. 2*C*, and *D*).

Ten-day studies

Fasting conditions: Overall there was a small but significant difference in fasting plasma glucose levels between baseline and pioglitazone studies (B10d = 221.08 ± 26.88 mg/dL vs. Pio10d = 195.31 ± 18.69 mg/dL; P = 0.04), but insulin levels did not differ (Supplementary Table 1). However, among the six subjects who participated in hyperinsulinemic clamp studies, fasting plasma glucose and insulin levels did not differ between the baseline and 10-day studies. There were also no differences in fasting C-peptide, FFA or glycerol levels between B10d and Pio10d.

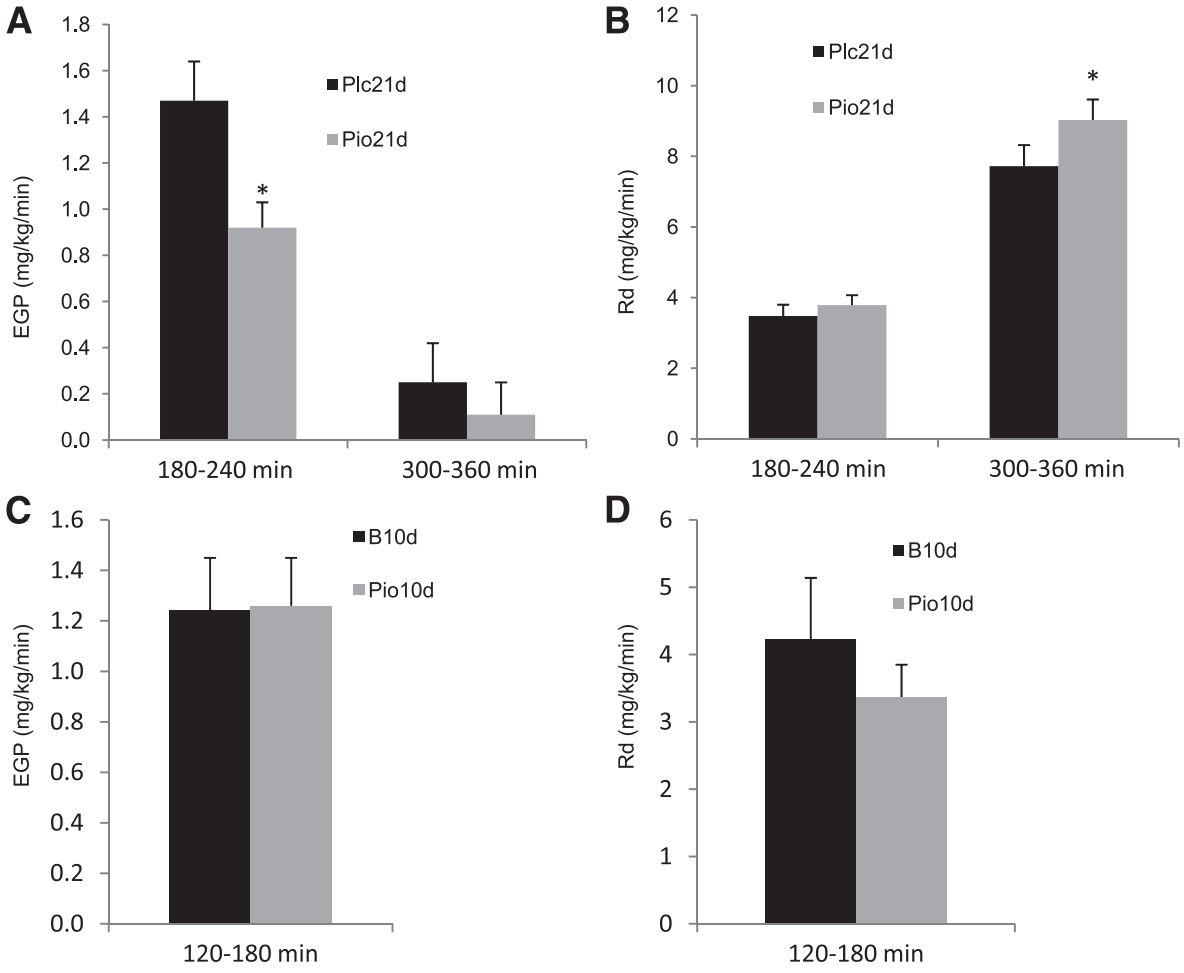


FIG. 2. EGP (*A*) and Rd of glucose (*B*) after Plc21d vs. Pio21d in response to low insulin (180–240 min) or high insulin (300–360 min) steps of the clamp. EGP (*C*) and Rd (*D*) at B10d and after Pio10d during last hour (120–180 min) of the clamp study. *$P < 0.05$.

Clamp conditions: Plasma glucose values did not differ from $t = 120$ to $t = 180$ min of clamp studies between the two study types ($P = 0.68$; Supplementary Table 1). C-peptide levels were suppressed in response to exogenous insulin infusion. FFA and glycerol levels did not differ significantly at the beginning of the clamp studies or for the duration of the clamp studies between the baseline and 10-day studies. Glucose-specific activity was constant after tracer equilibration during the final hour ($t = 120$–180 min) of the clamp studies and did not differ between the two study types. Pioglitazone administration for 10 days did not alter EGP ($P = 0.68$, Fig. 2*C*) or Rd ($P = 0.29$, Fig. 2*D*) at pharmacologic plasma insulin levels of ~80 μU/mL.

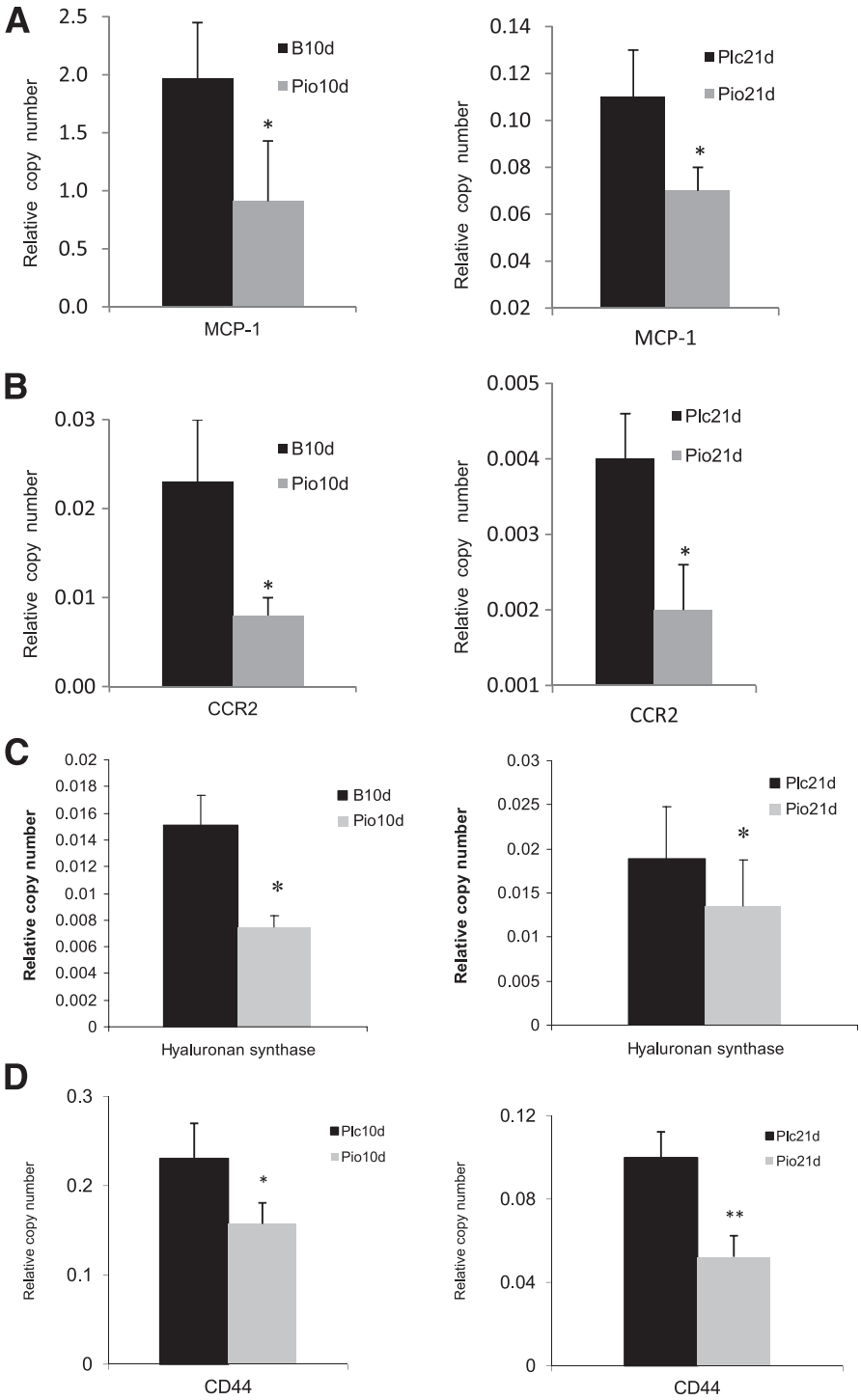


FIG. 3. Gene expression in whole adipose tissue of macrophage chemoattractant factors. *A*: MCP-1 expression in adipose tissue at B10d vs. after Pio10d (*left panel*) and after Plc21d vs. Pio21d (*right panel*). MCP-1 decreased by 30% (95% CI 16–44) after Pio21d. *B*: CCR2 expression in macrophages at B10d vs. after Pio10d (*left panel*) and after Plc21d vs. Pio21d (*right panel*). CCR2 decreased by 34% (95% CI 6–62) after Pio21d. *C*: Hyaluronan synthase expression in adipose tissue at B10d vs. after Pio10d (*left panel*) and after Plc21d vs. Pio21d (*right panel*). Hyaluronan expression decreased by 37% (95% CI 22–52) after Pio21d. *D*: Expression of the hyaluronan receptor CD44 in adipose tissue also decreased significantly after Pio10d and Pio21d, with a 42% decrease (95% CI 23–61) at 21 days (*right panel*). *$P < 0.05$; **$P < 0.01$.

Effects of pioglitazone on macrophage chemoattractant factors. We hypothesized that one of the mechanisms whereby pioglitazone reduces ATM content is by decreasing the expression of macrophage chemoattractant factors in adipose tissue, thus improving the inflammatory state via decreased macrophage recruitment (Fig. 3). Expression of MCP-1, hyaluronan synthase, and its receptor CD44 in whole fat were significantly reduced after pioglitazone treatment for 10 and 21 days. Adipose macrophage CCR2 expression was also significantly reduced after 10 and 21 days of pioglitazone, indicating that pioglitazone affected not only macrophage chemoattractant production by adipose tissue but also expression of specific chemoattractant receptors. Quantification of CCR2+ macrophages via FACS analysis in the SVF of subcutaneous adipose tissue also showed a significant decrease in CCR2+ cells after pioglitazone treatment (Fig. 4*E*).

Effects of pioglitazone on adipose macrophage content. At 21 days, ATM content was reduced by 69% as measured by the number of CD14+ cells in the SVF of adipose tissue quantified by FACS (Fig. 4). Furthermore, using expression of macrophage-specific markers as an alternate measure of ATM content, we observed a 28% reduction in CD14 expression and a 36% reduction in CD68 expression, whereas colony-stimulating factor 1 receptor (CSF-1R) expression decreased by 45% (95% CI 31–58). These results collectively indicate that pioglitazone treatment for 21 days reduced macrophage content in fat tissue. Conversely, there appeared to be no change in macrophage content in whole adipose tissue after 10 days of pioglitazone treatment, as quantified by FACS or by gene expression of the macrophage-specific markers CD14 and CD68.

Effects of pioglitazone on adipose tissue inflammatory markers and macrophage activation. Adipose tissue in obese subjects has been shown to contain increased numbers of classically activated (M1) macrophages, a major source of proinflammatory cytokines, including TNF-α, IL-1b, and IL-6 (32). Alternatively activated (M2) macrophages are associated with increased production of arginase-1 and IL-10 and may provide protection from obesity and insulin resistance, at least in part via peroxisome proliferator–activated receptor-γ (PPAR-γ) activation (32,33). We therefore examined multiple markers of inflammation and macrophage activation in adipose tissue (Fig. 5). After 10 days of pioglitazone treatment, gene expression of IL-6 and IL-1b was decreased in whole fat, with a downward trend in TNF-α. There was no change in expression of inducible nitric oxide synthase (iNOS) after this duration of treatment. However, all of these markers were decreased in whole fat after 21 days of pioglitazone. ATM activation was also significantly reduced after 10 and 21 days of pioglitazone treatment, as shown by the expression of TNF-α, IL-6, and iNOS. Interestingly, after 21 days of pioglitazone, expression of arginase-1 and IL-10 increased significantly in ATMs, consistent with a shift toward the M2 phenotype. Furthermore, pioglitazone induced an ~65% reduction in the quantity of cells with positive fluorescence for iNOS and CD68, indicating a substantial reduction in the amount of iNOS produced by macrophages in adipose tissue. Of note, the more pronounced decrease in inflammatory markers at 21 days corresponds with an observed reduction in ATM content at 21 days.

Effects of pioglitazone on adipose tissue regulatory T lymphocyte, neutrophil, and dendritic cell content. After both 10 and 21 days of pioglitazone treatment,

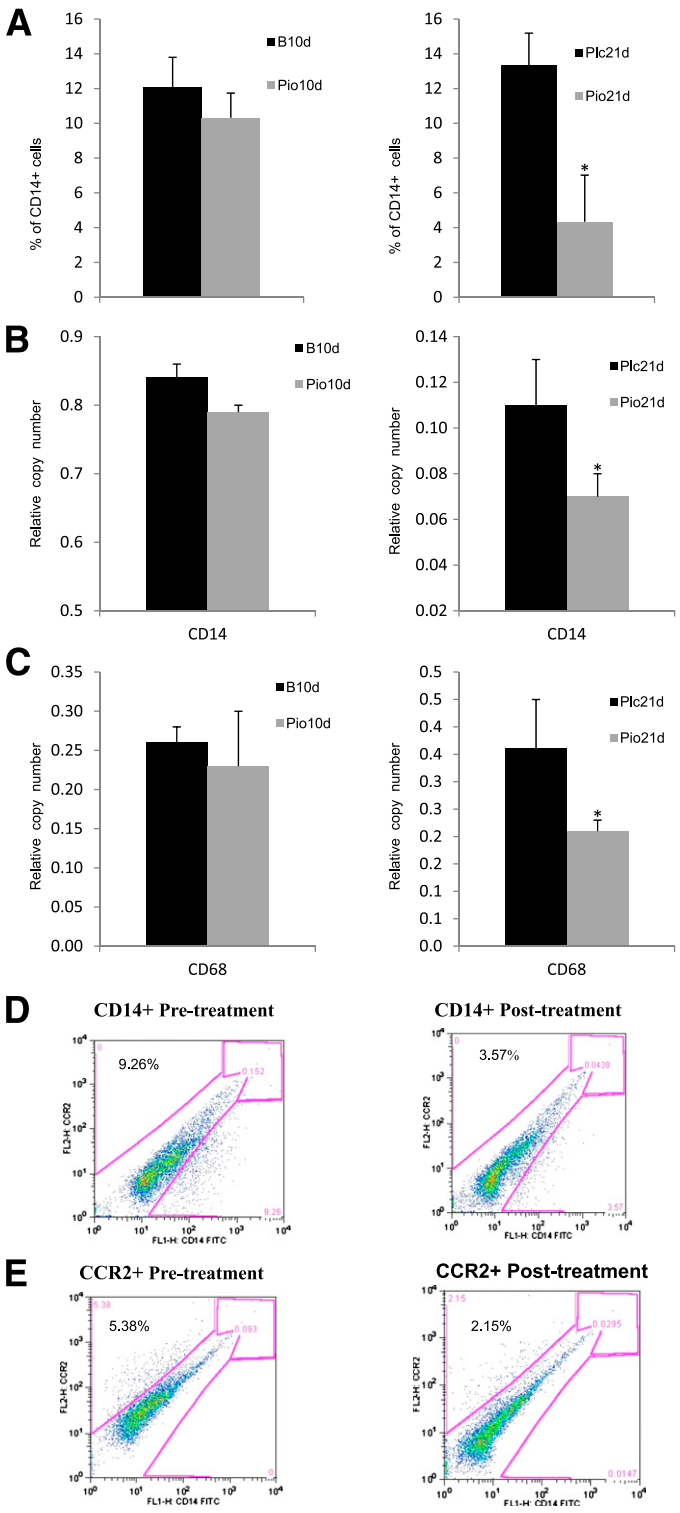


FIG. 4. *A*: FACS analysis of percentage of CD14+ cells in whole adipose tissue at B10d vs. after Pio10d (*left panel*) and after Pio21d vs. Plc21d (*right panel*). CD14+ cells decreased by 69% (95% CI 38–97). Gene expression in whole adipose tissue of CD14 (*B*) and CD68 (*C*) after Pio10d and Pio21d. At 10 days, no significant reductions in CD14 expression or CD68 expression were observed by FACS or by gene expression. At 21 days, a 28% reduction in CD14 expression (95% CI 20–37) and a 36% reduction (95% CI 24–37) in CD68 expression were observed. *D* and *E*: Quantification of CD14+ and CCR2+ cells in the SVF of subcutaneous adipose tissue from one diabetic subject by flow cytometry before and after Pio21d is shown. Fluorescein isothiocyanate–labeled CD14+ antibody and phycoerythrin-labeled CCR2+ antibody were used. Results were analyzed using FACSCalibur flow cytometer. *$P < 0.05$.

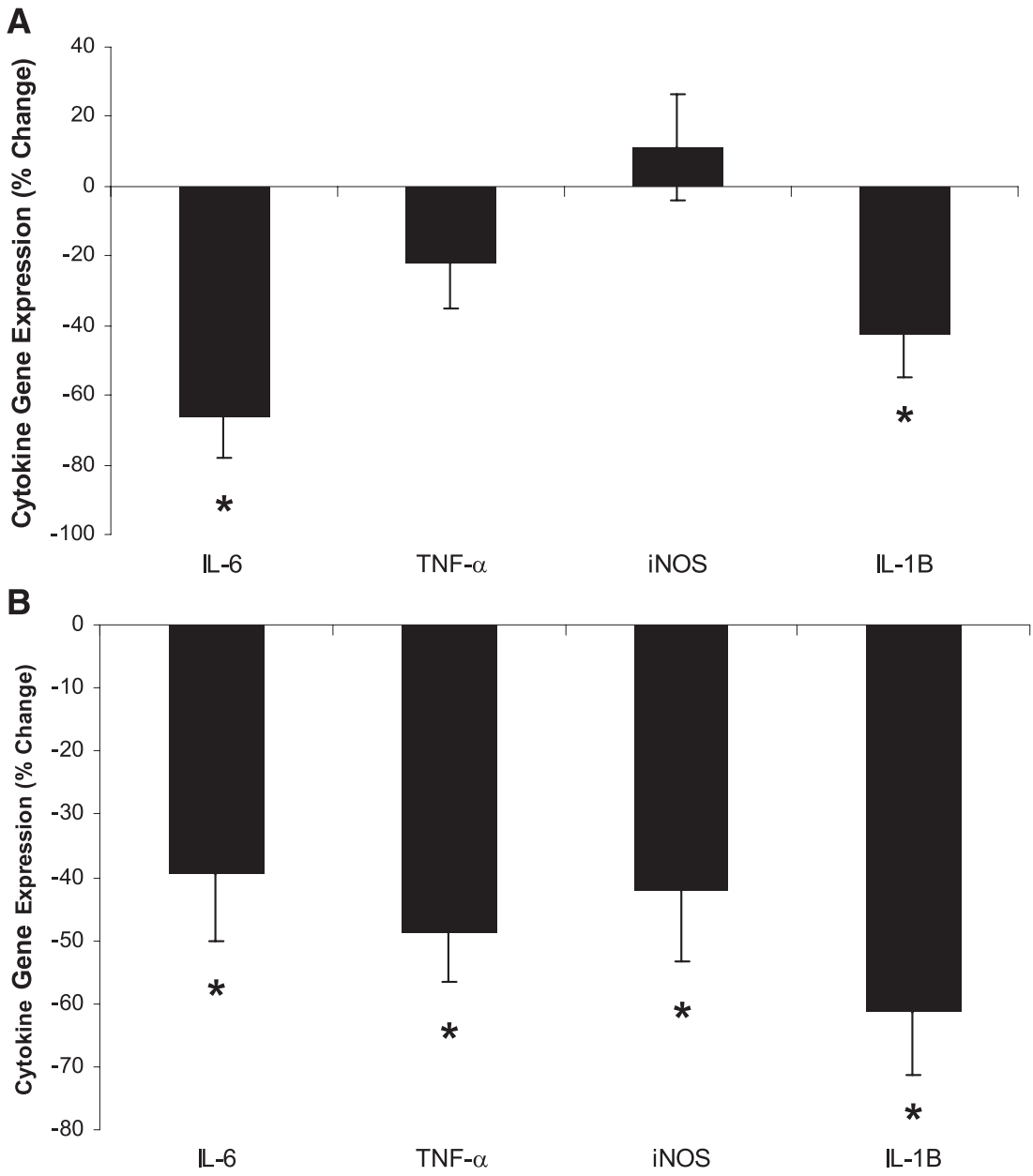


FIG. 5. Percent change in cytokine gene expression in whole fat after 10 (*A*) and 21 (*B*) days of pioglitazone. Percent change in cytokine gene expression in ATM after 10 (*C*) and 21 (*D*) days of pioglitazone. *Significance by $P < 0.05$ or CI. Double-immunofluorescence stains for iNOS expression (red), CD68 (green), and iNOS and CD68 coexpression (yellow, merged panel) in a placebo-treated subject. *E*: Bottom panels show adipose tissue from a subject treated with pioglitazone for 21 days. Note reduced iNOS (red), CD68 (green), and iNOS/CD68 coexpression (*overlay*) after pioglitazone treatment. *F*: iNOS+ CD68 coexpression was significantly reduced in 21 day pioglitazone-treated group compared with placebo; *$P = 0.03$ ($n = 5$).

expression of the dendritic cell markers DEC-205 and DC-SIGN were significantly decreased in whole fat (Fig. 6). In addition, expression of the neutrophil marker myeloperoxidase significantly decreased at both time points. There were no significant changes in expression of the Treg-specific markers FOXP3 or CD25 after 10 or 21 days of pioglitazone. Furthermore, the content of FOXP3+ cells in adipose tissue, as analyzed by IHC, did not differ with treatment of any duration.

DISCUSSION

To explore the relationship between adipose inflammation and insulin resistance in humans, we designed studies examining early effects of treatment with the PPAR-γ agonist pioglitazone on insulin action, macrophage chemoattractants, and adipose inflammatory cell content in subjects with type 2 diabetes. PPAR-γ is a nuclear receptor that regulates fatty acid storage and glucose metabolism and is known for its insulin-sensitizing and anti-inflammatory effects (34,35). In humans, these receptors are most highly expressed in adipocytes (36) but are also present in other cells types, including macrophages (37,38). After 21 days of pioglitazone treatment, a substantial decrease in macrophage content coincided with improved hepatic and peripheral insulin action. Although no significant changes were seen in macrophage content or insulin action after 10 days of pioglitazone, significant decreases in whole fat macrophage

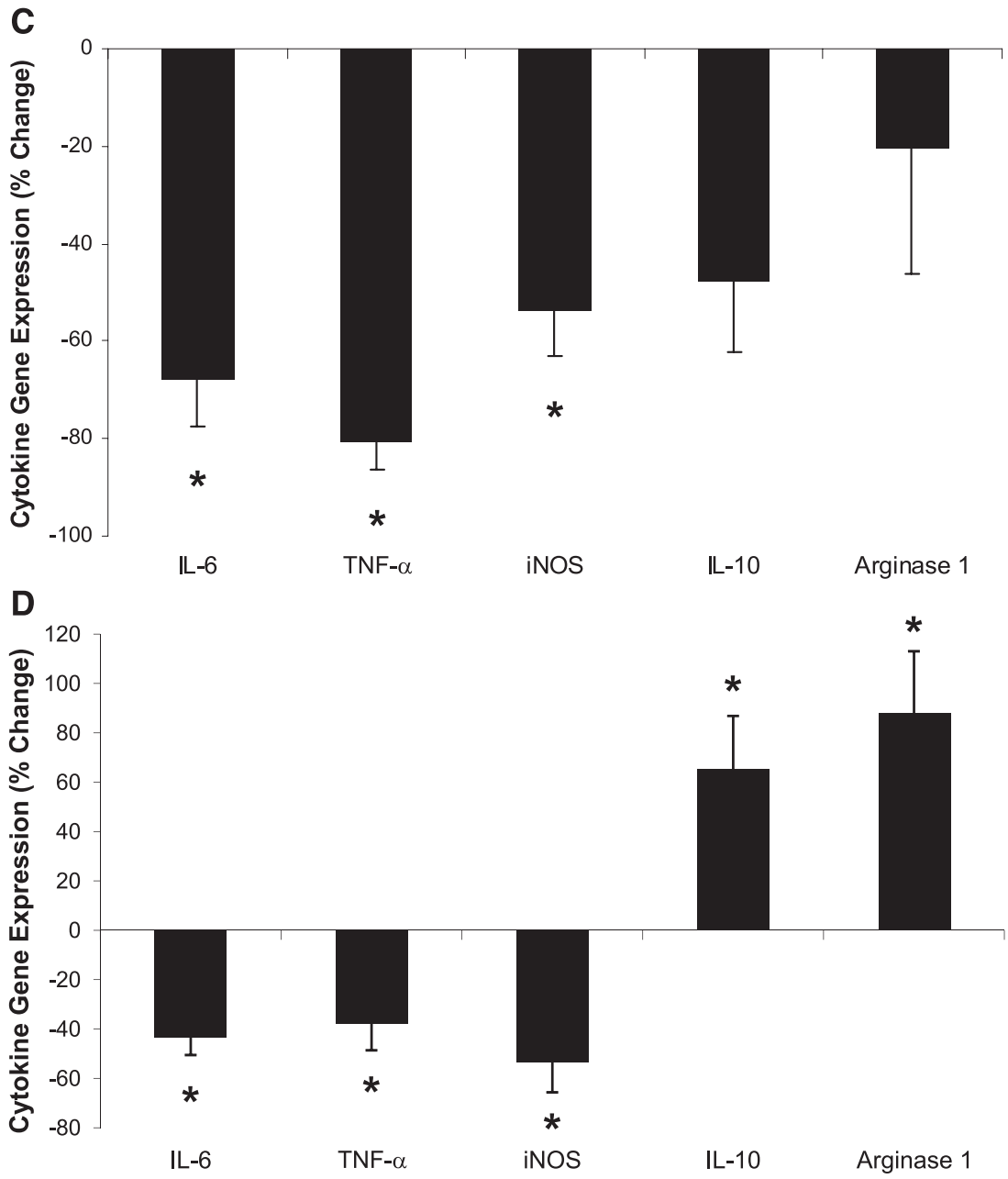
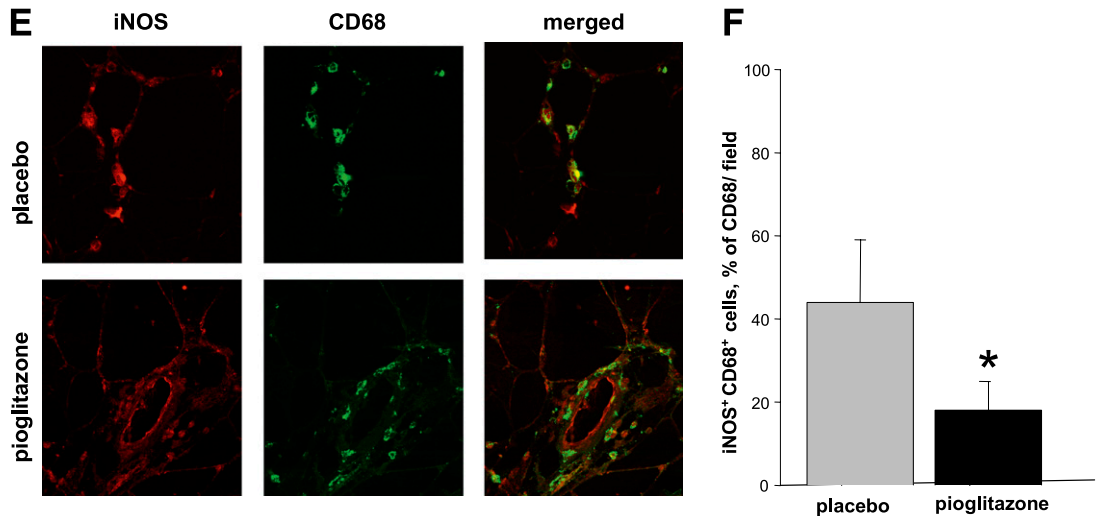


FIG. 5. Continued.

chemoattractant factors and their receptors, neutrophils, and dendritic cells at 10 days preceded the subsequent reduction in ATM content at 21 days. Conversely, there were no changes in FOXP3 or CD25 expression at 10 or 21 days, suggesting that changes in Tregs were not responsible for the observed changes in macrophage content.

Of note, consistent data are lacking to substantiate a relationship between ATM content and insulin sensitivity in humans. Cancello et al. (6) reported significant improvements in fasting glucose and insulin levels and in the quantitative insulin sensitivity check index 3 months after gastric bypass surgery in obese subjects, in association with an ~18% decrease in body weight. The number of subcutaneous ATMs was ~20% higher in obese subjects, and drastic postoperative weight loss resulted in a significant decrease (~11%) in macrophage number. Expression of a number of factors involved in macrophage attraction was also higher in the obese subjects and significantly decreased after surgery. In addition, Makkonen et al. (4) reported an inverse correlation between whole-body insulin sensitivity (M value) and adipose tissue expression of CD68 in overweight and obese humans.

By contrast, Tam et al. (14) reported that 28 days of feeding a high-fat diet to healthy individuals failed to increase the numbers of ATMs, T cells, and circulating immune cells, or expression of their surface activation markers, despite an 11% decrease in insulin sensitivity as measured by hyperinsulinemic-euglycemic clamp. That the subjects in this study group had only a modest weight gain (<3 kg) may explain the lack of increase in ATM content and MCP-1 expression, whereas the decreased insulin sensitivity may have been explained by a significant



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increase in liver fat content. Finally, a study of nondiabetic Pima Indians found that subcutaneous ATM content did not correlate with insulin action independent of adiposity, although subjects had a fairly wide range of BMI and different relationships may have applied in the overweight versus morbidly obese subjects (13).

Our studies assess the effects of pioglitazone on macrophage content, chemoattractant factors, cytokine expression, and immune cell activation at an early point in the treatment course, before glucose levels are affected and before decreases in adipose macrophage content become significant. This allows us to carefully study early factors that could contribute to the ultimate ~70% drop in macrophage content after 21 days. After only 10 days, pioglitazone therapy resulted in decreased expression of macrophage chemoattractant factors in whole fat, including MCP-1, hyaluronan, and the hyaluronan receptor CD44, as well as decreased expression of the MCP-1 receptor CCR2 in macrophages.

These studies also examined whether pioglitazone could affect activation of macrophages in adipose tissue. Indeed, after only 10 days of pioglitazone treatment, ATM activation was significantly reduced in adipose tissue of subjects with type 2 diabetes, as demonstrated by reductions in expression of iNOS, IL-1b, and IL-6. In addition, pioglitazone affected the quantity of iNOS associated with ATMs after 21 days of treatment, as quantified by immunofluorescence. Furthermore, whereas reductions in markers of adipose tissue activation were already observed after 10 days of pioglitazone, a shift toward the alternatively activated M2 phenotype was noted subsequently at 21 days. Given the likelihood that classically and alternatively activated macrophages represent a continuum (39–41), we have expressed our results in terms of degrees of inflammatory activation.

In addition to macrophages and adipocytes, T lymphocytes, neutrophils, and dendritic cells have recently received attention (22–24,42). High-fat feeding in mice causes early infiltration of neutrophils into adipose tissue (23), and dendritic cells have also been implicated in the development of obesity-associated inflammation by inducing macrophage infiltration into adipose tissue (24,42). Furthermore, several studies have shown that CD8+ and CD4+ T cells are both increased in obese adipose tissue (17) and may recruit and assist ATMs in promoting inflammation and insulin resistance (39,43). In our studies, significant decreases in neutrophil and dendritic cell markers were observed at 10 and 21 days, but there was no effect on Treg content, suggesting that decreases in regulatory T-cell content are not required for the changes in macrophages observed with pioglitazone treatment. Consistent with our inability to demonstrate a negative association between Tregs and ATM infiltration in humans, Zeyda et al. (22) reported that Tregs were significantly elevated in visceral and subcutaneous adipose tissue of morbidly obese subjects compared with lean control subjects. This does not exclude the possibility that Tregs play a role in the regulation of macrophages in fat.

To conclude, these are the first studies in humans to demonstrate a temporal relationship between ATM content and insulin sensitivity. Our studies confirm that improved insulin sensitivity is evident after only 21 days of pioglitazone treatment in subjects with type 2 diabetes. This effect may be at least partly mediated by decreased ATM content. Given the effects of pioglitazone on ATM chemoattractant factor expression, it is likely that the decrease in adipose macrophage content is mediated by decreased chemoattractant production. We also showed decreased expression of inflammatory markers of ATM activation, along with decreases in adipose tissue neutrophils and dendritic cells, although adipose Treg content did not change. Thus, results from this early time point of PPAR-γ activation provide further insights into mechanisms of macrophage recruitment in adipose tissue in humans. These extensive studies highlight the important role of macrophage chemoattractants and multiple inflammatory cells in adipose tissue inflammation and strongly suggest a role for ATMs in the metabolic consequences of obesity in humans.

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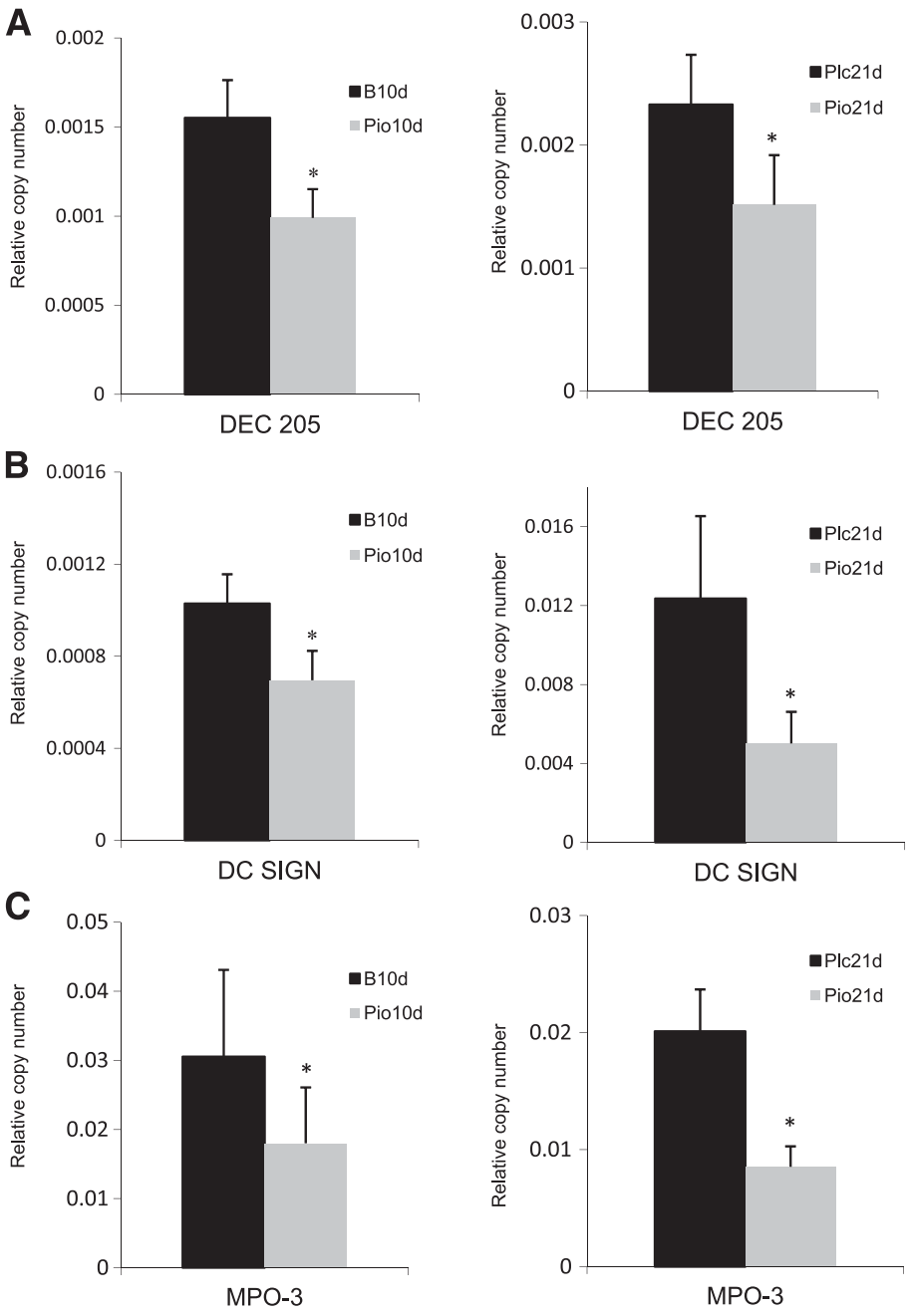

FIG. 6. Gene expression in whole adipose tissue of DEC-205 (*A, left and right panels*) and DC-SIGN (*B, left and right panels*) at B10d vs. after Pio10d and after Plc21d vs. Pio21d (*A* and *B*, *right panels*). *C*: Gene expression in whole adipose tissue of myeloperoxidase (MPO-3) after Pio10d (*left panel*) and Pio21d (*right panel*). Gene expression in whole adipose tissue of FOXP3 and CD25 after Pio10d (*D* and *E*, *left panels*) and Pio21d (*D* and *E*, *right panels*). *F*: Percentage of total nuclei per field of FOXP3 cells in whole adipose tissue samples of Plc21d vs. Pio10d as assessed by IHC. *G*: Representative histological staining for FOXP3. The arrows in subpanels B, C, and D represent FOXP3+ cells. Arrows indicate FOXP3+ cells.

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S.Ko. and S.Ke. performed the clamp studies and biopsies, analyzed and interpreted the data, and wrote the manuscript. M.C. contributed to data collection, analyzed and interpreted the data, and wrote the manuscript. W.L. and K.Z. processed the biopsy tissues, ran the PCR, and analyzed and interpreted the data. E.S. contributed to data collection and analyzed and interpreted the data. D.-E.L. and P.K. performed the clamp studies and biopsies and analyzed and interpreted the data. H.L. and E.C. performed the immunohistochemistry and analyzed and interpreted the data. J.C. processed the biopsy tissues, ran the PCR, contributed to data collection, and analyzed and interpreted the data. M.H. designed the experiments and participated in all aspects of data collection, analysis and interpretation, and manuscript preparation. M.H. 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.

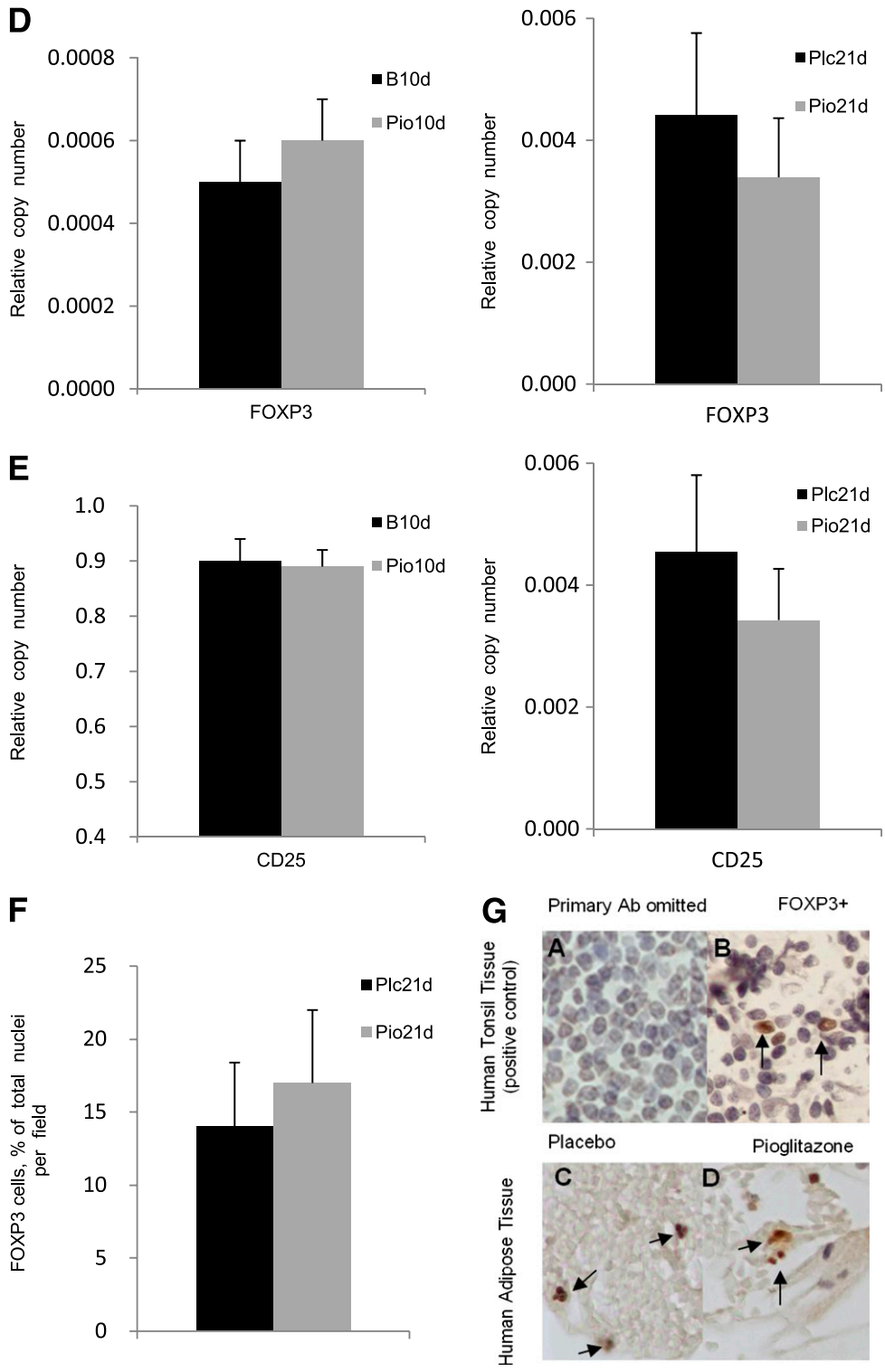

FIG. 6. Continued.

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