

Molecular Cardiology

T-Cell Accumulation and Regulated on Activation, Normal T Cell Expressed and Secreted Upregulation in Adipose Tissue in Obesity

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Background—Obesity is associated with chronic inflammation, which includes increased macrophage accumulation in adipose tissue (AT) and upregulation of chemokines and cytokines. T cells also play important roles in chronic inflammatory diseases such as atherosclerosis but have not been well studied in obesity.

Methods and Results—Flow cytometric analysis showed higher numbers of T cells and macrophages in AT of diet-induced obese insulin-resistant male mice than in lean mice and obese females ($P < 0.05$). RNase protection assay, ELISA, and flow cytometry indicated gender-dependent upregulation of mRNA and protein levels of regulated on activation, normal T cell expressed and secreted (RANTES) and its receptor CCR5 in AT of obese mice. Adipocytes, stromal/vascular cells from mouse AT, and human and murine adipocytes expressed RANTES. RANTES mRNA levels were negatively correlated with adiponectin in mouse AT. Adiponectin-deficient mice fed high-fat diet showed higher RANTES mRNA levels in AT than wild-type mice. Activated T cells cocultured with preadipocytes in vitro significantly suppressed preadipocyte-to-adipocyte differentiation. Obese humans with metabolic syndrome had higher mRNA levels of RANTES and CCR5 in subcutaneous AT than lean humans. RANTES and CCR5 mRNA levels were significantly higher in visceral than subcutaneous AT of morbidly obese humans. RANTES mRNA levels were positively correlated with CD3 and CD11b in human visceral AT.

Conclusions—Obesity is associated with increased accumulation of T cells and macrophages in AT, which may play important roles in obesity-related disease by influencing preadipocyte/adipocyte functions. RANTES is an adipokine that is upregulated in AT by obesity in both mice and humans. (*Circulation*. 2007;115:1029-1038.)

Key Words: inflammation ■ lymphocytes ■ obesity

Obesity increases risk for cardiovascular disease and diabetes mellitus.¹ In addition to the influence of obesity on cardiovascular risk factors such as dyslipidemia, glucose intolerance, and hypertension,¹ obesity is associated with chronic systemic inflammation, which could also directly contribute to development of cardiovascular disease and diabetes mellitus. Adipose tissue (AT) synthesizes and secretes inflammatory substances, which include cytokines such as tumor necrosis factor- α (TNF- α) and chemokines.^{2,3} In human and animal models, TNF- α is upregulated in AT in obesity and mediates obesity-linked insulin resistance.² Chemokines such as monocyte chemoattractant protein-1 (MCP-1) have also been shown to be elevated in AT of obese

animals and humans.³ Concurrently, macrophages, which are the primary targets of MCP-1, are increased in AT in obesity and are potentially involved in obesity-related insulin resistance.⁴⁻⁶ Macrophages, MCP-1, and other mediators related to chronic inflammation are important in the pathogenesis of cardiovascular disease⁷; therefore, chronic inflammation could also be a cardinal link between obesity and cardiovascular disease.

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Chronic inflammation is a complex process that involves altered T-cell function in addition to macrophage accumu-

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lation. T cells are major cellular components in human atherosclerotic plaques and may be important in the evolution of atherosclerotic lesions by communicating with macrophages through cell-cell interactions and/or secreting inflammatory mediators.⁸ However, the effects of diet-induced obesity on T-cell accumulation in AT and on chemokines involved with T-cell recruitment, such as regulated on activation, normal T cell expressed and secreted (RANTES, or CCL5) and its receptors, which also contribute to macrophage recruitment and are implicated in the development and progression of atherosclerotic lesions,^{9,10} have not been well characterized.

The purpose of the present study was to better characterize inflammatory leukocytes, including T cells, that accumulate in AT with obesity, the chemokines and chemokine receptors associated with T-cell recruitment, and the role of adiponectin, an adipokine with antiinflammatory and antiatherosclerotic activity.¹¹

Methods

Mouse Model of Diet-Induced Obesity

C57BL/6J (Jackson Laboratory, Bar Harbor, Me) and adiponectin-deficient (*adip*^{-/-}) mice were used. *Adip*^{-/-} mice were backcrossed onto C57BL/6J for 8 generations, with +/+ littermates used as wild-type (A-WT) controls.¹² All mice were fed standard chow diet (SCD; 4.5% w/w fat [12% of kcal from fat], 0.02% w/w cholesterol; PicoLab Rodent Chow 5053) after weaning until 8 weeks old. To induce obesity, mice were switched to high-fat diet (HFD; 21% w/w fat [41% of kcal from fat], 0.15% w/w cholesterol; Dyets Inc., Bethlehem, Pa) at 8 weeks of age and maintained on this diet for 24 weeks. Lean controls were fed SCD until the time of experiments. For each experiment, age- and gender-matched obese and lean mice were used; 154 mice were studied, which included 90 C57BL/6J (HFD: 34 males, 14 females; SCD: 28 males, 14 females), 32 *adip*^{-/-} (HFD: 8 males, 8 females; SCD: 8 males, 8 females), and 32 A-WT (same as for *adip*^{-/-}). All animal studies were approved by the Animal Protocol Review Committee of Baylor College of Medicine.

Tissue Collection and Fractionation of Adipose Tissue

Mouse perigonadal AT and liver were collected by dissection and weighed. Human visceral AT (VAT; perigastric omentum) and subcutaneous AT (SAT) were collected from 21 morbidly obese patients at the time of bariatric surgery. Additional SAT was collected by needle aspiration from 10 obese patients with metabolic syndrome and 3 lean controls. All human studies were approved by the Institutional Review Board of Baylor College of Medicine, and informed consent was obtained. Collagenase digestion was used to fractionate AT into adipocytes and stromal/vascular (S/V) cells, which were saved for RNA isolation or flow cytometric analysis, as described previously.¹³

Assessment of T Cells and CCR5+ Cells by Flow Cytometry

T cells and CCR5 expression were assessed in S/V fractions of mouse AT by flow cytometry.¹⁴ S/V cells were labeled with fluorescein isothiocyanate- or phycoerythrin-conjugated anti-mouse CD3, CCR5, or CD11c antibodies (BD Pharmingen, San Diego, Calif) and analyzed with a FACScan that used CellQuest software (Becton Dickinson, San Jose, Calif).

Immunofluorescence Staining

Mouse AT was fixed in Z-fix (Anatech Ltd, Battle Creek, Mich) for 24 hours at room temperature and embedded in paraffin. Sections (5 μ m thick) were mounted on charged glass slides and stained for T cells and RANTES with phycoerythrin-conjugated anti-mouse CD3 antibody (hamster IgG1; BD Pharmingen) and purified anti-mouse RANTES antibody (rat IgG2a; R&D Systems, Minneapolis, Minn), followed by fluorescein isothiocyanate-conjugated F(ab')₂ goat anti-rat IgG (Serotec, Raleigh, NC). The sections were counterstained for nuclei with DAPI (Molecular Probes Inc, Eugene, Ore) and analyzed with a deconvolution microscope (Deltavision).

Preadipocyte Culture and Differentiation to Adipocytes

3T3-L1 murine preadipocytes were cultured to confluence in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, Calif) supplemented with 10% calf serum. Two days after confluence (day 0), cells were stimulated with DMEM supplemented with 10% fetal bovine serum, 1 μ mol/L dexamethasone, 0.5 mmol/L isobutylmethylxanthine, and 1 μ g/mL bovine insulin (Sigma). On day 2, the media were changed to DMEM with 10% fetal bovine serum and 1 μ g/mL insulin. From day 4, the cells were fed every other day with DMEM supplemented with 10% fetal bovine serum. On day 8, >95% of the cells had differentiated to adipocytes by morphology. Then 3T3-L1 preadipocytes and adipocytes were incubated in DMEM with or without 10 ng/mL recombinant murine TNF- α (R&D Systems) for 24 hours. Cells were harvested in trizol reagent (Invitrogen) for RNA isolation.

Human adipocytes were differentiated in vitro from human preadipocytes (Cambrex Corporation) with adipocyte differentiation medium (Cambrex Corporation, East Rutherford, NJ) according to manufacturer's protocol. On day 15 after differentiation, >90% of the cells had differentiated to adipocytes by morphology. Preadipocytes and adipocytes were then incubated in preadipocyte growth medium-2 (Cambrex Corporation) with or without 10 ng/mL recombinant human TNF- α (R&D Systems) for 24 hours. Cells were harvested with trizol reagent, and RNA was isolated.

RANTES, CCR5, CD3, CD11b, and Adiponectin mRNA Expression

Total RNA was isolated from AT or cells with trizol reagent. RNase protection assay was performed with an RNase protection assay starter kit with mouse or human chemokine or chemokine receptor template sets (BD Pharmingen).¹⁵ mRNA quantity of RANTES, CCR5, and MCP-1, where appropriate, was determined by the intensity of the appropriately sized, protected probe fragment relative to that of housekeeping genes GAPDH and/or L32 visualized with a Storm 860 PhosphorImager (Molecular Dynamics). Adiponectin mRNA was examined by Northern blot with GAPDH as an internal control. RANTES, CD3, and CD11b mRNA were examined in human AT by quantitative reverse transcription polymerase chain reaction using predesigned primers and probes (Applied Biosystems, Foster City, Calif).

Determination of RANTES Protein by ELISA

To examine RANTES protein levels, mouse AT was homogenized in PBS with Complete Mini (Roche Applied Science, Indianapolis, Ind). To determine RANTES secretion, mouse AT was minced and incubated in DMEM/Ham's F12 (Invitrogen) with 1% bovine serum albumin and penicillin/streptomycin, with or without 10 ng/mL recombinant murine TNF- α , for 8 hours at 37°C. RANTES protein in mouse AT homogenate and AT culture media was measured with Quantikine ELISA kits (R&D Systems).

T-Cell Chemotaxis

T cells were purified from mouse splenocytes with a Pan T-cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as

described previously¹⁴ and cultured in vitro for 7 days with 400 U/mL murine interleukin 2 (IL-2) (R&D Systems) to increase expression of chemokine receptors for RANTES. The chemotaxis assay was performed as previously described.¹⁶ In brief, activated T cells were resuspended in RPMI with 3% fetal bovine serum. Conditioned media from mouse AT explants or control media were placed in the lower wells of a 48-cell chemotaxis chamber (Neuroprobe, Cabin John, Md) and separated from T-cell suspension in the top wells by a 5- μ m-pore size polyvinylpyrrolidone-free polycarbonate filter. After incubation at 37°C for 2 hours, T cells that reached the lower chamber were counted (transwell migration).¹⁶ To determine whether RANTES was responsible for T-cell migration, conditioned media from AT were preincubated with anti-RANTES antibody (Torrey Pines Biologicals, Houston, Tex) at a concentration of 2 μ g/mL before chemotaxis assay as previously described.¹⁶

Effect of T Cells on Preadipocyte Differentiation

T cells purified from mouse splenocytes as described above were cocultured with 3T3-L1 preadipocytes, separated by transwell inserts (Corning Inc, Lowell, Mass), in the presence of 400 U/mL murine IL-2, with or without 2 μ mol/L concanavalin A activation. After 2 days of coculture, preadipocytes were induced to differentiate into adipocytes in the presence of T cells. Differentiation was monitored visually under a microscope. Triglyceride (TG) accumulation was determined by Oil Red O staining after removal of T cells and quantified with a microQuant plate reader (Bio-Tek Instruments Inc, Winooski, Vt) after extraction with isopropanol.

Statistical Analysis

GraphPad Prism 4 (GraphPad Software Inc, San Diego, Calif) and Instate 3 were used to perform statistical analyses. Values are presented as mean \pm SEM. Student *t* test or 2-way ANOVA followed by Bonferroni multiple comparisons test, where appropriate, was used for statistical analyses; Spearman correlation coefficients were computed to examine correlations. Differences were considered significant at $P \leq 0.05$.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Physical and Biochemical Characteristics of Diet-Induced Obese and Lean Mice

Characteristics of the mouse model of HFD-induced obesity have been described previously.¹⁷ In the present study, body weights were significantly higher for obese males (49.15 \pm 0.62 g, $n=34$) and obese females (42.87 \pm 1.39 g, $n=14$), than for lean males (33.35 \pm 0.66 g, $n=28$, $P < 0.01$ versus obese males) and lean females (28.37 \pm 0.72 g, $n=14$, $P < 0.01$ versus obese females). Obese males were severely insulin resistant but not diabetic, and obese females tended to be insulin resistant.¹⁷

T Cells in Mouse Adipose Tissue

As assessed by flow cytometry, macrophage numbers were significantly increased in AT of obese mice compared with lean controls (data not shown). T-cell numbers per gram AT were also significantly increased in obese mice compared with lean controls ($P < 0.01$). Compared with lean counterparts, obese males, but not obese females, had significantly increased T-cell numbers in AT (Figure 1A). Obese males also had higher T-cell numbers in AT than obese females ($P < 0.05$). Immunofluorescence staining showed clusters of

CD3+ T cells between adipocytes in AT of obese males (Figure 1B), whereas T cells were dispersed and rarely seen in clusters in AT of lean males (Figure 1C).

RANTES and CCR5 Levels in Mouse Adipose Tissue and Liver

mRNA levels of RANTES (Figure 2A) and CCR5 (Figure 2B), a major receptor for RANTES, were significantly increased in AT of obese mice compared with lean controls ($P < 0.01$). This increase was significantly more pronounced in males than females ($P < 0.01$) as examined by diet-by-gender interaction. Compared with lean counterparts, obese males but not females had significantly increased RANTES and CCR5 mRNA in AT. CCR3, another RANTES receptor, was slightly increased in AT of obese males (relative mRNA levels: 21.8 \pm 0.6 versus 17.1 \pm 1.1 in lean males, $P < 0.05$), but the level was much lower than that of CCR5. mRNA levels for other RANTES receptors, CCR1 and CCR4, were not significantly different in AT of obese and lean mice (data not shown). RANTES mRNA level in the liver was significantly increased by ≈ 2 -fold in obese males compared with lean males, although RANTES mRNA level in the liver was significantly lower than in AT (data not shown). In contrast, obese females did not have significantly higher RANTES mRNA in the liver than lean females (data not shown).

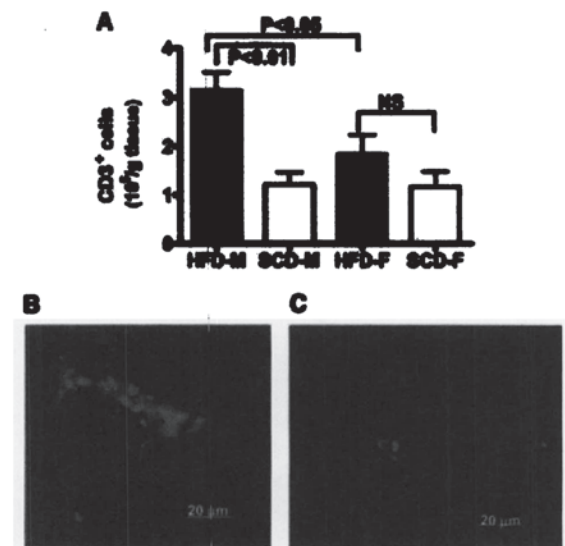


Figure 1. T (CD3+) cells in mouse AT. A, Two-way ANOVA showed that T cells in AT were significantly higher in the HFD than the SCD group ($P < 0.01$). The diet-by-gender interaction probability value was 0.07. Subsequent Bonferroni multiple comparisons test showed that T-cell numbers were higher in AT of obese males but not obese females than in lean counterparts ($n=7$ each for HFD-M, SCD-M, HFD-F, and SCD-F). Obese males also had significantly higher T cells in AT than obese females. Immunofluorescence staining showed clusters of CD3+ T cells (red) in AT of obese males (B) but not lean males (C). B and C are representative images of immunohistochemistry from 5 separate animals/group with similar results. HFD-M indicates diet-induced obese males; SCD-M, lean males; HFD-F, diet-induced obese females; SCD-F, lean females; and NS, not significant.

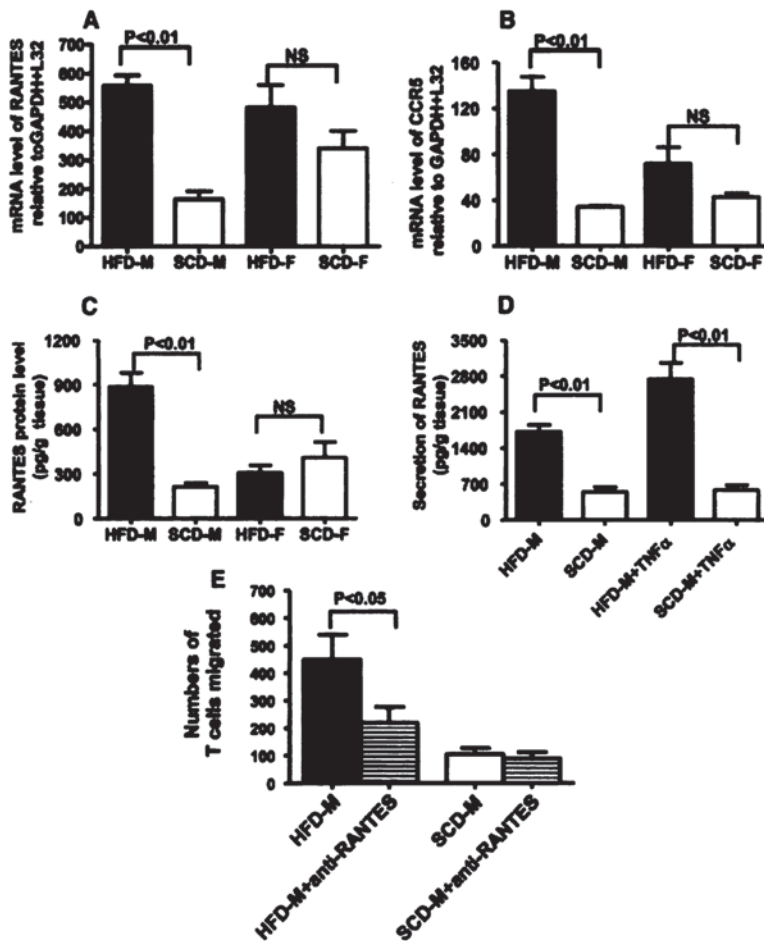


Figure 2. RANTES and CCR5 mRNA in mouse AT, RANTES protein level and secretion from mouse AT, and the role of RANTES in activated T-cell chemotaxis induced by mouse AT explant medium. Two-way ANOVA showed that diet main effect was highly significant ($P < 0.01$) for panels A through E, and diet-by-gender interaction was highly significant ($P < 0.01$) for panels A through C. Subsequent Bonferroni multiple comparisons tests showed that, compared with lean counterparts, obese males showed significantly increased mRNA levels of (A) RANTES ($n = 12$ each for HFD-M and SCD-M, $n = 7$ each for HFD-F and SCD-F) and (B) CCR5 ($n = 4$ /group) in AT as detected by RNase protection assay. C, Obese males had higher RANTES protein levels in AT than lean controls ($n = 4$ /group). D, AT from obese males secreted higher levels of RANTES than AT from lean males when cultured *ex vivo* with or without 10 ng/mL TNF- α ($n = 4$ /group) for 8 hours. Conditioned medium from AT of obese males induced more T-cell migration than that from lean males, and E, neutralization of RANTES significantly inhibited T-cell migration induced by conditioned medium from AT of obese males ($n = 3$ each for HFD-M and SCD-M).

Protein levels of RANTES were significantly higher in AT of obese males, but not females, than in lean counterparts ($P < 0.01$; Figure 2C). RANTES secretion from AT was significantly higher in obese males than in lean controls with or without TNF- α stimulation (Figure 2D).

Flow cytometric analysis showed that CCR5 expression was increased in AT from obese males compared with lean males (geometric mean fluorescence intensity of CCR5: 59 ± 3 in obese versus 34 ± 5 in lean mice, $P < 0.05$, $n = 3$ /group). Dual labeling showed that CCR5 was expressed on subsets of macrophages and T cells; in AT from obese males, $32.6 \pm 3.0\%$ of T cells and $55.3 \pm 1.2\%$ of CD11c+ macrophages expressed CCR5.

Chemoattractant Activity of Conditioned Medium of Mouse Adipose Tissue Explant Culture

Few T cells migrated in response to culture medium alone used as a negative control. Conditioned culture medium from AT of obese males induced significantly more T-cell migration than that of lean controls (Figure 2E). Antibody neutralization of RANTES markedly inhibited T-cell migration induced by conditioned medium from AT of obese males

(Figure 2E), whereas the isotype control of the blocking antibody did not significantly affect conditioned medium-induced T-cell migration (data not shown).

RANTES Expression in Mouse Adipocytes and S/V Fractions and in Mouse and Human Preadipocytes and Adipocytes

Consistent with a previous report,¹⁸ fractionation with collagenase digestion upregulated mRNA levels of proinflammatory molecules in mouse AT (data not shown). Both adipocytes and S/V cells expressed RANTES mRNA, with higher levels in S/V cells (Figure 3A). Immunofluorescence staining confirmed RANTES expression in mouse AT (Figure 3B). RANTES expression was abundant in AT and colocalized with T cells as indicated by dual staining of antibodies to RANTES and CD3 (Figures 1B and 3C). Although adipocytes did not show abundant RANTES expression by immunostaining (Figure 3B), the dehydration procedure required for immunostaining depleted cytoplasm from adipocytes.

In vitro studies with cultured cells demonstrated that mature 3T3-L1 adipocytes had higher mRNA levels of

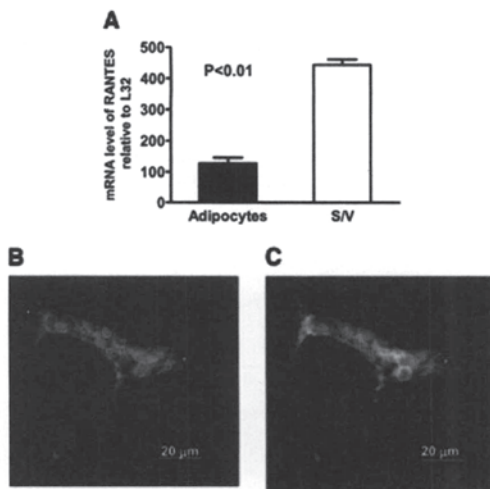


Figure 3. RANTES expression in S/V and adipocytes from mouse AT. A, Significantly higher RANTES mRNA levels were detected in S/V than in adipocytes from mouse AT by RNase protection assay ($n=6$ /group). Immunofluorescence staining showed RANTES expression (green) in mouse AT (B) and its colocalization (yellow) with CD3 expression (C). B and C are representative images of 5 animals with similar results.

RANTES but lower mRNA levels of MCP-1 than preadipocytes (Figure 4A). Compared with preadipocytes, adipocytes had significantly increased upregulation of RANTES (Figures 4A and 4B) but decreased upregulation

of MCP-1 (Figures 4A and 4C) with TNF- α stimulation. Both human mature adipocytes and preadipocytes showed abundant expression of RANTES and MCP-1 with TNF- α stimulation (Figure 4D).

Adiponectin and RANTES Expression

Adiponectin mRNA in mouse AT was significantly lower in obese mice than in lean counterparts ($P<0.01$). Compared with lean counterparts, obese males but not females had significantly reduced adiponectin mRNA (Figure 5A). Obese males also had decreased adiponectin mRNA compared with obese females. Adiponectin mRNA levels were inversely correlated with RANTES mRNA levels in mouse AT (Figure 5B).

In contrast to a previous finding,¹² both male and female obese adip^{-/-} mice were significantly heavier and had larger perigonadal fatpads than A-WT (Table). Livers of obese adip^{-/-} mice tended to be larger than those of A-WT, but the difference was not statistically significant (Table). Consistent with the previous report,¹² adip^{-/-} mice, on either SCD or HFD, did not have significantly different plasma glucose levels than A-WT; however, insulin levels and homeostasis model assessment for insulin resistance of obese adip^{-/-} mice were significantly higher in females but not males than in A-WT counterparts (Table).

Compared with A-WT, lean adip^{-/-} mice did not show significant differences in RANTES mRNA levels in AT (data not shown). However, obese adip^{-/-} mice had significantly higher mRNA levels of RANTES in AT than A-WT ($P<0.01$) (Figure 5C). Male obese adip^{-/-} mice had

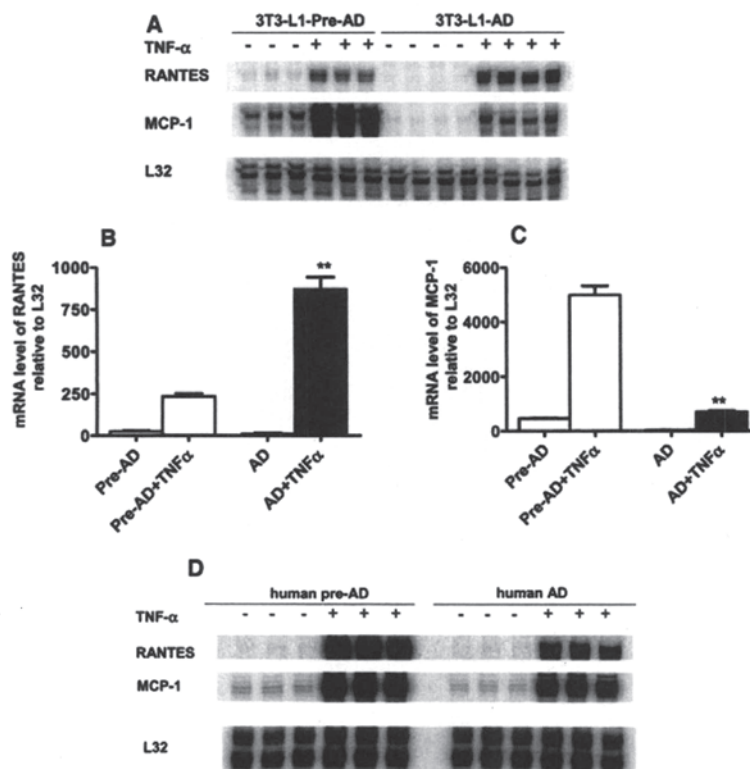


Figure 4. RANTES mRNA in murine and human preadipocytes (pre-AD) and adipocytes (AD). Two-way ANOVA showed significantly increased RANTES mRNA levels (A and B), but decreased MCP-1 mRNA (A and C) in AD compared with pre-AD ($P<0.01$). Upregulation of RANTES by TNF- α (10 ng/mL) stimulation for 24 hours was significantly higher in AD than in pre-AD (A and B; $P<0.01$), but upregulation of MCP-1 by TNF- α was significantly lower in AD than in pre-AD (A and C; $P<0.01$) as examined by the interaction of AD (versus pre-AD) and TNF- α stimulation. $n=3$ for pre-AD, $n=4$ for AD. D, Human AD and pre-AD expressed both RANTES and MCP-1 mRNA with TNF- α stimulation. ** $P<0.01$ for comparisons of the difference with and without TNF- α between AD and pre-AD.

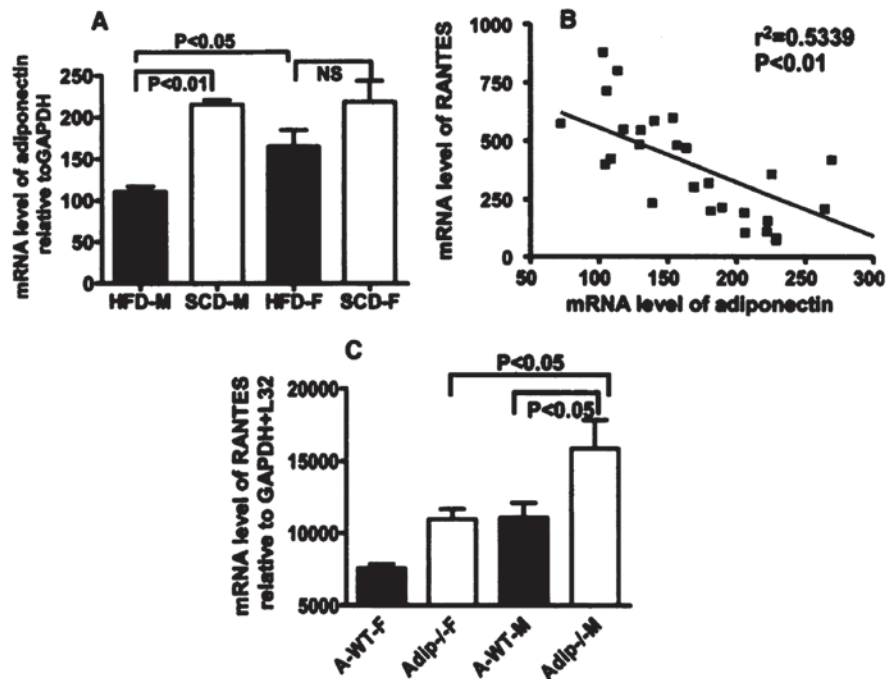


Figure 5. Correlation of RANTES mRNA levels with adiponectin mRNA in mouse AT and RANTES levels in *adip^{-/-}* mice. Two-way ANOVA showed significantly lower adiponectin mRNA in AT of HFD than SCD ($P < 0.01$). A, Bonferroni multiple comparisons tests showed adiponectin mRNA levels were significantly reduced in AT of obese males but not females compared with lean counterparts, and also lower in obese males than obese females ($n = 7$ /group). B, mRNA levels of RANTES and adiponectin were inversely correlated in mouse AT ($n = 28$). C, Two-way ANOVA showed that obese *adip^{-/-}* mice had significantly higher RANTES mRNA levels in AT than A-WT controls ($n = 7$ /group). Bonferroni multiple comparisons tests showed higher RANTES mRNA in AT of *adip^{-/-}* males than *adip^{-/-}* females and A-WT males.

significantly higher RANTES levels in AT than female obese *adip^{-/-}* mice ($P < 0.05$). Obese *adip^{-/-}* mice did not show a significant difference in RANTES mRNA in the liver compared with A-WT.

Effect of T Cells on Preadipocyte Differentiation

On day 8 after differentiation, most 3T3-L1 preadipocytes (Figure 6a) differentiated into adipocytes with abundant TG accumulation (Figure 6b). Coculture with T cells in the presence of murine IL-2 significantly inhibited preadipocyte differentiation with less TG accumulation inside cells (Figures 6c and 6g). Activation of T cells with concanavalin A

dramatically enhanced inhibition of preadipocyte differentiation with even less TG accumulation (Figures 6d and 6g). Murine IL-2 alone (data not shown) or with concanavalin A in the absence of T cells did not significantly affect 3T3-L1 preadipocyte differentiation (Figures 6e and 6g). As reported previously,¹⁹ TNF- α strikingly suppressed preadipocyte-to-adipocyte differentiation (Figures 6f and 6g).

RANTES, CCR5, CD3, and CD11b mRNA in Adipose Tissue of Obese Humans

Obese humans with metabolic syndrome (age, 49.5 ± 3.6 years; body mass index, 41.3 ± 2.1 kg/m²) had significantly higher mRNA

Physical and Biochemical Characteristics of Adiponectin-Deficient Mice on High-Fat Diet or Normal Diet

	High-Fat Diet				Normal Diet			
	Adiponectin ^{-/-}		Adiponectin-WT		Adiponectin ^{-/-}		Adiponectin-WT	
	Male (n=8)	Female (n=8)	Male (n=8)	Female (n=8)	Male (n=8)	Female (n=8)	Male (n=8)	Female (n=8)
Body weight, g	53.6 \pm 2.9*	48.9 \pm 2.5*	46.3 \pm 1.4	42.2 \pm 1.5	33.2 \pm 1.1	27.1 \pm 1.0	30.2 \pm 0.9	23.7 \pm 0.8
Fatpad weight, g	3.05 \pm 0.19†	4.36 \pm 0.51*	2.40 \pm 0.12	3.03 \pm 0.35	0.86 \pm 0.16	0.57 \pm 0.11	0.57 \pm 0.10	0.30 \pm 0.07
Liver weight, g	5.25 \pm 0.46	3.37 \pm 0.51	4.41 \pm 0.42	2.86 \pm 0.32	1.23 \pm 0.07	0.92 \pm 0.09	1.44 \pm 0.06	0.86 \pm 0.04
Glucose, mg/dL	137.3 \pm 9.8	134.2 \pm 7.4	139.0 \pm 6.7	126.8 \pm 6.1	109.3 \pm 5.0	116.9 \pm 8.0	116.6 \pm 7.4	122.2 \pm 14.0
Insulin, pmol/L	1150 \pm 319.6	739.9 \pm 181.8*	1331 \pm 279.8	271.5 \pm 43.1	157.8 \pm 34.8	116.8 \pm 22.0	154.8 \pm 56.6	98.0 \pm 29.0
HOMA-IR	69.8 \pm 22.1	39.2 \pm 9.4*	78.1 \pm 17.9	14.2 \pm 2.4	7.5 \pm 2.0	6.1 \pm 1.6	8.2 \pm 3.5	5.5 \pm 1.9

* $P < 0.05$, † $P < 0.01$ for *adiponectin^{-/-}* vs *adiponectin-WT*.

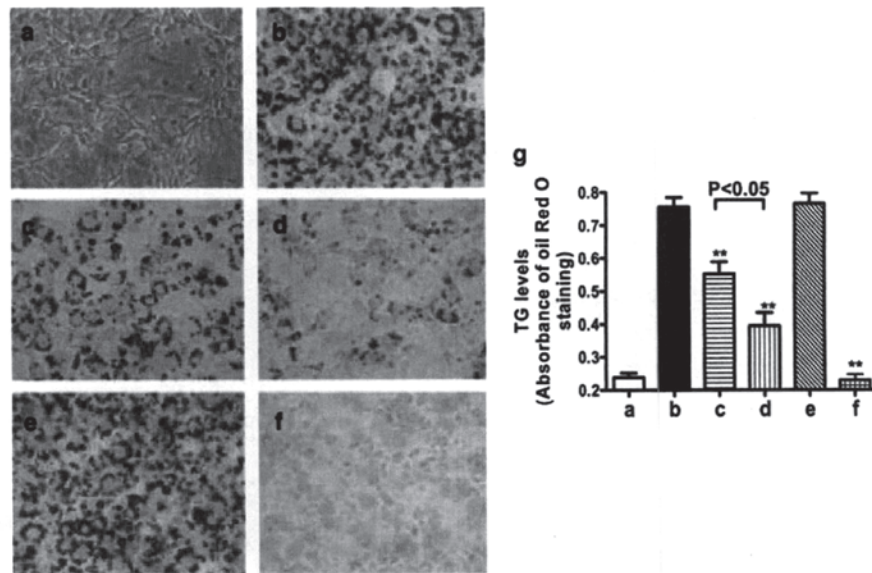


Figure 6. Effect of T cells on 3T3-L1 preadipocyte differentiation. On day 8 after differentiation, adipocytes and preadipocytes were stained with Oil Red O. a, 3T3-L1 preadipocytes with no induction of differentiation; b, 3T3-L1 adipocytes; c, 3T3-L1 adipocytes cocultured with T cells with 400 U/mL murine IL-2; d, 3T3-L1 adipocytes cocultured with T cells with 400 U/mL murine IL-2 and 2 μ mol/L concanavalin A; e, 3T3-L1 adipocytes with 400 U/mL murine IL-2 and 2 μ mol/L concanavalin A; f, 3T3-L1 adipocytes with 10 nmol/L mTNF- α . Panels a through f are representatives from 6 culture wells in 2 separate experiments. g, Quantification of triglyceride accumulation in adipocytes; X-axis labels indicate the same conditions as in a through f, and data are from 6 culture wells in 2 separate experiments. ** $P < 0.01$ versus b.

levels of RANTES and CCR5 in SAT than lean controls (age, 35.0 ± 8.1 years; body mass index, 22.4 ± 1.0 kg/m²; Figure 7A). mRNA levels of RANTES and CCR5 in SAT were positively correlated with body mass index (Figures 7B and 7C). VAT from morbidly obese subjects (age, 38.3 ± 1.8 years; body mass index, 49.9 ± 1.7 kg/m²) had higher mRNA levels of RANTES and CCR5 than SAT (Figure 7D). In human VAT, RANTES mRNA was positively correlated with the T-cell marker CD3 (Figure 7E, $r^2 = 0.85$, $P < 0.01$) and the macrophage marker CD11b (Figure 7F, $r^2 = 0.53$, $P < 0.01$).

Discussion

We found a gender-dependent increase in the number of T cells and macrophages in AT of mice with diet-induced obesity, with higher numbers of T cells and macrophages in AT of obese males than in AT of obese females or lean males. Previous studies in mice have shown that obesity caused by diet or genetic factors such as deficiency of leptin (*ob/ob*) or leptin receptor (*db/db*) leads to increased macrophage accumulation in AT associated with increased MCP-1 and CCR2.⁴⁻⁶ In the model of diet-induced obesity used in our study, which had 41% of caloric intake from fat, similar to a high-fat Western diet, T cells were significantly increased in male obese mice but not female mice compared with lean controls. Most previous studies have presented data on male mice only⁵ or assumed that molecular processes responded to AT mass changes independent of sex and the mechanism of obesity.⁶ Xu et al did not observe increased CD3+ T cells in stromal cells from *ob/ob* mice.⁵ However, mice with genetic deficiencies in leptin or leptin receptors may not be optimal for study of the immune system, because of the role of leptin

in inflammatory processes that involve T cells²⁰ and the observation of thymus atrophy in *db/db* mice.²¹

Both mRNA and protein levels of RANTES were also increased in a gender-dependent fashion with obesity. RANTES secretion from AT of obese males was increased, and monoclonal antibodies to block RANTES significantly reduced T-cell chemotaxis induced by media conditioned by AT from obese males. The most striking increase was in CCR5; CCR3 was modestly increased, and CCR1 and CCR4 were not changed. Mice deficient in CCR2 or MCP-1 were recently reported to have modestly reduced macrophages in perigonadal AT, which implies that other chemokines/receptors, such as RANTES/CCR5, may be important in leukocyte accumulation.^{4,22}

RANTES mRNA was expressed in both adipocytes and S/V fractions, with higher levels in S/V fractions. Immunostaining showed that RANTES was primarily colocalized with T cells in AT of obese male mice. However, both human and murine mature adipocytes expressed RANTES with TNF- α stimulation, which shows that RANTES is a true adipokine under inflammatory conditions. In contrast to a previous study in which mRNA expression of inflammatory genes was barely detectable in the liver and essentially unchanged in obese mice,⁵ we found that RANTES mRNA in livers was significantly increased in obese male mice compared with leans.

The mechanisms for upregulation of RANTES in AT of obese mice may be multiple and remain to be elucidated. Although T-cell and macrophage accumulation could be contributing factors, it is not known whether RANTES

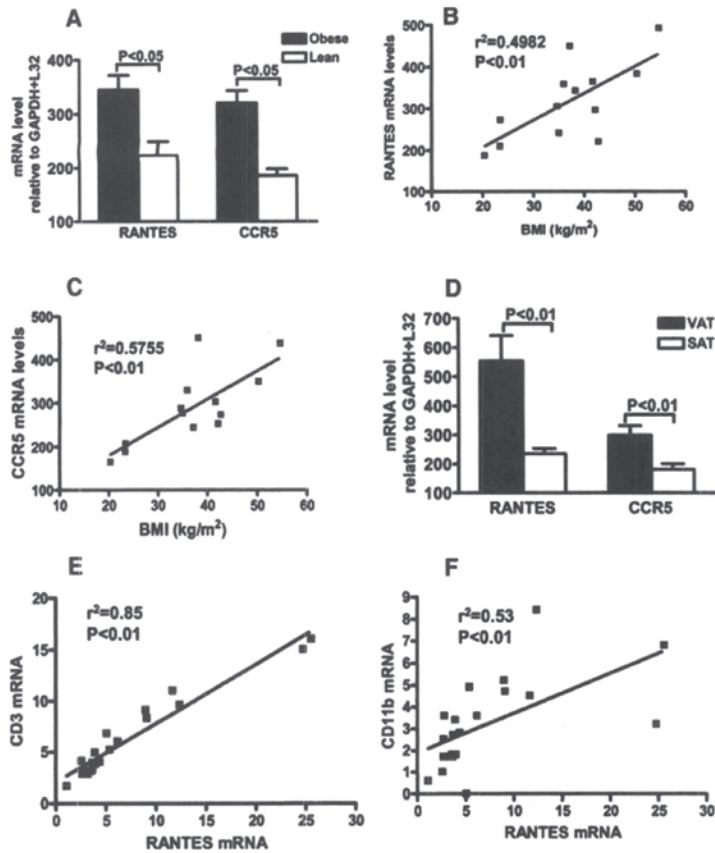


Figure 7. RANTES, CCR5, CD3, and CD11b mRNA levels in human AT. A, mRNA levels of RANTES and CCR5 detected by RNase protection assay in SAT of obese subjects with metabolic syndrome ($n=10$) and lean controls ($n=3$); correlation of (B) RANTES and (C) CCR5 mRNA in SAT with body mass index; D, mRNA levels of RANTES and CCR5 in VAT and SAT from morbidly obese subjects ($n=19$); correlation of RANTES mRNA with (E) CD3 and (F) CD11b in VAT detected by quantitative reverse transcription polymerase chain reaction ($n=21$).

upregulation or T-cell accumulation comes first, and what pathways initiate this process in AT during diet-induced obesity. TNF- α , which is upregulated in AT of obese mice and humans,² induces RANTES expression in human and murine adipocytes, as seen in the current study, and is therefore a potential candidate for upregulation of RANTES in AT of obese mice. However, a recent report that induction of chemokines such as MCP-1 in AT preceded that of TNF- α in a mouse diet-induced obesity model²³ makes TNF- α a less likely "initiator."

Adiponectin is an adipocyte-specific molecule that is downregulated with adiposity and positively regulates insulin sensitivity through an AMP-activated protein kinase pathway.²⁴ Adiponectin also has antiinflammatory and antiatherosclerotic activity.¹¹ We found downregulation of adiponectin in obese male but not female mice, and the significant inverse correlation of adiponectin with RANTES mRNA levels in mouse AT suggests a potential role of adiponectin in regulation of RANTES expression in AT. Obese *adip*^{-/-} mice had significantly increased RANTES mRNA levels in AT compared with A-WT. Although the increased insulin resistance in *adip*^{-/-} females compared with A-WT females could contribute to the tendency of higher RANTES levels, the effects of adiponectin on RANTES cannot be explained solely by changes in insulin resistance. Homeostasis model assessment for insulin resistance was not significantly different between *adip*^{-/-} males and A-WT males, yet mRNA

levels of RANTES were significantly higher in *adip*^{-/-} males, which indicates that adiponectin plays a distinct role in RANTES expression in AT. Furthermore, the gender differences in RANTES expression with HFD cannot be explained solely by adiponectin levels, because in *adip*^{-/-} mice, mRNA levels for RANTES were higher in males than in females even though both lacked adiponectin. We were unable to examine whether this gender difference was present in human VAT because only 1 of the 21 patients was male.

The pathophysiological significance of the increased T cells in AT in obesity is not understood. In our *in vitro* study, activated T cells significantly inhibited preadipocyte-to-adipocyte differentiation (adipogenesis), with reduced TG accumulation. The suppression of TG accumulation in AT may increase ectopic TG deposition in liver and skeletal muscle, and thus cause insulin resistance in these organs.

Finally, upregulation of RANTES and CCR5 in SAT of obese subjects with metabolic syndrome provides additional evidence for the association of inflammation with human metabolic syndrome. The higher levels of RANTES and CCR5 mRNA in VAT than SAT supports the link between visceral obesity and inflammation. The strong positive correlation of RANTES with CD3 and CD11b mRNA in human VAT strongly suggests the involvement of RANTES in T-cell as well as macrophage recruitment in human VAT. Because we could not get VAT from lean

controls, we were unable to compare mRNA levels of RANTES in VAT between obese and lean subjects. Systemic RANTES concentrations were reported to be higher in individuals with type 2 diabetes (who were also obese) or impaired glucose tolerance than in nondiabetic controls.²⁵

The diet we used to induce obesity also increases atherosclerosis, which is characterized by accumulation of lymphocytes and macrophages and increased expression of chemokines and chemokine receptors. The development of chronic inflammation in AT with diet-induced obesity may promote insulin resistance by causing preadipocyte/adipocyte malfunction and accelerate atherosclerosis by endocrine mechanisms, with increased secretion of cytokines and chemokines by AT and decreased secretion of adiponectin that leads to systemic activation of leukocytes and/or upregulation of endothelial cell adhesion molecules, which thus promotes atherogenesis. An alternative paracrine model has been suggested in which chemokines produced by perivascular AT that surrounds arteries could promote accumulation of T cells and macrophages at the interface of arterial adventitia and AT.²⁶ New therapies that target chemokines and chemokine receptors, such as inhibitors of CCR5 for HIV, which have also shown a benefit of reducing atherosclerosis in LDL receptor-deficient mice,²⁷ will provide opportunities to examine whether modulation of inflammation in AT is associated with metabolic or cardiovascular benefit.

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Disclosures

Dr Sweeney has served as an expert witness on malpractice cases for laparoscopic surgery. The other authors report no conflicts.

References

- Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX, Eckel RH. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss. *Circulation*. 2006;113:898-918.
- Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF- α function. *Nature*. 1997;389:610-614.
- Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. *Proc Natl Acad Sci U S A*. 2003;100:7265-7270.
- Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW Jr. CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest*. 2006;116:115-124.
- Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest*. 2003;112:1821-1830.
- Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest*. 2003;112:1796-1808.
- Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell*. 1998;2:275-281.
- Melian A, Geng YJ, Sukhova GK, Libby P, Porcelli SA. CD1 expression in human atherosclerosis: a potential mechanism for T cell activation by foam cells. *Am J Pathol*. 1999;155:775-786.
- Appay V, Rowland-Jones SL. RANTES: a versatile and controversial chemokine. *Trends Immunol*. 2001;22:83-87.
- Schober A, Manka D, von Hundelshausen P, Huo Y, Hanrath P, Sarmbock JJ, Ley K, Weber C. Deposition of platelet RANTES triggering monocyte recruitment requires P-selectin and is involved in neointima formation after arterial injury. *Circulation*. 2002;106:1523-1529.
- Goldstein BJ, Scalia R. Adiponectin: a novel adipokine linking adipocytes and vascular function. *J Clin Endocrinol Metab*. 2004;89:2563-2568.
- Ma K, Cabrero A, Saha PK, Kojima H, Li L, Chang BH, Paul A, Chan L. Increased β -oxidation but no insulin resistance or glucose intolerance in mice lacking adiponectin. *J Biol Chem*. 2002;277:34658-34661.
- Vasudevan AR, Wu H, Xydakis AM, Jones PH, Smith EO, Sweeney JF, Corry DB, Ballantyne CM. Eotaxin and obesity. *J Clin Endocrinol Metab*. 2006;91:256-261.
- Wu H, Rodgers JR, Perrard X-YD, Perrard JL, Prince JE, Abe Y, Davis BK, Dietsch G, Smith CW, Ballantyne CM. Deficiency of CD11b or CD11d results in reduced staphylococcal enterotoxin-induced T cell response and T cell phenotypic changes. *J Immunol*. 2004;173:297-306.
- Wu H, Prince JE, Brayton CF, Shah C, Zeve D, Gregory SH, Smith CW, Ballantyne CM. Host resistance of CD18 knockout mice against systemic infection with *Listeria monocytogenes*. *Infect Immun*. 2003;71:5986-5993.
- Garcia GE, Xia Y, Harrison J, Wilson CB, Johnson RJ, Bacon KB, Feng L. Mononuclear cell-infiltrate inhibition by blocking macrophage-derived chemokine results in attenuation of developing crescentic glomerulonephritis. *Am J Pathol*. 2003;162:1061-1073.
- Thakker GD, Frangogiannis NG, Bujak M, Zymek P, Gaubatz JW, Reddy AK, Taffett G, Michael LH, Entman ML, Ballantyne CM. Effects of diet-induced obesity on inflammation and remodeling after myocardial infarction. *Am J Physiol Heart Circ Physiol*. 2006;291:H2504-H2514.
- Ruan H, Zarnowski MJ, Cushman SW, Lodish HF. Standard isolation of primary adipose cells from mouse epididymal fat pads induces inflammatory mediators and down-regulates adipocyte genes. *J Biol Chem*. 2003;278:47585-47593.
- Xu H, Sethi JK, Hotamisligil GS. Transmembrane tumor necrosis factor (TNF)- α inhibits adipocyte differentiation by selectively activating TNF receptor 1. *J Biol Chem*. 1999;274:26287-26295.
- Otero M, Lago R, Lago F, Casanueva FF, Dieguez C, Gomez-Reino JJ, Gualillo O. Leptin, from fat to inflammation: old questions and new insights. *FEBS Lett*. 2005;579:295-301.
- Fantuzzi G, Faggioni R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. *J Leukoc Biol*. 2000;68:437-446.
- Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, Egashira K, Kasuga M. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J Clin Invest*. 2006;116:1494-1505.
- Chen A, Mumick S, Zhang C, Lamb J, Dai H, Weingarth D, Mudgett J, Chen H, MacNeil DJ, Reitman ML, Qian S. Diet induction of monocyte chemoattractant protein-1 and its impact on obesity. *Obes Res*. 2005;13:1311-1320.
- Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB,

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- Kadowaki T. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med.* 2002; 8:1288–1295.
25. Herder C, Haastert B, Muller-Scholze S, Koenig W, Thorand B, Holle R, Wichmann HE, Scherbaum WA, Martin S, Kolb H. Association of systemic chemokine concentrations with impaired glucose tolerance and type 2 diabetes: results from the Cooperative Health Research in the Region of Augsburg Survey S4 (KORA S4). *Diabetes.* 2005;54:S11–S17.
26. Henrichot E, Juge-Aubry CE, Pernin A, Pache JC, Velebit V, Dayer JM, Meda P, Chizzolini C, Meier CA. Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? *Arterioscler Thromb Vasc Biol.* 2005;25:2594–2599.
27. van Wanrooij EJ, Happe H, Hauer AD, de Vos P, Imanishi T, Fujiwara H, van Berkel TJ, Kuiper J. HIV entry inhibitor TAK-779 attenuates atherogenesis in low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol.* 2005;25:2642–2647.

CLINICAL PERSPECTIVE

Obesity is associated with chronic inflammation, which includes macrophage accumulation in adipose tissue (AT) and upregulation of chemokines and cytokines. T cells also play important roles in chronic inflammatory diseases such as atherosclerosis but are not well studied in obesity. With a mouse model of obesity induced by a high-fat “Western” diet, we found higher numbers of T cells and macrophages in AT of obese insulin-resistant males than in obese females and lean counterparts. Gender-dependent upregulation of mRNA and protein levels of the chemokine regulated on activation, normal T cell expressed and secreted (RANTES) and its receptor CCR5 was observed in AT of obese mice. Importantly, both human and murine adipocytes expressed RANTES. Adiponectin-deficient mice fed a high-fat diet showed higher RANTES mRNA levels in AT than wild-type mice. Activated T cells cocultured with preadipocytes in vitro suppressed differentiation of preadipocytes to adipocytes. Obese humans with metabolic syndrome had higher mRNA levels of RANTES and CCR5 in subcutaneous AT than lean counterparts. Furthermore, RANTES and CCR5 mRNA levels were higher in visceral than subcutaneous AT from morbidly obese patients. RANTES mRNA levels were highly correlated with the T-cell marker CD3 in human visceral AT. Obesity was associated with increased accumulation of both T cells and macrophages in AT, which may play important roles in obesity-related disease through the influence of preadipocyte/adipocyte functions. RANTES is an adipokine that is upregulated in AT by obesity in both mice and humans. Ongoing development of therapies that target chemokines and chemokine receptors will provide new opportunities to examine whether modulation of inflammation in AT has metabolic or cardiovascular benefit.