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Aging Is Associated with an Increase in T Cells and Inflammatory Macrophages in Visceral Adipose Tissue

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Age-related adiposity has been linked to chronic inflammatory diseases in late life. To date, the studies on adipose tissue leukocytes and aging have not taken into account the heterogeneity of adipose tissue macrophages (ATMs), nor have they examined how age impacts other leukocytes such as T cells in fat. Therefore, we have performed a detailed examination of ATM subtypes in young and old mice using state of the art techniques. Our results demonstrate qualitative changes in ATMs with aging that generate a decrease in resident type 2 (M2) ATMs. The profile of ATMs in old fat shifts toward a proinflammatory environment with increased numbers of $CD206^{-}CD11c^{-}$ (double-negative) ATMs. The mechanism of this aging-induced shift in the phenotypic profile of ATMs was found to be related to a decrease in peroxisome proliferator-activated receptor-γ expression in ATMs and alterations in chemokine/chemokine receptor expression profiles. Furthermore, we have revealed a profound and unexpected expansion of adipose tissue T cells in visceral fat with aging that includes a significant induction of regulatory T cells in fat. Our findings demonstrate a unique inflammatory cell signature in the physiologic context of aging adipose tissue that differs from those induced in setting of diet-induced obesity. *The Journal of Immunology*, **2011, 187: 6208–6216.**

Aging is increasingly recognized as being associated with a proinflammatory state that plays an important role in the development of chronic diseases (1). Although a healthy acute inflammation response is a crucial part of the body's defense against foreign pathogens, chronic low-grade inflammation may be detrimental to health. To date, published works have largely focused on correlating serum level of proinflammatory cytokines with aging or aging syndromes in cross-sectional analyses. For example, significant positive correlation between human frailty and serum IL-6, C-reactive protein, hemoglobin, T cell CCR5 expression, peripheral blood monocyte number, and total WBC count have all been reported (2–4). However, the source of the proinflammatory cytokines in aging has not been definitively determined.

Advancing age is also accompanied by general obesity (increased total body fat percentage) and in particular central obesity that contributes to a number of important health problems, such as insulin resistance, cardiovascular disease, sarcopenia, and disability (5–8). White adipose tissue has been proposed to be a key regulator of life span. In several model organisms, genetic manipulations that modify fat mass also impact on life expectancy, in part through sirtuin 1 and suppression of the nuclear receptor protein peroxisome proliferator-activated receptors (PPAR)-γ (9–11).

Whereas the underlying mechanism for the association between age-related obesity and disease is incompletely understood, adipose tissue inflammation has been shown to be a critical regulator of the systemic inflammatory phenotype in diet-induced and genetic obesity models (12, 13). In this context there is a renewed appreciation that adipose tissue is composed of diverse cell populations besides adipocytes that contribute to metabolism and adipocyte function. The nonadipocyte cells can be purified from fat after collagenase digestion and are known as the stromal vascular fraction (SVF). The dominant cells in the SVF are leukocytes (e.g., macrophages and lymphocytes) and adipose tissue stromal cells (ATSCs) that include preadipocytes and fibroblasts. The coordinated inflammatory response to obesity is complex and involves numerous leukocytes such as T cells, B cells, mast cells, and NKT cells in communication with adipocytes and stromal cells (14–17). Of these adipose tissue leukocytes, adipose tissue macrophages (ATMs) are the most abundant and have been shown to be a critical link between obesity and metabolic dysfunction and disease (18). Based on this role, ATMs have been proposed to play a potential pathogenic role in "inflamm-aging" (19, 20).

ATMs are present in multiple fat depots in lean states, and obesity induces both quantitative and qualitative changes in ATMs that impact the inflammatory state in fat (21–23). A resident population of ATMs (type 2) expresses multiple markers of an alternative activation state (M2) with anti-inflammatory properties that include CD206, CD301, and increased production of IL-10

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Abbreviations used in this article: ATM, adipose tissue macrophage; ATSC, adipose tissue stromal cell; ATT, adipose tissue T; CBA, Cytometric BeadArray; CM, conditioned medium; DN, double-negative; FALC, fat-associated lymphoid cluster; PM, peritoneal macrophage; PPAR, peroxisome proliferator-activated receptor; SVF, stromal vascular fraction; Tconv, conventional T cells; Treg, regulatory T cell.

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(21, 23, 24). Maintenance of type 2 ATMs is dependent on local IL-4 production in adipose tissue in lean animals (25) and the activity of lipid nuclear receptors such as PPAR-γ and PPAR-δ (26–28). Obesity is associated with the accumulation of a distinct ATM subtype (type 1) that expresses CD11c and is associated with dead and dying adipocytes (29, 30). These ATMs have a gene expression profile that resembles a classical or M1 activation state induced by LPS and IFN-γ stimulation that may relate to the ability of free fatty acids to activate TLR4 (31, 32). At least two other ATM subtypes have been described with a mix of M1 and M2 gene expression profiles (21) and include $CD206^{-}CD11c^{-}$ double-negative (DN) ATMs (type 4), which are induced with obesity (33).

Overall, current data suggest that the balance between ATM subtypes regulates the global inflammatory environment in fat. Obesity induces the activation and recruitment of proinflammatory ATMs (21, 34) that overwhelm the homeostatic function of the resident type 2 ATMs. Impairment of M2 activation tilts the balance toward a proinflammatory environment that can directly negatively influence nutrient metabolism (25, 35). Such heterogeneity in ATMs has been observed in human obesity as well (22), and the balance of ATMs is shifted away from inflammatory ATMs by weight loss (36).

Our understanding of how aging-induced obesity influences adipose tissue leukocytes is incomplete. Macrophage content in s.c. fat increases with age in humans (37), but there are limited data on the inflammatory events in visceral adipose tissue ATMs that may drive much of the systemic changes in inflammatory mediators with age (38). In rat models, there is evidence of depot-specific changes in ATMs (39) and an increase in ATM content with age (40). In mice, Wu et al. (41) showed that total ATM content was not altered with age, although inflammatory cytokine expression was different between ATMs from young and old mice. Despite this, they argued that adipocytes, and not ATSCs, had the most profound alterations in inflammatory cytokine expression with age.

Given the interest in the intersection among aging, inflammation, and adipose tissue, we investigated the hypothesis that aging would lead to qualitative changes in ATMs that would resemble those seen in obesity models. Using mouse models of aging, we performed a comprehensive evaluation of ATM subsets, distribution, and inflammatory function in visceral adipose tissue. These studies demonstrate that whereas there are few quantitative changes in ATMs in visceral fat with age, there are significant qualitative changes in the ratio of ATM subtypes, chemokine expression, and chemokine receptor expression consistent with a proinflammatory environment in old fat. The mechanisms behind these changes are related to an age-dependent downregulation of PPAR-γ expression in ATMs that bias the system away from an M2 profile. Furthermore, we observed that adipose tissue T (ATT) cells are significantly and specifically induced in visceral fat with aging, which may influence ATM phenotypes. Overall, our studies suggest a complex regulation of adipose tissue leukocytes with age that has unique features compared with the events that occur with obesity.

Materials and Methods

Mice

Young (3–4 mo) and old (18–22 mo) C57BL/6 mice were obtained from the National Institute on Aging aged rodent colonies through Harlan Sprague Dawley (Indianapolis, IN). All mice were maintained in a pathogen-free environment provided by the Unit for Laboratory Animal Medicine at the University of Michigan (Ann Arbor, MI) until they were used. All the experimental research in the current study has been approved by the University of Michigan University Committee on Use and Care of Animals.

Isolation of adipose tissue cell fractions

Careful inspection was done to exclude aged animals with cancer or lymphoma. Mice were euthanized by CO_2 asphyxiation or cervical dislocation. Visceral fat from mice was excised under sterile conditions, and gonadal/epididymal, mesenteric, and renal fat depots weighed as a group to determine visceral fat weight. Epididymal adipose tissue was then fractionated into adipocyte and SVF, as described (24). For chemokine studies, epididymal adipose tissue from several mice was pooled together (7–10 mice for young, and 3–5 for old), weighed, and minced into small pieces. For flow cytometry analysis, individual mice were assessed ($n = 5$) as independent data points. Three adipose tissue cell populations, adipocytes, ATM, and $CD11b^{-}$ ATSCs were purified. $CD11b^{+}$ ATMs were positively selected from the total SVF using the MACS Microbeads technology (Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the manufacturer's instructions. Briefly, the macrophages were magnetically labeled with the CD11b Microbeads (10 μl/10^7 total cells) and passed through the MS^{+} separation column while placed in the magnetic field of a MidiMACS separator (Miltenyi Biotec). The columns were then washed three times with 1 ml MACS buffer, and the $CD11b^{-}$ ATSCs were collected. The $CD11b^{+}$ cells were removed from the column by washing twice with 2 ml MACS buffer away from the magnetic field. Purity of the isolated cells was determined by staining with the FITC-conjugated anti-CD11b and PE-conjugated anti-F4/80 (eBioscience, San Diego, CA) and was consistently between 94 and 99%.

Isolation of splenic monocytes

Spleens were removed from young and old mice. A single-cell suspension was prepared by passing the spleens through a 40-μm cell strainer filter (BD Falcon). $CD11b^{+}$ monocytes were then isolated by the MACS Microbeads technology (Miltenyi Biotec), according to the manufacturer's instructions. $CD11b^{+}$ cells were positively selected using $CD11b^{+}$ Microbeads. Purity of the isolated cells was determined by staining with the FITC-conjugated anti-CD11b and PE-conjugated anti-CD14, and the cells were confirmed to be >90% CD11b and CD14 double positive.

RNA extraction and real-time RT-PCR

ATMs, the ATSCs ($CD11b^{-}$), and splenic monocytes were placed directly in RNA lysate buffer, and RNA was extracted using the RNeasy kit (Qiagen). The adipocytes were added to the DIAzol lysis reagent (Qiagen), and RNA was extracted using RNeasy Lipid Tissue Midi Kit (Qiagen). The following PCR primers were designed using the online software Primer3 (v. 0.4.0) (42): CCR1, 5′-AGG GCC CGA ACT GTT ACT TT-3′ (forward) and 5′-TAT AAG CCA GGC ATG GAA GC-3′ (reverse); CCR2, 5′-AGG CTC ATC TTT GCC ATC AT-3′ (forward) and 5′-AAG GAT TCC TGG AAG GTG GT-3′ (reverse); CCR3, 5′-GGA ACA CAC TCT CCT CAT AAT GC-3′ (forward) and 5′-CAC CTG GAC TTC TCA ATA CAG ATG-3′ (reverse); CCR4, 5′-GCT GCC CAC ATC CAC TTA TT-3′ (forward) and 5′-CAT TAA CTT GGG GCA GGA AA-3′ (reverse); CCR5, 5′-TAG ATG AGG GCT GTT TCC ATA G-3′ (forward) and 5′-CTT CCA GAG ATG ATG ACT GCT AAG-3′ (reverse); CCR7, 5′-GAC GGA TAC CTA CCT GCT CAA C-3′ (forward) and 5′-TGC CAA AGA TGC CCT TAC AC-3′ (reverse); CCR8, 5′-TTC CTG CCT CGA TGG ATT AC-3′ (forward) and 5′-GAG GAG GAA CTC TGC GTC AC-3′ (reverse); CCR9, 5′-TGG TCA ATG GAT GTT CCA GA-3′ (forward) and 5′-TGC ACA TGA TGA GAA GCA CA-3′ (reverse); CXCR2, 5′-AGC AGA GGA TGG CCT AGT CA-3′ (forward) and 5′-TCC ACC TAC TCC CAT TCC TG-3′ (reverse); CXCR3, 5′-CCA TGC CCT ATC TTG CTG TT-3′ (forward) and 5′-ACA CAG GGA TGG CTG AGT TC-3′ (reverse); CXCR4, 5′-GAA ACT GCT GGC TGA AAA GG-3′ (forward) and 5′-CTG TCA TCC CCC TGA CTG AT-3′ (reverse); CXCR5, 5′-CCA AGC AGA AAG CTG AAA CC-3′ (forward) and 5′-CTT CTG GAA CTT GCC CTC AG-3′ (reverse); CX3CR1, 5′-GTG GTG CCT TCA TCC ATT CT-3′ (forward) and 5′-CCA GCT CCA TTT CTC AGA GG-3′ (reverse); MIP-1α, 5′-GTG TAG AGC AGG GGC TTG AG-3′ (forward) and 5′-AGA GTC CCT CGA TGT GGC TA-3′ (reverse); MIP-1β, 5′-GAG CCC TGG GTC ACT GAG TA-3′ (forward) and 5′-GAG GAG GCC TCT CCT GAA GT-3′ (reverse); PPAR-γ, 5′-GAT GGA AGA CCA CTC GCA TT-3′ (forward) and 5′-AAC CAT TGG GTC AGC TCT TG-3′ (reverse); guanine nucleotide-binding protein (G protein), β polypeptide 2 like 1 (GNB2L1), 5′-GGACAAGC-TGGTCAAGGTGT-3′ (forward) and 5′-GGGATCCATCTGGAGAGA-CA-3′ (reverse). Real-time PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen), according to the manufacturer's protocol. Relative expression of inflammatory cytokine/chmokine and PPAR-γ was assessed and normalized with mouse housekeeping gene GNB2L1.

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FACS staining and flow cytometry

Before Ab staining, all the cells were incubated with Mouse BD Fc Block (BD Pharmingen, San Diego, CA). To detect macrophage infiltration in adipose tissue, total SVF cells were double stained with macrophage-specific PE-conjugated anti-mouse F4/80 (eBioscience) and FITC-conjugated anti-mouse CD11b (eBioscience) or the appropriate isotype controls. To detect CCR5 chemokine receptor cell surface expression, total SVF cells were double stained with PE-conjugated anti-mouse CCR5 (eBioscience) and FITC-conjugated anti-mouse F4/80 (eBioscience). Labeled cells were then washed twice with FACS buffer (1% BSA and 0.1% sodium azide in 1× PBS), fixed in 1% paraformaldehyde in PBS, and analyzed for fluorescence on a FACSCalibur Canto II (BD Biosciences, San Jose, CA). Intracellular staining was performed after fixation and permeabilization (BD Biosciences). Data analyses were done based on examination of 30,000 cells/sample and performed using FCS express (De Novo Software) or Weasel (Walter and Eliza Hall Institute of Medical Research). The following Abs were used for flow cytometry for ATM subset and ATT cell analysis: anti-CD3 allophycocyanin-Alexa780, anti-CD4 PE-Cy7, anti-CD8 FITC, anti-Foxp3 allophycocyanin, anti-CD11c PE-Cy7, anti-CD11b allophycocyanin-Alexa780, anti-F4/80 PE, anti-7/4 PE, anti-CD115 allophycocyanin, anti-Ly6c PerCP5.5, and anti-CD206 Alexa647 (all Abs were from eBioscience, except anti-CD206 from Abd Serotec).

Adipose tissue cells and cytokine production

Adipose tissue from young and old mice was fractioned into adipocytes, ATSCs ($CD11b^-$), and ATMs ($CD11b^+$), as stated above. ATMs and ATSCs ($CD11b^-$) were plated into 24-well plates at the density of 1.0×10^6 cells/well and incubated at 37°C, 5% CO_2 for 24 h. Equal volume of young or old adipocytes (200 μl) was cultured in 1 ml DMEM plus 10% FBS in 24-well plates separately for 24 h. For ATMs, the supernatants were directly collected after 24 h; for SVF, the cell suspensions were collected after 24 h and centrifuged at 360 × *g* at 4°C for 5 min to pellet the floating cells, and the supernatants were collected. For adipocytes, the culture media below the floating cells were collected. All the supernatants were stored at −80°C until use. The supernatants were simultaneously assayed for IL-6, IL-10, IL12-p70, MCP-1, TNF-α, and IFN-γ cytokines using the mouse inflammatory Cytometric BeadArray (CBA) kit (BD Biosciences), following the manufacturer's instructions. This assay kit provides a mixture of six microbeads with distinct fluorescence intensity that are precoated with capturing Abs specific for the inflammatory proteins. When the beads were incubated with the corresponding PE-conjugated detection Abs and the test sample, sandwich complexes were formed. The fluorescence produced by the beads was measured using a FACSCalibur flow cytometer.

Incubation of peritoneal macrophages with conditioned medium

To obtain peritoneal macrophages, young and old mice were euthanized by CO_2 asphyxiation, and peritoneal cells were immediately collected by injecting and washing the peritoneal cavity with 2 × 5 ml ice-cold PBS. The peritoneal cells were spun down, washed twice with DMEM containing 10% FBS, and resuspended in DMEM with 10% FBS. The cells were then seeded on 96-well plates at a density of 0.3×10^6 cells/well, and incubated for 2 h at 37°C, 5% CO_2. Floating cells were removed by gentle washing with DMEM plus 10% FBS; the adherent cells are peritoneal macrophages. ATM-conditioned medium (CM) was made by culturing 1×10^6 freshly isolated young and old ATMs for 24 h at 37°C, 5% CO_2, and the supernatants were obtained as ATM-CM. ATSC-CM was made by culturing 1×10^6 freshly isolated young and old ATSCs for 24 h at 37°C, 5% CO_2, respectively, and the supernatants were obtained as ATSC-CM. The CM were stored at −80°C until use. ATM-CM and ATSC-CM from young or old mice were then added to the young or old peritoneal macrophages and incubated for additional 24 h. The culture media were used for analysis of IL-6, TNF-α, MCP-1, IL-12, and IL-10 by mouse inflammatory CBA kit.

Treatment of old mice with PPAR-γ–specific agonist rosiglitazone

Old mice were injected i.p. with 2 mg/kg/day rosiglitazone (potassium salt; Cayman Chemical) or vehicle (sterile ddH_2O) for 2 wk, and ATMs were harvested from the mice. RNAs were extracted from the isolated ATMs, and real-time PCR was performed to compare cellular markers between rosiglitazone- and vehicle-treated groups.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed, as described (30). Abs included anti-MGL1 and anti-CD4 with species-specific secondary Abs (Jackson ImmunoResearch Laboratories). Alexa568-conjugated Isolectin (Molecular Probes) was used to highlight blood vessels. Fat-associated lymphoid cluster (FALC) and adipocyte sizing was performed using ImageJ software.

Statistical analysis

Results are expressed as means ± SEM. Statistical analyses were performed using Student *t* test, and a *p* value <0.05 was considered to be statistically significant.

Results

The nonadipocyte microenvironment contributes to the age-related adipose tissue inflammation

In obesity, the majority of proinflammatory mediators are produced by the SVF, but it is unclear whether this is also true in aging. To examine this directly, the mouse inflammatory CBA assay was used to quantify inflammatory cytokines produced by ATMs ($CD11b^+$), nonmacrophage ATSCs ($CD11b^-$), and adipocytes (Fig. 1). Comparing the different adipose tissue cell types, ATMs were found to be the dominant source for TNF-α and the source for ~10% of IL-6 production (Fig. 1*A*), whereas ATSCs are the primary source for IL-6 (representing 90% of the cytokine production) (Fig. 1*B*). With age, there was a significant increase in TNF-α, IL-6, and MCP-1 production in ATMs as well as a significant increase in IL-6 production in ATSCs. In contrast, we did not find any significant changes in the cytokine/chemokine production by adipocytes from young and old mice (Fig. 1*C*). These results indicate that, similar to that seen in diet-induced obesity,

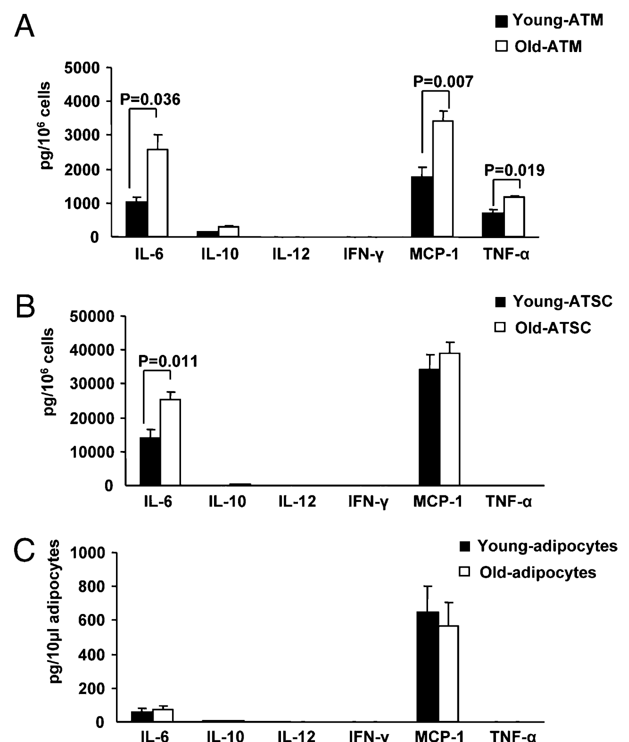

FIGURE 1. Cytokine production by adipose tissue cells. SVFs and adipocytes from young and old mice were cultured for 24 h. The CM was collected to measure cytokine release using the mouse inflammatory CBA (BD Biosciences). Cytokines release from total ATMs ($CD11b^+$) (*A*), ATSCs ($CD11b^-$) (*B*), and adipocytes (*C*). The results represent the mean ± SEM; *n* = 4–6 experiments, representing a total of 15 old and 40 young mice.

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nonfat cells, including ATMs and ATSCs, but not adipocytes, are the major sources of proinflammatory mediators that promote age-related adipose tissue inflammation. In addition, it suggests that age shifts the inflammatory cytokine production toward a proinflammatory M1 profile with enhanced TNF-α and IL-6 production. IL-12 and IFN-γ are both part of the commercial CBA kit. Our results show that murine visceral ATMs express very low levels of IL-12 and IFN-γ, and there is no age difference detected.

Total ATM content is unchanged in old mice

To better assess the macrophage-derived changes in adipose tissue with age, we isolated ATMs from the epididymal fat tissue from young and old mice and found that, consistent with other reports, aging is associated with increased total visceral fat content (gonadal, mesenteric, and renal depots) and body weight (Table I). Total number of SVF cells per gonadal fat pad was increased in old mice and persisted even when normalized for fat pad weight (Fig. 2*A*, 2*B*). CD11b$^+$F4/80$^+$ ATMs were quantified by flow cytometry and were found to represent a smaller proportion of the SVF in old mice when expressed as a percentage of total cells (Fig. 2*C*). However, when normalized to fat mass, total ATM content (cells/g) did not change with age in contrast to what is observed with high-fat diet and genetic obesity models (23). These results were supported by immunofluorescence microscopy, which did not demonstrate any significant qualitative differences in the distribution or density of ATMs in fat comparing young and old mice (Fig. 2*D*).

Aging alters the balance of ATM subsets toward a proinflammatory (M1) macrophage profile

Because ATMs exist in many distinct subtypes with differential surface marker expression, we next proceeded to examine the possibility that the proportion of ATM subtypes may change with age independent of differences in total ATM content. We were able to delineate three main subsets of CD11b$^+$F4/80$^+$ ATMs in epididymal fat pads from young and old male mice that parallel prior studies: resident type 2 ATMs (CD206$^+$CD11c$^-$), inflammatory type 1 ATMs (CD206$^-$CD11c$^+$), and DN ATMs (CD206$^-$CD11c$^-$). When the proportion of these ATM subsets was enumerated, there was a significant decrease in the percentage of type 2 ATMs with age, no change in type 1 ATMs, and a trend toward an increase in DN ATMs with age (Fig. 3*A*, 3*B*). The appearance of DN ATMs was substantial in some old mice and was surprising, as these ATMs are typically induced with obesity and express proinflammatory genes such as *Ccr2*, *Ccr5*, and *Il1b* (33).

With age, the ratio of proinflammatory (type 1 and DN ATMs) to resident type 2 (M2) ATMs was significantly increased, demonstrating age-induced qualitative changes in ATMs that are similar to the proportional changes in ATM subtypes seen in diet-induced obesity (Fig. 3*C*). These data supported the cytokine data (Fig. 1) and suggest that aging generates a shift in the properties of ATMs toward an M1 profile due to the accumulation of DN ATMs and a loss of resident type 2 (M2-polarized) ATMs. This altered ratio occurred in the absence of any significant changes in the distribution or quantity of crownlike structures (Fig. 2*D*) that are hallmarks of high-fat diet-induced obesity.

Table I. Old mice have greater amount of visceral fat

	Fat (g)/Mice	Fat (g)/Body Weight (g)
Young	0.27 ± 0.02	1.06 ± 0.11
Old	0.66 ± 0.06*	2.16 ± 0.21[a]

[a] $p < 0.001$ versus young mice; the results represent the mean ± SEM; $n = 5$ experiments.

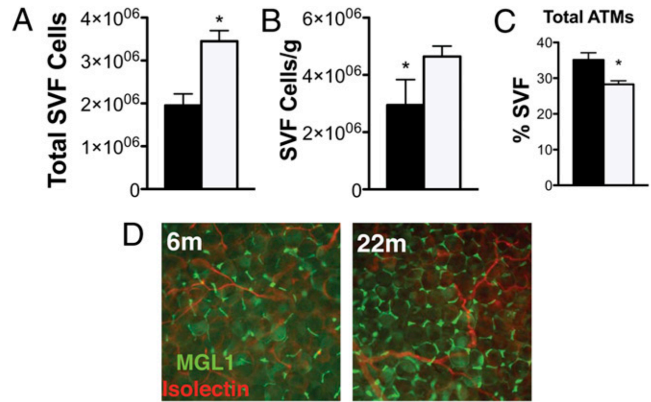

FIGURE 2. Analysis of ATM content in old and young mice. *A*, Quantitation of total SVF cells isolated from epididymal fat pads from 6-mo-old (■) and 22-mo-old (□) C57 mice. *B*, SVF cells normalized to fat pad mass. *C*, ATMs (F4/80$^+$CD11b$^+$) were quantified by flow cytometry. *D*, Immunofluorescence localization of resident type 2 ATMs (MGL1$^+$; green) in young and old mice. Vasculature labeled with isolectin (red). Original magnification ×200. The results represent the mean ± SEM; n = 3–5 experiments, representing a total of 12 old and 35 young mice. *$p < 0.05$.

Paracrine signals from old ATMs and ATSCs are sufficient to induce the M1 macrophage polarization

Because ATMs and ATSCs from old animals have significantly increased production of M1-promoting cytokines, we next determined whether these soluble factors are capable of altering mac-

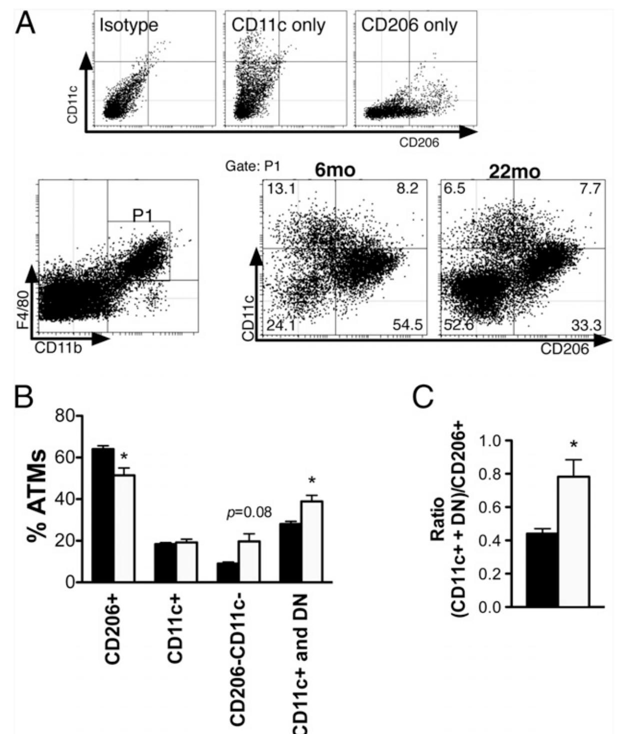

FIGURE 3. Alterations in ATM subtypes with age. *A*, Flow cytometry delineation of ATM subtypes. Definition of control gates is shown (*upper panels*). After gating for ATMs (F4/80$^+$CD11b$^+$; P1), subtypes were differentiated by CD11c and CD206 staining in young and old mice (representative plots shown in *lower panels*). *B*, ATM subtypes quantified as a percentage of the total ATM population in 6-mo-old (■) and 22-mo-old (□) mice. *C*, Ratio of CD11c$^+$ and DN ATMs relative to resident CD206$^+$ ATMs. *$p < 0.05$.

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rophage phenotypes via paracrine action. To test this, we incubated peritoneal macrophages (PM) from young and old mice with CM from ATMs and ATSCs and found that ATM-CM from old animals induced significantly greater CCL2 (MCP-1) production in PM compared with ATM-CM from young mice (Fig. 4*A*). Additionally, ATSC-CM induced higher IL-6 and TNF-α production in PM (Fig. 4*B*), indicating that paracrine signals derived from both ATMs and ATSCs are capable of influencing the polarization state of ATMs.

Increase in selected chemokine and chemokine receptor gene expression in ATMs

Multiple studies have identified a critical role of the CCR2/CCL2 chemokine axis in the recruitment of inflammatory ATMs to fat in obesity (43, 44). The data above suggest that CCR2-dependent chemokine pathways may also be involved the unique pattern of ATM recruitment observed with age. Consistent with this, ATMs from aged animals expressed twice the level of CCR2 (Fig. 5*A*), a finding consistent with the increase in DN ATMs, which have high CCR2 expression. In contrast, the expression of the fractalkine receptor CX3CR1 in ATMs was decreased, which differs from data demonstrating an induction of CX3CR1 in adipose tissue with obesity (45). ATMs from old mice also express greater amount of CCR5, CXCR3, and CXCR5, but lower amount of CCR7 (Fig. 5*B*, 5*C*). In contrast, young and old splenic monocytes had similar CCR2 and CX3CR1 expression (Supplemental Fig. 2), suggesting that these changes are specific to the adipose tissue compartment.

In diet-induced obesity, the increase in CCR2 expression in fat is related to an increase in the quantity and the trafficking of inflammatory $CCR2^+Ly6c^{high}$ monocytes from the circulation (43, 46). Therefore, we hypothesized that the age-induced imbalance between ATM subsets may relate to alterations in the circulating pools of $Ly6c^{high}$ and $Ly6c^{low}$ monocytes. $CD115^+$ monocytes were profiled in the bone marrow and blood of young and old male mice by flow cytometry (Supplemental Fig. 1). There was no change in total $CD115^+$ monocytes with age. In addition, no significant alterations in the ratio or quantity of $Ly6c^{high}$ and $Ly6c^{low}$ monocytes were observed within the $CD115^+$ monocyte pool.

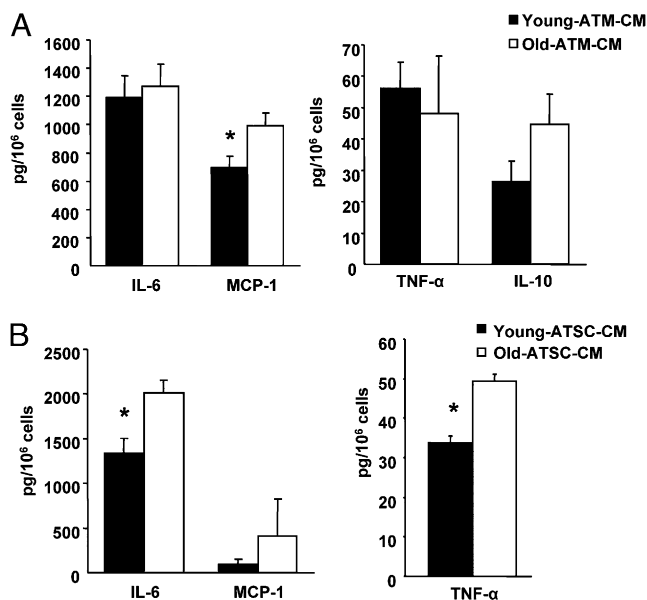

FIGURE 4. Paracrine signals from ATMs and ATSCs. CM from young and old ATMs (*A*) and ATSCs ($CD11b^-$) (*B*) was incubated with peritoneal macrophages for 24 h, and the cytokines released were measured by CBA. The results represent the mean ± SEM; $n = 4$ experiments, representing a total of 20 old and 28 young mice. $*p < 0.05$.

PPAR-γ controls alternative activation of adipose tissue macrophage in aging

The maintenance of an alternative M2 activation state of type 2 ATMs is linked to the activity of nuclear receptors important in lipid metabolism that include PPAR-γ and PPAR-δ (26, 47). Decreased PPAR-γ expression has been reported in the aging kidney (48), brain (49), and spleen (50). Because aging led to a suppression of type 2 ($CD206^+$) ATMs, we examined whether the observed changes in ATMs with old age are associated with altered PPAR-γ expression. PPAR-γ expression was significantly decreased in old ATMs (total) compared with those from younger animals (Fig. 6*A*) consistent with the decrease in type 2 ATMs ($CD206^+$). Treatment with the PPAR-γ agonist rosiglitazone has been shown to promote type 2 ATMs in diet-induced obesity in mice (51), but the drug's effect in aging ATMs is unknown. Old mice were treated for 14 d with rosiglitazone and ATMs isolated for gene expression analysis. As expected, rosiglitazone increased PPAR-γ expression in ATMs from old mice (Fig. 6*B*). Expression of M1 cytokines *Tnfa* and *Il6* was decreased, whereas *Il10* expression was increased in isolated ATMs (Fig. 6*C*). These effects were observed to be independent of any changes in body weight or total ATM content with rosiglitazone treatment.

Aging is associated with a significant induction of ATT cells in visceral fat depots

Current models of adipose tissue inflammation suggest that alterations in ATMs are coordinated with alterations in other adipose tissue leukocytes such as T cells. To date, no studies have examined the effect of old age on ATT cells. We hypothesized that old age may modify T cell content in fat and performed an analysis of ATT cells by flow cytometry. Total lymphocytes ($CD3^+$) in visceral (epididymal) fat were increased ~2-fold in old mice even when normalized for fat weight (Fig. 7*A*). This finding was unique to adipose tissue, as $CD3^+$ cells were unchanged in the spleen with age (Fig. 7*D*).

Within the $CD3^+$ ATT cell population, $CD4^+$, $CD8^+$, and DN ($CD4^-CD8^-$) $CD3^+$ cells were quantified (Fig. 7*B*). No change in DN cells was observed; however, there was a significant increase in the percentage of both $CD4^+$ (4-fold) and $CD8^+$ (7-fold) cells in adipose tissue in old mice. Further analysis of the $CD4^+$ T cell population indicated that adipose tissue regulatory T cells (Tregs; $CD3^+CD4^+Foxp3^+$) were increased 11-fold, whereas conventional T cells (Tconv; $CD3^+CD4^+Foxp3^-$) were increased 2-fold with age (Fig. 7*C*). This increase in $CD4^+$ and $CD8^+$ T cells was specific to visceral fat, as there were no significant changes in $CD4^+$ and $CD8^+$ cells in the spleen or blood with old age (Fig. 7*D*).

The location of T cells in adipose tissue in nonobese states is unclear. Recently, FALCs have been identified as adipose tissue regions that are enriched for lymphocytes in multiple fat depots (52). To examine whether the increase in ATT cell was related to alterations in the architecture of FALCs, we examined milky spots in omental fat pads identified by $CD4^+$ T cell content. FALCs were found in young and old mice in similar density in the omental fat pads (Fig. 8*A*, 8*B*). However, many of the FALCs in old mice were significantly larger than those seen in young mice. Sizing of the FALCs confirmed a shift in the distribution of FALC size in omental fat toward larger $CD4^+$ FALCs with old age (Fig. 8*C*, 8*D*). This indicates that the expansion of $CD4^+$ T cells in adipose tissue with age relates to an increase in the size of FALCs.

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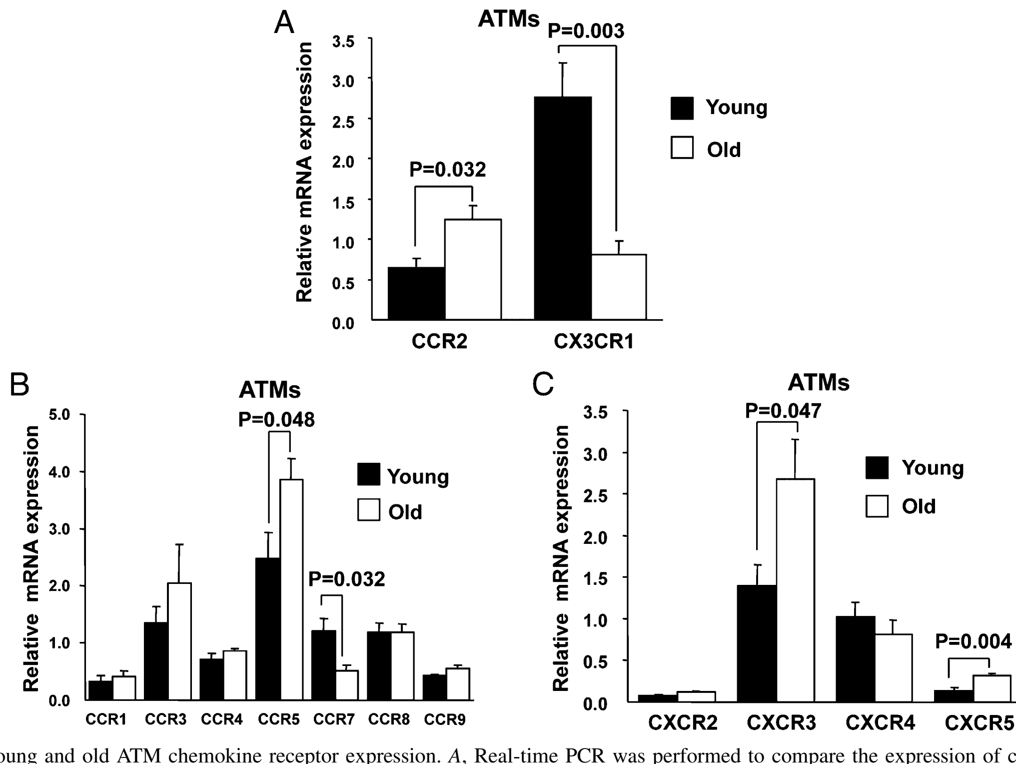

FIGURE 5. Young and old ATM chemokine receptor expression. *A*, Real-time PCR was performed to compare the expression of chemokine receptor CCR2 and CX3CR1 expression in young and old ATMs. Other CCR (*B*) and CXCR (*C*) chemokine receptors were also examined. The results represent the mean ± SEM; *n* = 4–5 experiments, representing a total of 25 old and 40 young mice.

Discussion

Our data demonstrate that aging induces a unique profile of ATMs and ATT cells in visceral adipose tissue that differs significantly from what is observed with high-fat diet-induced and genetic models of obesity. The key observations in this study are as follows: 1) aging induces a decrease in the resident CD206$^+$ ATM population in visceral fat that leads to a decrease in the ratio between these cells and two inflammatory ATM subtypes (CD11c$^+$ and CD206$^-$ CD11c$^-$ DN); 2) nonmacrophage stromal cells from old mice are able to activate macrophages via paracrine action; 3) a decrease in PPAR-γ expression in ATMs is associated with the change in the balance between inflammatory and noninflammatory ATMs with age; and 4) aging is associated with a robust expansion of CD4$^+$ and CD8$^+$ T cells in adipose tissue that correlates with the enlargement of FALCs.

Compared with the report by Wu et al. (41), we saw similar decreases in ATMs as a percentage of the SVF with age without an overall change in ATM numbers per gram due to an increase in the total number of SVF cells with age. In agreement with this study, we found qualitative changes in the inflammatory profile of old ATMs that fall in line with our current knowledge about ATM subtypes derived from obesity models. Interestingly, we have found that aging decreases the proportion of resident CD206$^+$ ATMs. Because CD206 is a marker of alternatively activated M2 macrophages, our data that PPAR-γ is coordinately downregulated in ATMs with age are consistent with this observation. Current evidence suggests that defining ATMs along a simple M1/M2 dichotomy is imprecise (53), and our data support this, as a prominent DN (CD206$^-$CD11c$^-$) ATM population was identified as increased in old mice. DN ATMs have been shown to be in-

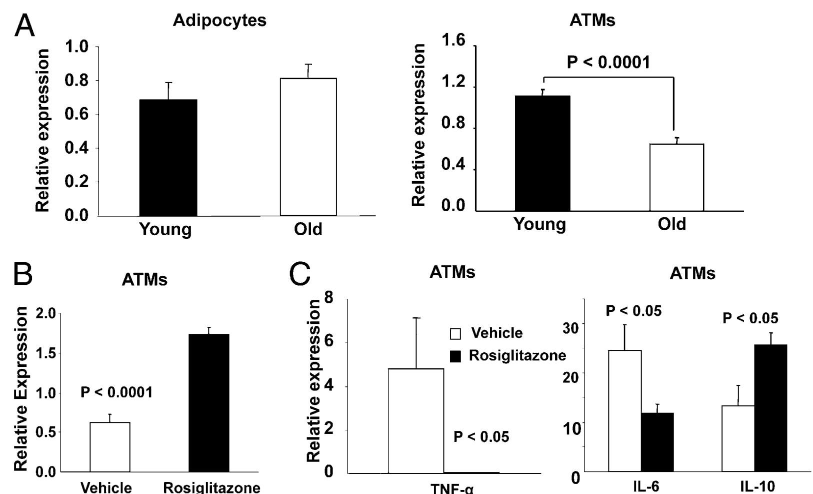

FIGURE 6. Effects of rosiglitazone on PPAR-γ gene expression by PCR. Real-time RT-PCR analysis of PPAR-γ expression, relative to the control gene guanine nucleotide-binding protein (G protein), β polypeptide 2 like 1 (GNB2L1). *A*, Decreased PPAR-γ expression is seen in old ATMs, but not adipocytes. *B*, Short-term (14-d) treatment of rosiglitazone increased PPAR-γ expression in old ATMs. *C*, Rosiglitazone treatment increased *Il10* and reduced *Tnfa* and *Il6* expression in old ATMs. The results represent the mean ± SEM; *n* = 7–10 experiments, representing a total of 30 old and 49 young mice.

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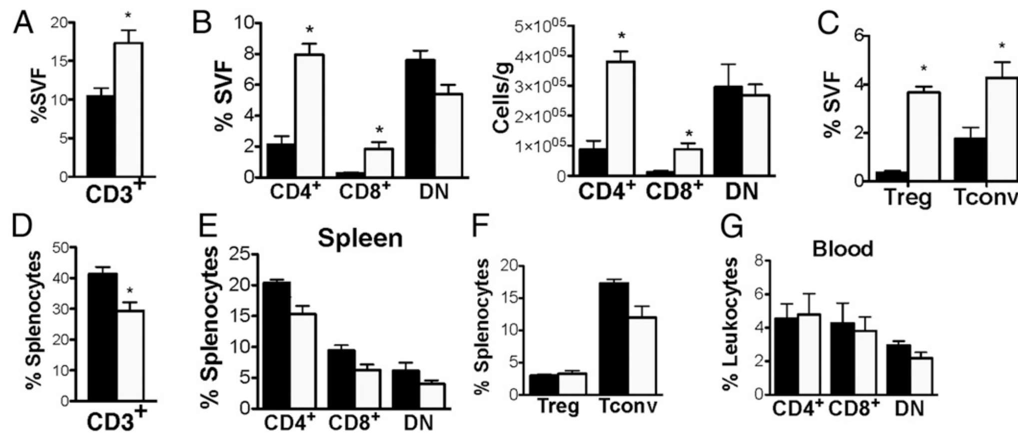

FIGURE 7. Increase in ATT cells with age. Flow cytometry analysis of ATT cells from SVF of epididymal fat from 6-mo-old (■) and 22-mo-old (□) mice. *A*, Percentage of CD3$^+$ cells in fat. *B*, Percentage of T cell subtypes in adipose tissue. Cells were gated on side scatter^{low}CD3$^+$ cells in adipose tissue prior to analysis. *C*, Quantitation of adipose tissue Tregs. CD3$^+$CD4$^+$Foxp3$^+$ (Treg) and CD3$^+$CD4$^+$Foxp3$^-$ (Tconv) were quantitated in young and old mice. *D*, Splenic CD3$^+$ cells. T cell subsets (*E*) and Treg content (*F*) in spleens of young and old mice. *G*, Blood T cell subsets (gated on CD3$^+$ cells). Results represent the mean ± SEM, $n = 5$ per group. *$p < 0.05$.

duced with obesity and express genes prominent in both M1 and M2 macrophage activation (33). Importantly, along with type 1 CD11c$^+$ ATMs, DN ATMs express high levels of CCR2 and CCR5, which matches our observation of the induction of these genes in ATMs with aging. The net result of these changes is an alteration in the balance between resident M2-polarized ATMs and the other subtypes with age biasing against an M2 environment with age. The importance of this balance is well established in obesity-associated adipose tissue inflammation (25), and to our knowledge, this is the first evidence that aging can alter ATM biology in a similar manner.

Our findings also suggest a role for the stromal vascular fraction cells in the induction of the inflammatory ATM phenotype in aging. The altered ratio of inflammatory to noninflammatory ATMs occurred independent of any significant differences in the number of crownlike structures that are a hallmark of inflamed obese fat, although a moderate increase in adipocyte size was observed with age. Further work will have to be done to assess whether the properties of these ATM subtypes defined by surface marker expression are identical to or different from the ATMs induced with high-fat diet. Our observations suggest a different architecture of ATMs with age-induced adipocyte hypertrophy.

Given the evidence showing that T cell activation occurs in adipose tissue with high-fat diet-induced obesity, which may alter ATM phenotype (54, 55), we examined the dominant ATT cell populations in visceral fat with age. This led to the striking observation of an age-dependent accumulation of CD4$^+$ (Tconv and Treg) and CD8$^+$ T cells in visceral fat. Despite this drastic in-

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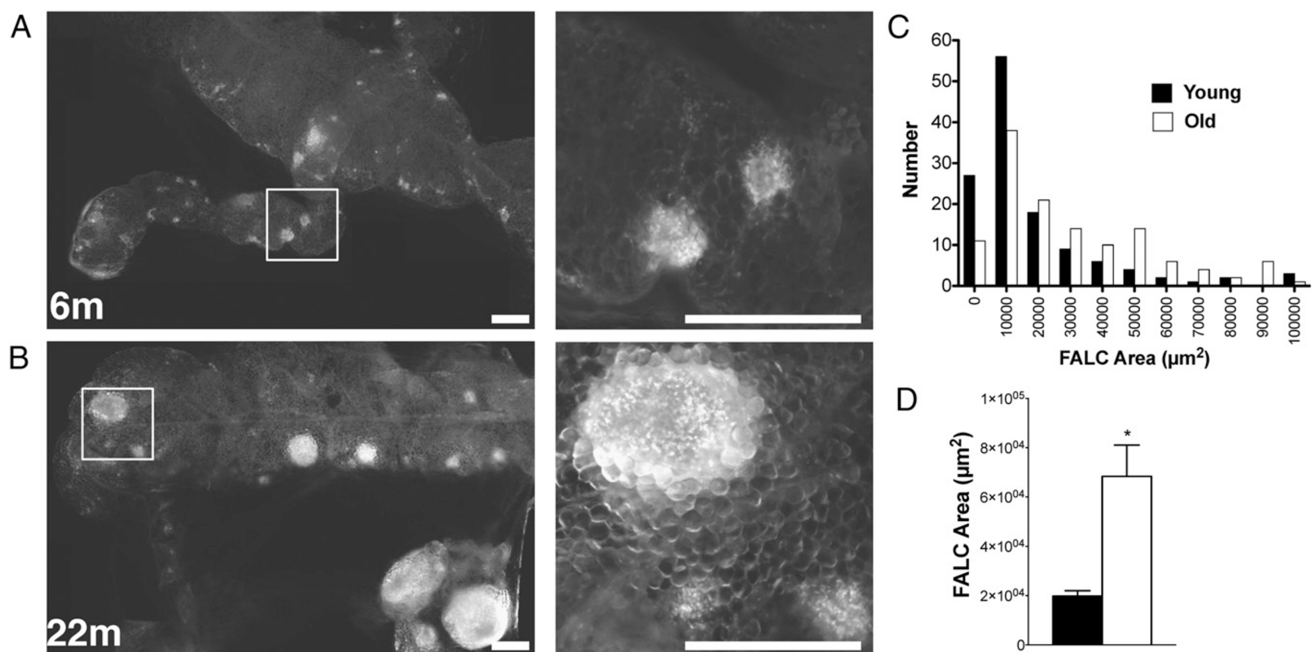

FIGURE 8. Enlargement of fat-associated lymphoid clusters with age. Omental fat pads were dissected and stained with anti-CD4 Abs to identify FALCs/milky spots. Images from representative samples from young (*A*) and old (*B*) mice. Low- (*left panels*) and high-power (*right*) images are shown. Scale bars, 500 μm. *Right panels*, Enlargements of the boxed area. Similar results obtained from five independent samples. *C*, FALC size frequency distribution in omental fat with age. CD4$^+$ FALCs were sized from four mice. *D*, FALC size represented as mean ± SEM. *$p < 0.001$.

duction, there was only a modest change in ATMs. This does not fully support the model that T cell accumulation is sufficient to drive ATM recruitment to fat. However, it is important to realize that the changes in adipose tissue leukocytes in physiologic contexts (e.g., fasting and aging) are likely to be divergent from those induced with nonphysiologic disease states such as obesity. The role that ATT cells play in regulating ATM function in age-associated obesity is currently unclear, and will need to be addressed in future research.

Based on the obesity literature, the massive induction of $CD8^+$ and Tconv ($CD4^+$) cells in fat would be expected to promote adipose tissue inflammation and the accumulation of proinflammatory ATMs (54, 55). This may in fact explain some of the ATM alterations observed with age. However, we also observed a parallel increase in Tregs in fat with age that would be predicted to suppress proinflammatory stimuli and maintain a low inflammation state (56, 57). These findings are consistent with recent reports demonstrating age-related increase in Treg number and function in central and peripheral T cell compartments (58–60). Expansion of ATT appears to be due to an age-dependent increase in the size of FALCs, which are highly specialized structures enriched for T cells, B cells, and macrophages (52). These lymphoid structures are not well characterized, but appear to be a site of immune surveillance in the peritoneal cavity (61). Further work will be required to detail the exact mechanism of this expansion and what it may mean for systemic immune challenges, especially in the visceral compartment. Our results also highlight the fact that for T cells, adipose tissue represents a unique environment with different regulatory controls compared with secondary lymphoid organs such as the spleen.

We have much to learn and clarify about the function of adipose tissue leukocytes, and care must be taken to differentiate ATT cell and ATM responses in different physiologic contexts. Much of what we know about adipose tissue inflammation is driven by the examination of nonphysiologic disease states such as obesity and insulin resistance. It is important to understand that the presence of a resident pool of macrophages and lymphocytes in fat provides the means for innate immune components to sense and respond to metabolic cues in many physiologic contexts. Recent studies have highlighted an important contribution of ATMs to the response to fasting and lipolysis, which are critical adipocyte functions required to preserve metabolic homeostasis (62). Our study provides crucial details about the physiologic changes induced in fat with aging that have broad implications to many disease states.

Disclosures

The authors have no financial conflicts of interest.

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