T CELL ALTERATIONS IN ADIPOSE TISSUE DURING OBESITY, HIV AND CANCER

EDITED BY: Dorothy Ellen Lewis, Joanne Lysaght and Huaizhu Wu
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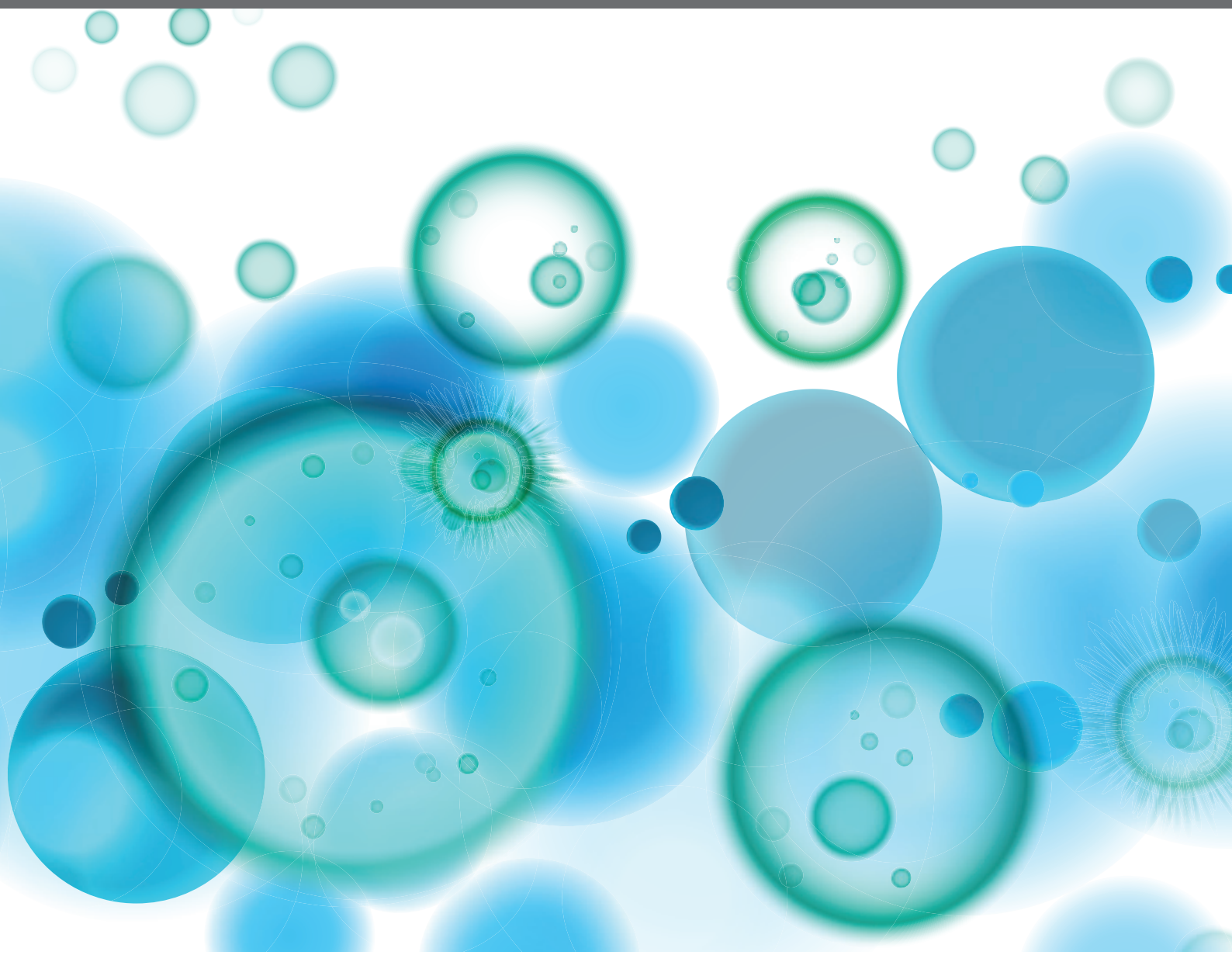

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T CELL ALTERATIONS IN ADIPOSE TISSUE DURING OBESITY, HIV AND CANCER

Topic Editors:
Dorothy Ellen Lewis, University of Texas Houston, United States
Joanne Lysaght, Trinity College Dublin, Ireland
Huaizhu Wu, Baylor College of Medicine Houston, United States

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EDITORIAL
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Editorial: T Cell Alterations in Adipose Tissue During Obesity, HIV, and Cancer

Dorothy E. Lewis[1]*, *Joanne Lysaght*[2] and *Huaizhu Wu*[3]

[1] McGovern Medical School, The University of Texas Health Science Center, Houston, TX, United States, [2] Department of Surgery, Trinity Translational Medicine Institute St. James's Hospital, Dublin, Ireland, [3] Section of Cardiovascular Research, Department of Medicine, Baylor College of Medicine, Houston, TX, United States

Keywords: T cells, obesity, cancer, HIV, inflammation

Editorial on the Research Topic

T Cell Alterations in Adipose Tissue during Obesity, HIV and Cancer

This collection of articles is designed to foster understanding of the variety and function of T cells in adipose from different species in different disease states. This work originally developed from the obesity epidemic. As the obesity epidemic grew, initial work recognized that adipose was dysfunctional. Work focused on inflammatory macrophages that increase in adipose during over-nutrition to produce inflammatory cytokines (1–3), which also serve to increase the dysfunction of adipose. However, it was soon realized especially in mouse studies that T cells had an increased role, specifically that obesity was associated with an increase in Th1 and Th17 T cells that make inflammatory cytokines (4–9), which also drive adipose dysfunction (4, 10, 11). Recent studies suggest that obesity in cancer patients may be a major risk factor for acquisition Lauby-Secretan et al. (12) but a positive feature in response to certain therapies for certain tumors Wang et al. (13). Because there is widespread adipose dysfunction in HIV infected people, there was also focus on how and why adipose changed, whether it was in response to antiretroviral therapy or due to infection itself (14, 15). This collection of articles are mainly reviews which cover what is known about T cells in obesity, in cancer, and in HIV.

Two articles Wang and Wu, Zhou and Liu and examine the role of T cells in metabolism as well as the interactions with antigen presenting cells in adipose. The review by Wang integrates what we know about T cells and macrophages in adipose and how they function and differ in obesity vs. the lean state. The Liu review nicely shows what happens after over feeding as the cells and resulting molecular consequences in adipose tissue changes. There is an initial response by adipocytes in the first days, followed by induction of pro-inflammatory cytokines and recruitment of T cells and macrophages in the first 2–3 weeks, followed by reduction in Foxp3 at 12 weeks in the mouse. How this develops in humans is not clear, although constant high fat feeding takes very little time to cause major changes in appearance and lipid and liver enzymes as seen in the movie, Super Size Me released in 2004. In the movie, Morgan Spurlock ate only at McDonald's for a month. He gained 11 lbs that took him 14 months to lose.

Another article examines how and why the T cells in aging animals, which are also usually obese, might be different than those found in young obese animals Antony et al. In particular, the differences seem to lie in differences in T reg numbers or function in aging. In particular the numbers of T regs increase in aging and their function changes perhaps as a response to the prolonged lipophilic environment found in aging mice. Work on human adipose in aging remains to be done, but from mouse studies it is clear that the changes in adipose are different in aging than in mere obesity.

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Edited by:
Remy Bosselut, National Cancer Institute (NCI), United States

Reviewed by:
Valerie Dardalhon, UMR5535 Institut de Génétique Moléculaire de Montpellier (IGMM), France

***Correspondence:**
Dorothy E. Lewis
Dorothy.e.lewis@uth.tmc.edu

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Two articles focus on the role of T regs in regulation of metabolism in obesity and in different species. The review by Zeng et al. covers what is known about the function of T regs in adipose in humans and mice, noting that several studies in humans associated Foxp3 expression with obesity. However, measuring T regs by flow cytometry has produced inconsistent results. There is some concern that variations might be due to fresh vs. frozen tissue observations, since T reg staining is reduced by freezing in some cases. In addition, human CD4 T cells express Foxp3 when they are activated, it is normally transient, therefore results of Foxp3 mRNA studies are confounded by that caveat. Cold also induces T regs in mouse adipose, but it is unknown if the same is true in humans. Finally, the authors present a nice summary of attempts to exploit T regs for obesity therapy. A comparison of adipose from different species (humans, mice, and cynomolgus monkeys) was done by Laparra et al. which clearly indicates that most animals except for the C57 mouse do NOT have increased Foxp3 T cells in the lean state. Adipocyte size is also markedly different between the three species. The dramatic conclusion is that mouse and human adipose tissue have markedly different immune cell compositions which should offer caution when interpreting mouse studies. More studies need to be done on young humans and on feral mouse populations to know whether environmental differences could contribute to these differences.

Two articles concern characteristics of T cells in cancer patients. One (Cornò) examines the information about innate lymphocytes in adipose and how they differ in obesity, as these cells likely act as sentinels to control tumor development Del Corno et al. Most of the work has been done in the peripheral blood and indicates that there is a decrease in many innate lymphocytes in the blood in obesity. In adipose there is some evidence for a decrease but more work is needed to directly link these cells to development of cancer. Another paper Conroy et al. reports novel data for the chemokine fractalkine found in omentum as well as its receptor (CX3CR1) in cancer patients and links it to memory CD8 T cell recruitment to the omentum perhaps bypassing the tumor and leading to a mechanism whereby tumor cells are allowed to go unchecked.

A single article reviews human T cells found in HIV and compares the information to the T cell changes in obesity Wanjalla et al. A key difference in HIV is that there are clonal populations of CD8 T cells and those cells predominate with reduced CD4 T cells. In obesity, although CD8 T cells have greater increases in adipose compared to lean (6, 16) there are higher levels of polyclonal memory CD4 T cells than CD8 T cells (16). In addition, in HIV there are fewer Tregs, with more in obese humans. Finally a key recent observation is that CD4 T cells in human adipose express more PD-1 which has implications for adipose as a reservoir for HIV.

In summary, this collection of articles provide the background to focus efforts on preventing the consequences of adipose dysfunction seen in so many disease states.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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The Frequencies of Immunosuppressive Cells in Adipose Tissue Differ in Human, Non-human Primate, and Mouse Models

*Ariane Laparra[1], Sabine Tricot[1], Mélanie Le Van[1], Abderaouf Damouche[1], Jennifer Gorwood[1], Bruno Vaslin[1], Benoit Favier[1], Stéphane Benoist[2], Raphael Ho Tsong Fang[1], Nathalie Bosquet[1], Roger Le Grand[1], Catherine Chapon[1], Olivier Lambotte[1,3,4] and Christine Bourgeois[1]**

[1] CEA - Université Paris Sud 11 - INSERM U1184, Immunology of Viral Infections and Autoimmune Diseases, IDMIT Department, IBFJ, Fontenay-aux-Roses, France, [2] Assistance Publique Hôpitaux de Paris, Hôpital Bicêtre, Service de Chirurgie Digestive et Oncologique, Le Kremlin-Bicêtre, France, [3] Université Paris Sud, Le Kremlin Bicêtre, France, [4] Assistance Publique-Hôpitaux de Paris, Service de Médecine Interne et Immunologie Clinique, Groupe Hospitalier Universitaire Paris Sud, Hôpital Bicêtre, Le Kremlin-Bicêtre, France

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Edited by:
Huaizhu Wu,
Baylor College of Medicine,
United States

Reviewed by:
James B. Hoying,
Cardiovascular Innovation Institute (CII), United States
Dorothy Ellen Lewis,
University of Texas, United States

***Correspondence:**
Christine Bourgeois
christine.bourgeois@u-psud.fr

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Although the metabolic properties of white adipose tissue have been extensively characterized, the tissue's immune properties are now attracting renewed interest. Early experiments in a mouse model suggested that white adipose tissue contains a high density of regulatory T cells (Tregs), and so it was assumed that all adipose tissue has an immunosuppressive profile—even though the investigation was limited to visceral body fat in relatively old male mice. This observation was also corroborated by high frequencies of other cell subsets with immunoregulatory properties, such as anti-inflammatory M2 macrophages, and regulatory B cells. Many studies have since evidenced the persistence of pathogens (trypanosomes, *Mycobacterium tuberculosis*, HIV, etc.) in adipose tissue. However, a recent report identified adipose tissue as a reservoir of memory T cells capable of protecting animals upon rechallenge. The immune potential of lean adipose tissue thus remains to be further investigated. Here, we compared the relative proportions of immune cells (and Tregs in particular) in lean adipose tissue collected from humans, a non-human primate (the cynomolgus macaque), and three mouse models. We demonstrated that the proportion of Foxp3+ Tregs in visceral adipose tissue was low in all models other than the C57Bl/6 mouse. These low values were not linked to correspondingly low proportions of effector cells because T lymphocytes (a main target of Treg suppression) were more frequent in cynomolgus macaques than in C57Bl/6 mice and (to a lesser extent) humans. In contrast, the proportions of macrophages and B cells were lower in cynomolgus macaques than in C57Bl/6 mice. We also observed a higher proportion of CD34+CD45- cells (which predominantly correspond to mesenchymal stem cells) in C57Bl/6 mouse and cynomolgus macaques than in humans and both for subcutaneous and visceral adipose tissues. Lastly, a microscopy analysis confirmed predominant proportion of adipocytes within adipose tissue, and highlighted a marked

difference in adipocyte size among the three species studied. In conclusion, our study of lean, middle-aged, male individuals showed that the immune compartment of adipose tissue differed markedly in humans vs. mice, and suggesting the presence of a more inflammatory steady-state profile in humans than mice.

Keywords: adipose tissue, Treg-regulatory T cell, immunosuppression/immune modulation, human–animal relationships, healthy adipose tissue, age, metabolism and obesity

INTRODUCTION

Adipose tissue (AT) is composed of both adipocytes and a stromal vascular fraction (SVF) that contains a highly heterogeneous range of immune cells (1, 2). A growing body of evidence has revealed the close interplay between the immune cells and metabolic cells in AT, which appear to have synergistic roles in the development and control of obesity and in thermostasis (1–3). Adipose tissue also underpins the regenerative functions (4–6) performed by mesenchymal stromal cells [also referred to as adipose stromal cells (ASCs)] and hematopoietic stem cells. Hence, it is now clear that AT has multiple immune and regenerative functions as well as crucial metabolic activities.

Adipose tissue shows a strikingly high level of plasticity when affected by metabolic disorders–either quantitatively (e.g., with the infiltration of pathogenic cells) or qualitatively (e.g., following functional changes in the resident AT cells). With regard to immune functions, most studies of lean, healthy individuals have designated lean fat as an immunosuppressive site (1). Indeed, the vast majority of immune cells in AT appear to exert an immunosuppressive function. This profile favors the AT's metabolic activity (by limiting inflammation and ensuring tissue-remodeling) but might be less beneficial when considering immune responses to infections. The metabolic and immunosuppressive properties of Tregs in AT have been extensively described in mouse models. It has been found that obesity affects both the proportions and transcriptional profiles of AT Tregs (7–12). In clinical settings, the impact of obesity on the proportion of Tregs in human AT has been indirectly assessed by detecting *FOXP3* mRNA; these studies have given more heterogeneous results (13–16). In lean animals, AT contains a low proportion of M1 macrophages and a high proportion of M2 (anti-inflammatory) macrophages (17–21). The accumulation of macrophages and the change in macrophage phenotype are robust markers of obesity in AT, and are observed both in humans and mice (22, 23). Eosinophils (a subset present in lean AT) also exhibit anti-inflammatory properties by favoring the persistence of M2 macrophages and the maturation of adipocytes (24, 25). Studies of other immune cell subsets in AT [such as B cells, natural killer (NK) T cells, γδ-T cells, and innate lymphoid cells (ILCs)] are also now being performed–principally in mouse models. In lean animals, the B cells in AT include a regulatory B fraction (26), whereas obesity is associated with a greater proportion of B cells with a pathogenic profile (12, 27, 28). It has also been shown that the NK T cells in lean AT have immunomodulatory activities, and protect the AT from metabolic disorders (29–32). Natural killer T cells and γδ-T cells reside in the AT of lean individuals, and accumulate when metabolic disorders occur (1, 33, 34). Innate lymphoid cells have been studied in both murine and human ATs (35–37). Type 1 ILCs cells can be triggered by signals induced by metabolic stress and are involved in adipose inflammation, whereas type 2 ILCs appear to provide regulatory signals. Murine and human ASCs also exhibit strong immunosuppressive functions (38, 39). Lastly, the immune activity of adipocytes is also under scrutiny. Adipokine production by adipocytes is clearly associated with the development of an anti- or pro-inflammatory environment in AT (40, 41), as assessed, respectively by the secretion of adiponectin and leptin (41–44). Resolvin and other lipid mediators are also involved in the anti- or pro-inflammatory profile (45–48). Adipocytes also express MHC class II, and may therefore have a key role in immune activation (49–51). If metabolic stress is present, the immune properties of adipocytes also change because the cells upregulate their expression of stress markers and can thus generate pro-inflammatory signals (33).

Based on these observations, one can question the ability of AT immune cells to mount an effective local immune response. Although steady-state immune activity might be controlled by the immunosuppressive environment, AT immune cells might be capable of rapid mobilization once danger signals or pathogen have been detected. This type of plasticity (which has been described for metabolic regulation) might efficiently combine immunomodulation (guaranteeing metabolic homeostasis) and a rapid immune response when pathogens are encountered. Alternatively, the striking persistence of various pathogens (52) [e.g., trypanosomes (53, 54), HIV (55–58), and *M. tuberculosis* (59)] in AT in different species strengthens the hypothesis whereby lasting anti-infectious responses are suppressed in AT. We studied this topic in the context of HIV infection by analyzing the composition of the AT in SIV-infected cynomolgus macaques (55) and then in HIV-infected patients (58). Modest changes in the AT immune compartment were detected: a higher proportion of SVF cells and CD8 T cells, and a modest change in the macrophages' phenotype and T cell activation in SIV-infected animals. In fact, one of our most striking observations was that the basal composition of AT in the cynomolgus macaque and in humans did not fully corroborate the data obtained in mice. We have observed remarkably low frequencies of AT Tregs in lean, non-human primates (NHPs) (55), and non-obese patients (58). More recently, it has also been found that AT is a reservoir for memory T cells capable of protecting the host upon infectious re-challenge after adoptive transfer (60). The objective of the present study was to evaluate the basal immune properties of "healthy" AT as a prerequisite for evaluating AT's

anti-infectious responses. To this end, we compared five different experimental models: three murine models (C57Bl/6, the most frequently used model of obesity, CBA and Balb/c strains), the cynomolgus macaque, and healthy human donors. Given that the AT's composition is thought to be strongly influenced by sex, age, and the metabolic context (10, 12), we confirmed these observations in various murine models and then performed comparative analyses of middle-aged male individuals from all species. After using the same protocol for AT dissociation, we performed FACS analyses on the SVF and thus defined the relative contribution of the main immune cell subsets in AT for all the groups tested. We found that a high proportion of AT Tregs was observed in middle-aged male C57Bl/6 mice but not in middle-aged male CBA mice, Balb/c mice, cynomolgus macaques, or humans. Similarly, C57Bl/6 mice had higher proportions of the immune subsets generally associated with immunosuppression (i.e., macrophages, B cells, and γδ-T cells) and ASCs (but not T cells), relative to humans. These discrepancies suggest that the steady-state immunosuppressive environment in AT differs in the three models. Our present observations may have a major impact on the choice of the most appropriate model for studying immune phenomena in the AT.

MATERIALS AND METHODS

Biological Materials

Samples From Mice

C57Bl/6 Rj, CBA, and Balb/c mice (Janvier Labs, Saint Berthevin, France), and Foxp3-GFP mice (Charles River, Saint-Germain-sur-l'Arbresle, France) were maintained under pathogen-free conditions in the central animal facility at the Paris-Sud Faculty of Medicine (Paris, France) (approval reference: D92-032-02). All procedures were conducted in compliance with French and European Union regulations on animal welfare (agreement B-94-043-12 and license 94-440, delivered by the French veterinary authorities) and had been validated by the local animal care and use committee (*Comité d'éthique en experimentation animale* (CEEA) 27, Paris-Sud University, Paris, France).

We studied male mice aged 2–3, 6, 12 months, and (for Foxp3-GFP mice only) 18–22 months. The impact of sex on the AT's immune properties was investigated by studying 2–3 months-old male vs. female C57Bl/6 Rj mice. To assess the impact of obesity on the AT's immune properties, 8 week-old C57Bl/6 Rj mice were fed a high fat diet (HFD, Research Diets, Broogarden, Lynge, Denmark), or standard chow for 48 weeks. The animals' bodyweight was recorded weekly. At sacrifice (at 12 months of age), the mean ± standard deviation bodyweight was, respectively 52.0 ± 2.8 and 33.1 ± 3.8 g. Six month-old mice were used in comparisons of C57Bl/6, CBA, and Balb/C animals.

Samples of subcutaneous AT (SCAT) and gonadal AT (as a source of VAT) were collected from all animals. All comparative experiments included appropriate control animals at the time of sacrifice. Different animals were used to study the respective impacts of sex, age, and metabolic disorders. For 2-3 month-old animals, AT samples were collected from two to four mice and pooled in order to obtain sufficient quantities of tissue.

Samples From Cynomolgus Macaques

Adult cynomolgus macaques were imported from Mauritius and housed in the animal facility at the *Commissariat à l'Energie Atomique et aux Energies Alternatives* (CEA, Fontenay-aux-Roses, France). The NHPs are housed and handled in accordance with French national regulations, and are subject to inspection by the veterinary authorities (CEA permit number: A 92-032-02). The CEA facility complies with the Standards for Human Care and Use of Laboratory Animals published by the US Office for Laboratory Animal Welfare (reference: OLAW #A5826-01). The use of NHPs at the CEA also complies with the European Directive 2010/63/EU. The animals were used under the supervision of the veterinarians in charge of the animal facility. Animals were housed in adjoining, individual cages (allowing social interactions) and under controlled humidity, temperature and light conditions (12 h light/12 h dark cycles). Water was available *ad libitum*. Animals were monitored and fed (by trained personnel) with commercial macaque chow and fruits once or twice daily. Samples of SCAT and VAT from 10 non-obese, healthy, middle-aged, male cynomolgus macaques were collected at sacrifice. For ethical reasons, the animals were not sacrificed for the sole purpose of collecting AT. Samples from control groups designated in various other studies were made available for the present work. The study protocols were reviewed by the CEA's Animal Care and Handling Committee (*Comité d'Ethique en Expérimentation Animale*, registered with the French Ministry of Research). At the time of the experiments, the median [interquartile range] age was 6.4 years [5.1–7.5], and the median [interquartile range] bodyweight was 7.5 kg [6.3–8.3].

Samples From Humans

Subcutaneous AT and/or VAT samples from 10 middle-aged male individuals were collected during elective abdominal surgery. The exclusion criteria were inflammatory bowel diseases, ischemic colitis, cancer, HIV infection, obesity and/or metabolic disorders, weight loss surgery, and the administration of medications with metabolic effects. At the time of surgery, the men's median [interquartile range] age was 53 years (47–59), and the median [interquartile range] BMI was 23 (22–25). Written, informed consent was provided by all participants. The study protocol was approved by the local investigational review board (*Comité de Protection des Personnes Ile-de-France VII*, PP12-021, Paris, France). Samples of abdominal SCAT, VAT, and (when available) blood were collected. We rarely had access to both human SCAT and VAT from the same patient (collection of both SCAT and VAT samples in sufficient quantity was only obtained in 3 out of 10 patients).

Dissociation of Adipose Tissue

Samples from all species (summarized in **Table 1**) were processed according to the same protocol. Stromal vascular fraction was isolated from fresh samples. Subcutaneous AT and VAT samples were weighed, washed twice in PBS 1x supplemented with 5% fetal bovine serum (FBS), cut into pieces of 2–3 mm, and then digested in a bath of collagenase (C2139, Sigma-Aldrich, Saint Quentin Fallavier, France) at a concentration of 0.33 mg/mL in

TABLE 1 | Age, sex, and characteristics of the models studied.

	Nber	Age (median)	Sex	Metabolic profile	Body weight (median)
MICE					
C57Bl/6 Rj	40	2–3 months	M/F	Lean (standard chow)	ND
	10	6 months	M	Lean (standard chow)	25.0 g
	30	12 months	M	Lean (standard chow)	33.1 g
	8	12 months	M	Obese (high-fat diet)	52.0 g
CBA	10	6 months	M	Lean (standard chow)	35.0 g
Balb/C	10	6 months	M	Lean (standard chow)	25.0 g
Foxp3 GFP	12	2–3 months	M	Lean (standard chow)	ND
	6	12 months	M	Lean (standard chow)	ND
	6	18–22 months	M	Lean (standard chow)	ND
CYNOMOLGUS MACAQUES					
	10	6.4 years	M	Non-obese	7.5 kg
HEALTHY HUMAN DONORS					
	10	53 years	M	Non-obese (BMI 23)	nd

ND, not determined.

Samples of SCAT and VAT were collected from C57Bl/6, CBA and Balb/c mice, cynomolgus macaques, and humans. The quantity of AT obtained differed as a function of the site (SCAT or VAT) and the species. Human SCAT or VAT were predominantly collected from different individuals. In contrast, SCAT and VAT were collected from the same individuals in mice and macaques. If cell numbers recovered from murine samples (especially the younger animals) were not sufficient, samples from 2 to 4 animals were collected for performance of the FACS analysis.

DMEM with 5% FBS for 30 min and at 37°C, with continuous shaking. The same batch of collagenase enzyme was used for all species. Next, the tissue was mechanically dissociated by repeated suction into and discharge from a 10 mL syringe. The adipose suspension was then filtered through a 100-micron mesh, and treated with ammonium-chloride-potassium lysing buffer. Cells in the SVF (i.e., all cells other than adipocytes) were treated with Trypan blue (to exclude dead cells) and then counted in Malassez chambers (C-chip, NanoEntek, Seoul, Korea) under the microscope.

Staining for Fluorescence-Activated Cell Sorting (FACS)

The following antibodies were used to stain the cynomolgus macaque and human samples [target molecule (clone)]: CD16 (3G8)/CD90 (5E10)/CD45 NHP (D058-1283)/CD45 Hu (HI30)/CD4 (RPA-T4)/CD8 (RPA-T8)/CD20 (2H7)/CD3 (SP34-2)/TCR GD (B1.1)/NKG2A (Z199)/HLA-DR (G46-6)/CD14 (M5E2)/CD11b (Bear1)/CD34 (563)/CD146 (P1H12)/CD73 (AD2)/CD235a (HI264)/CD31 (WM59)/PD-1 (EH12-2H7)/CD25 (2A3)/Ki67 (B56)/CD39 (eBioA1)/CD127 (MB15-18C9)/Foxp3 236A/E7)/CD1a (SK9)/CD34 (581)/CD123 (7G3)/BDCA2 (AC144)/CD161 (HP-3G10)/CD94 (DX22)/CD117 (104D2)/IL33R/CD69 (FN50)/TCR Vδ2 (B6)/αβTCR (T10B9-1A-31)/αβTCR NHP (R73)/CD45RA (clone 5H9)/CD27 (O323).

The following antibodies were used to stain the murine samples: CD45 (30F11)/F4/80 (BM8)/CD4 (RM4-5)/CD8 (53-6.7)/TCR GD (GL6)/NK1.1 (PK136)/CD11c (HL3)/TCR beta (H57-597)/CD11b (M1/70)/B220 (RA3-6B2)/PD-1 (29F.1A12)/CD106 (429)/CD90 (OX-7)/CD73 (TY/11.8)/CD31 (390)/CD34 (MEC14.7)/CD44 (IM7)/TCR β chain (57-597)/CD127 (A7R34)/CD25 (PC61.5)/CD3 (17A2)/Foxp3 (FJK-16s)/CD69 (H1.2F3)/CD14 (rmC5-3)/CD19 (1D3)/CD11c (HL3)/CD11b (M1/70)/CD25 (PC61.5)/CD161 (PK136)/CD117 (2B8)/IL33R (RMST2-33).

The staining protocol was identical for all three species. Staining was performed after the saturation of Fc receptors by incubation with Fc block reagent (BD) for 30 min at 4°C. Amine-reactive blue dye (LIVE/DEAD^{TM} Fixable, Life Technologies, Carlsbad, CA, USA) was used to assess cell viability. Cells were incubated with monoclonal antibodies for 15 min at 4°C, washed in PBS 1X/10% FBS, and fixed in commercial fixation solution or permeabilized when required [Intracellular Fixation and Permeabilization Buffer Set (eBiosciences/ThermoFisher Scientific, Waltham, MA, USA)]. The FACS data were acquired on an LSR Fortessa system (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo software (Treestar, Ashland, OR, USA).

Immunohistofluorescence

Adipocyte tissue (volume: 1 cm^3) was fixed with 4% PFA in phosphate buffer (pH 7) for 6 h, dehydrated overnight in 30% sucrose PBS at 4°C, embedded in optimal cutting temperature compound, and quickly frozen in isopentate with nitrogen liquid. Cryostat sections (thickness: 20 μm) were then prepared, and incubated at 4°C overnight with antibodies against FABP-4, CD45, CD34, and CD4. The anti-FABP-4 antibody was covalently linked to Alexa Fluorochrome AF55 using a microscale labeling kit (Life Technologies) and NHS ester conjugation. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). An isotype antibody was used as a negative control for each

staining. Immunofluorescence images were examined using an SP8 confocal microscope (Leica, Germany).

Statistical Analysis

The FACS data from mouse-only experiments are quoted as the median ± SD, and the data from all other experiments are quoted as the median [interquartile range]. The microscopy data are quoted as the median ± standard error or the mean (SEM). All statistical analyses were carried out with GraphPad Prism software (version 7.02, GraphPad Software Inc.). A non-parametric Kruskal-Wallis test was used to compare species. For the mouse-only experiments, a paired, non-parametric Wilcoxon test was used to compare SCAT vs. VAT for each species or strain, and an unpaired, non-parametric Mann–Whitney test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) was used to compare groups. Student's *t*-test was used to compare microscopy data. In graphs, the *p*-value is indicated as follows: $^{*} < 0.05$; $^{**} < 0.01$, $^{***} < 0.001$.

RESULTS

The Proportion of Foxp3 Tregs Among CD4 T Cells in Adipose Tissue Is Low in All Models Other Than the Lean Male C57Bl/6 Mouse

We and others have previously demonstrated that Foxp3+ CD4 T cells are extremely scarce in VAT from humans and cynomolgus macaques (55, 58, 61), when compared with the corresponding data obtained in mice (8, 11, 62). To fully ascertain the potential differences in immune cell composition between humans and the other animal models, we analyzed SCAT and VAT samples from three mouse strains, a non-human primate (the cynomolgus macaque) and humans. The method used to dissociate the AT (i.e. the type, batch and concentration of collagenase, and the duration of incubation) was the same for all species. Likewise, the criterion used to identify Tregs (Foxp3 expression by CD4 T cells) was the same for all species. Given that the proportion of Tregs has been shown to differ markedly as a function of sex, age and metabolic context (e.g., obese vs. lean mice) (8, 10), we chose to study mouse models in which all these conditions can be easily evaluated. We first compared the frequency of Foxp3+ CD4 T cells in 2- to 3-month-old male and female C57Bl/6 mice (**Figure 1A**). The Treg frequency was significantly higher in the VAT of male animals, whereas all other samples (female SCAT and VAT, male SCAT) exhibited values that were similar to those usually reported for the murine spleen. We next studied the impact of age on male C57Bl/6 mice by comparing 2- to 3-month-old and 11- to 13-month-old animals (**Figure 1B**). In line with the literature data, we confirmed the drastic impact of age on the percentage of Foxp3+ cells in the VAT of male C57Bl/6 mice. We also used Foxp3-GFP C57Bl/6 mice to evaluate the long-term persistence of Tregs in VAT (**Supplementary Figure 1**). As observed in B6 mice, the percentages of GFP+/Foxp3+ cells in both VAT and SCAT were significantly higher in older animals (12 months of age and 18–22 months of age) than in young animals (i.e., 2–3 months of age); the difference was less striking for SCAT, however. Lastly, as a control, we also evaluated the impact of a metabolic disorder on the Treg frequency in AT by comparing HFD-induced obese animals with lean animals. 8 week-old mice were fed a HFD or standard chow, and sacrificed at 10–12 months of age. As described previously (11, 12), obese male animals had a significantly lower proportion of Tregs in the VAT than their lean counterparts (**Figure 1C**). Taken as a whole, our results confirmed the literature reports of a high frequency of Tregs in the AT in a very restricted context (i.e., in relatively old, non-obese, male animals) and in a relatively specific tissue (i.e., VAT, rather than SCAT). Given that these observations did not match those reported for cynomolgus macaque and human VAT samples, we next evaluated the proportion of Tregs in the SCAT and VAT of 6 month-old C57Bl/6, CBA and Balb/c male mice. Interestingly, the proportions of Tregs in the SCAT and VAT were much lower in both CBA and Balb/c mice (**Figure 1D**). We next assessed the proportions of Foxp3+ CD4 T cells in AT samples collected from mice, NHPs, and humans. On the basis of our initial set of experiments, we selected individuals with the following characteristics: male gender, middle age (12 months of age for mice, 6 years of age for cynomolgus macaques, and 53 years of age for humans) and no obesity (mice fed standard chow, a median bodyweight of 7.5 kg for cynomolgus macaques, and a median BMI of 23 for humans). The C57Bl/6 mouse [the best-characterized murine model in studies of obesity and AT homeostasis (11, 63)] was used as control for this comparison. The high proportion of Tregs observed in the VAT of C57Bl/6 mice was not observed in the VAT or SCAT of cynomolgus macaques or humans (**Figure 1E**).

Overall, we found that C57Bl/6 mouse appears to be a rather peculiar model with regard to the high proportion of Foxp3+ CD4 T cells in the VAT. This high proportion was not observed in two other strains of mice (CBA or Balb/c) or in two other species (the cynomolgus macaque and the human).

The Low Proportion of Foxp3 Tregs in Cynomolgus Macaques and Humans Impacts the Effector/Treg Ratio in Adipose Tissue

The immunosuppressive activity of Tregs is also defined by the ratio between the Tregs and the potentially suppressed effector cells. One could therefore hypothesize that the low proportion of Tregs in human and cynomolgus macaques AT (relative to C57Bl/6 mice) essentially reflects the low proportion of effector T cells without affecting the Treg/effector ratio and the AT's immunosuppressive potential. We therefore looked at whether the low proportion of Foxp3 Tregs reflected a difference (i.e., a relative reduction) in the proportion of effector cells in cynomolgus macaques and humans. We first determined the proportions of hematopoietic CD45+ cells (and macrophages and T cells, the two most represented subsets among CD45+ cells) in the SCAT and VAT of the three groups. The three species did not differ with regard to the proportion of hematopoietic cells (**Figure 2A**). In each species, the proportion of hematopoietic cells was similar in SCAT and VAT. The data were more dispersed within the group of humans,

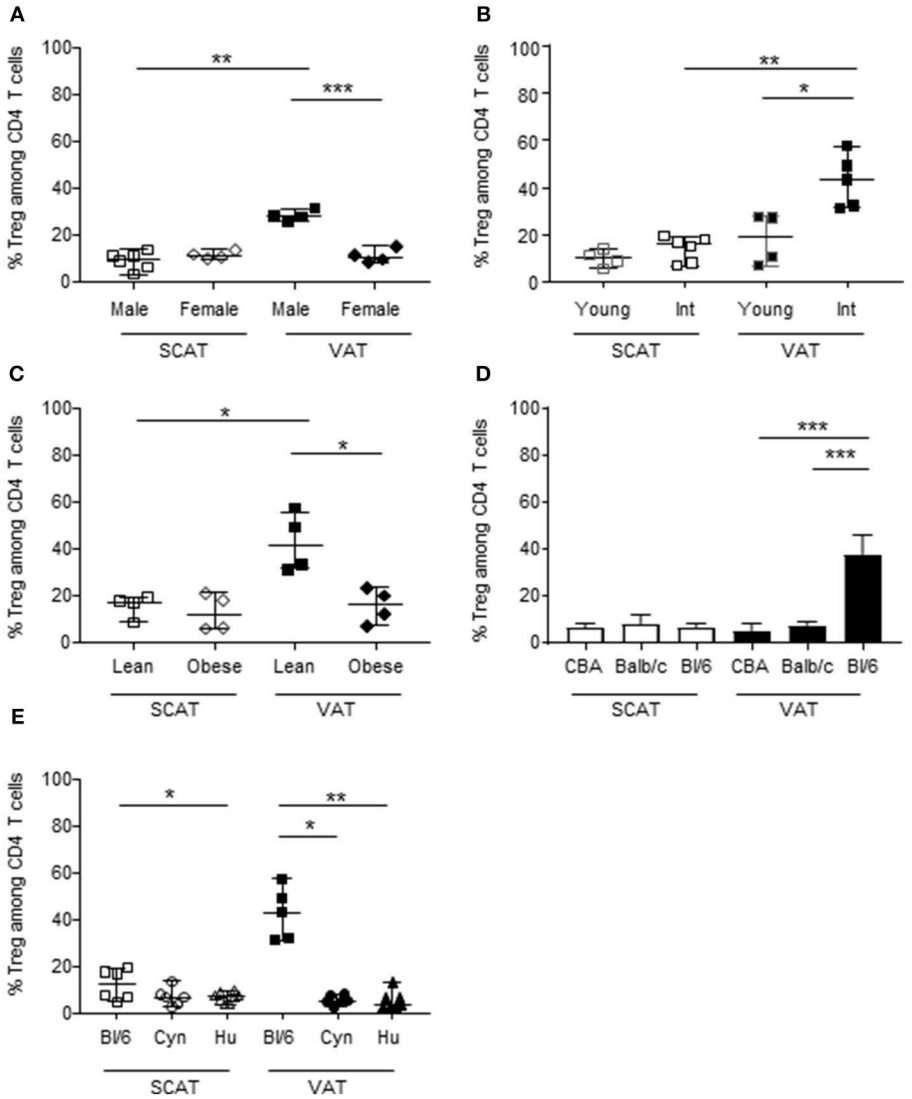

FIGURE 1 | The proportion of Tregs in AT, as a function of sex, age, metabolic context, strain, and species.The frequencies of Foxp3+ cells (calculated as a proportion of CD4 T cells) were quantified in SVF from murine, NHP and human SCAT (open symbols), and VAT (closed symbols). In C57Bl/6 mice **(A–C)**, the frequency of Foxp3+ cells was evaluated as a function of the animals' sex, age, and metabolic context. **(A)** The impact of sex was studied by comparing female with male 8- to 12-week-old C57Bl/6 mice. **(B)** The impact of aging was studied by comparing 8- to 12-week-old vs. 12-month-old male C57Bl/6 mice. **(C)** The impact of the metabolic context was studied by comparing lean vs. obese 12-month-old male C57Bl/6 mice. The data correspond to two representative experiments comparing 4–6 animals. **(D)** Comparison of the frequencies of Foxp3+ cells among CD4 T cells in SCAT and VAT from 6 month-old-male CBA, Balb/C and C57Bl/6 mice. The graphs showed data from 10 mice for each strain. **(E)** Comparison of the frequencies of Foxp3+ cells among CD4 T cells in SCAT and VAT from adult male C57Bl/6 mice (age: 10–14 months), cynomolgus macaques (age: 5–7 years), and humans (age: 47–59 years). Bars represent median values. Statistical significance was determined with a Kruskal–Wallis test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) when performing comparisons of SCAT vs. VAT within a group, and a non-parametric Wilcoxon test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) when comparing groups.

which presumably reflects greater genetic heterogeneity. We next evaluated the proportion of T lymphocytes in SVF cells–a subset generally targeted by Tregs (64). The proportion of T cells in VAT was higher in cynomolgus macaques than in C57Bl/6 mice (**Figure 2B**). A similar trend was observed when comparing humans with mice. The low proportion of Tregs was thus not attributable to the low proportion of effector T cells. Lastly, we evaluated the proportion of macrophages, which can potentially be modulated by Tregs (65–68) but may also favor Treg accumulation (69, 70) The proportion of macrophages in the SVF fraction was not defined in the same way in all the three species: F4/80 expression in mice, and CD14 expression in cynomolgus macaques and humans. The percentage of monocytes/macrophages in middle-aged male cynomolgus macaques was significantly lower than the percentage of macrophages in C57Bl/6 mice (**Figure 2C**). A

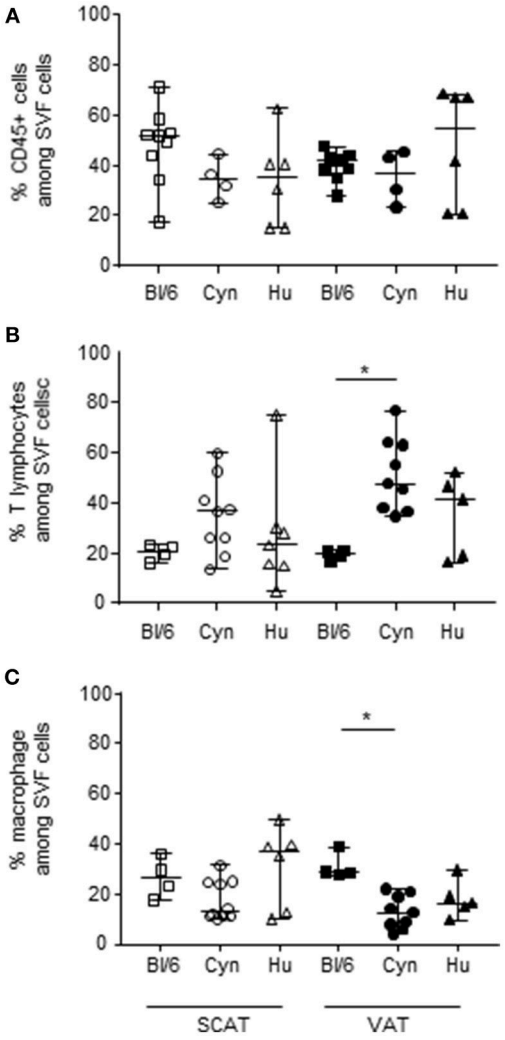

FIGURE 2 | Frequencies of hematopoietic CD45+ cells, macrophages, and T lymphocytes in SVF. The frequencies of CD45+ cells **(A)**, T cells **(B)**, and macrophages **(C)** in the SVF are shown. The comparison was performed on samples of SCAT (open symbols) and VAT (closed symbols) collected from 4 to 10 mice or groups of mice, 10 cynomolgus macaques, and 9 human donors. Macrophage subsets were defined as F4/80+ cells in mice, and CD14+ cells in cynomolgus macaques and humans. (In humans and cynomolgus macaques, HLA-DR and CD11b expression were confirmed on CD14+ cells). T cells were defined as CD3- and/or TCRβ-expressing cells. Bars represent median values. Statistical significance (*$p < 0.05$ in a non-parametric Wilcoxon test) is shown as appropriate.

similar difference was observed when comparing human and mice VAT samples, although it was not statistically significant. Although the detection of macrophages may be less stringent in cynomolgus macaques and humans, the proportion in these species was significantly lower than in C57Bl/6 mice. The low proportion of macrophages may indirectly reflect the elevated proportions of T cells observed in humans and macaques, relative to C57Bl/6 mice.

In conclusion, 12 month-old male C57Bl/6 mice differed from middle-aged male cynomolgus macaques and humans with regard to the macrophage and T lymphocyte profiles in VAT. The VAT in C57Bl/6 mice featured a higher proportion of macrophages, whereas the VAT in cynomolgus macaques and humans featured a higher proportion of T lymphocytes. When comparing Treg and effector cell fractions in the three species, we observed that the low number of Tregs (as a proportion of all CD4 T cells) in SCAT and VAT from cynomolgus macaques and humans was associated with a higher proportion of T cells (the usual target of Treg suppression).

The Relative Contributions of Immune Subsets Among CD45+ Cells in VAT From Middle-Aged Male C57Bl/6 Mice, Cynomolgus Macaques, and Humans.

Given the observed intergroup differences in the proportions of Tregs, macrophages and T cells, we next performed an overall analysis of the main immune subsets commonly studied in AT. In addition to macrophages and αβ T cells, we determined the relative proportions of B cells, γδ T cells, NK cells, and ILCs (**Figure 3A**). The identification criteria for each subset in each species are given in **Supplementary Table 1**. In both SCAT and VAT, the frequency of B cells (as a proportion of CD45+ cells) was markedly higher in C57Bl/6 mice than in humans. A similar trend was observed when comparing C57Bl/6 mice and cynomolgus macaques. In both SCAT and VAT, the percentage of γδ T cells was also higher in middle-aged male C57Bl/6 mice than in cynomolgus macaques. Again, a similar trend was observed in humans. The proportions of NK cells and the ILCs (data not shown) were similar in all groups considered. The relative proportions of the various CD45+ immune cells in the SVF from VAT are shown in **Figure 3B**. Arbitrary colors were given to each subset, based on the immune function described by the data literature in mice, i.e., an immunosuppressive function (in green) or an effector function (in blue). Macrophages, B cells, γδ T cells, and NK cells have been linked to modulatory properties in lean AT (1, 26), and so were assigned a green color in **Figure 3B**. Although the functional activity of the various immune subsets was not evaluated in the present study, the relative proportions suggested that the immune cells' environment differ in middle-aged, male C57Bl/6 mice vs. humans, and cynomolgus macaques.

The High Proportion of CD34+CD45- Cells in SVF Collected From Middle-Aged Male C57Bl/6 Mice Was Not Observed in NHPs and Humans

To provide an exhaustive characterization of the potential immunosuppressive environment in AT, we also evaluated the percentage of CD34+CD45- cells in the SVF of the three species considered. The CD34+CD45- fraction is enriched in AT mesenchymal stromal cells (i.e., ASCs). These cells are multipotent progenitors that have attracted great interest in the context of regenerative therapies based on AT extracts. However, ASCs are also characterized by their high immunosuppressive potential. Although the ASCs' immunosuppressive mechanism

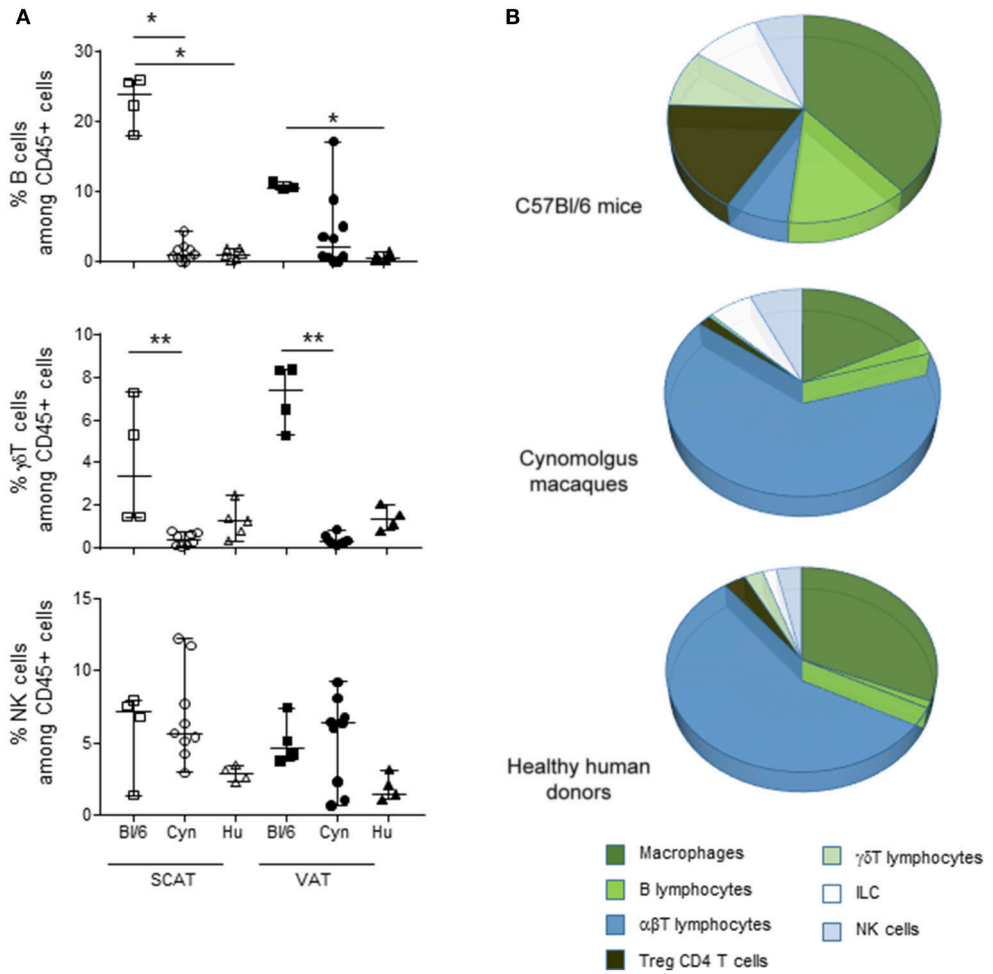

FIGURE 3 | Frequencies of immune cell subsets among the hematopoietic CD45+ SVF cells in VAT. The cell subsets in SCAT and VAT SVF in adult, non-obese, male C57Bl/6 mice, cynomolgus macaques, and healthy human donors were studied using flow cytometry. When considering CD45+ cells, we identified macrophages, B lymphocytes, αβ T lymphocytes, Foxp3 + CD4 Tregs, γδ T cells, ILCs, and NK cells. **(A)** Comparison of the percentages of B cell, γδ T cells, and NK cells among CD45+ cells. **(B)** Pie chart of the cell subsets composing the CD45+ fraction in the SVF for each species. The comparison was performed on VAT collected from 4 groups of mice, 10 cynomolgus macaques, and 9 healthy human donors. Statistical significance (*$p < 0.05$, **$p < 0.01$, in a non-parametric Wilcoxon test) is shown as appropriate.

has not been fully characterized, these cells express CD73—an ectonucleosidase that triggers immunosuppressive pathways. We thus evaluated the proportion of CD34+CD45- in the SVF from SCAT and VAT of the three groups studied (**Figure 4**). Interestingly, the percentages of CD34+CD45- cells in SCAT and VAT (ranging from 40 to 60%) were similar in middle-aged male C57Bl/6 mice and cynomolgus macaques but were significantly lower in middle-aged male humans. We also determined CD73 expression by CD34+CD45- cells in the SVF in cynomolgus macaques and humans. As previously described in mice, we observed that most of the CD34+CD45- cells expressed CD73 (data not shown). Our analysis of the CD34+CD45- fraction identified a high proportion of non-hematopoietic cells with potential immunosuppressive properties in C57BL/6 mice and cynomolgus macaques but not in humans.

Adipocyte Size Also Differed When Comparing Middle-Aged Male C57Bl/6 Mice, Cynomolgus Macaques, and Humans.

To more accurately evaluate the immune environment within AT, we performed an immunohistofluorescence analysis of VAT tissue sections. In fact, flow cytometry analysis of the SVF requires prior tissue dissociation and thus eliminates the adipocyte fraction–the main cell type in AT. Our immunohistofluorescence analyses detected FABP-4-expressing adipocytes in VAT collected from lean, middle-aged, male C57BL/6 mice, cynomolgus macaques, and humans (**Figure 5A**) but also simultaneously detected CD45-expressing cells and CD34-expressing cells. We found that the proportions of CD45- and CD34-expressing cells in all three groups were extremely

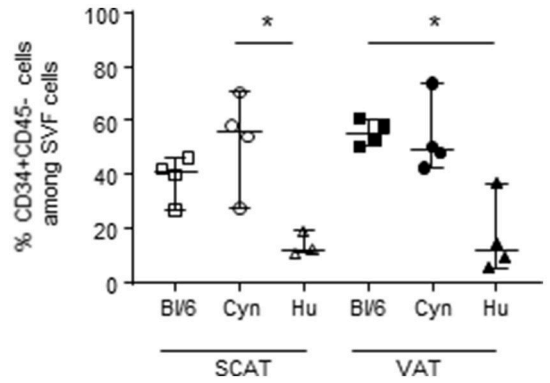

FIGURE 4 | Frequencies of CD34+ CD45- cells in SVF. The frequency of CD34+CD45- cells in the SVF from SCAT and VAT in adult, non-obese, male C57Bl/6 mice, cynomolgus macaques, and healthy human donors. We assessed SCAT and VAT collected from 4 groups of mice, 4 cynomolgus macaques, and 5 human donors. Statistical significance was determined with a Kruskal Wallis test when performing comparisons of SCAT vs. VAT within a group, and a non-parametric Wilcoxon test (*$p < 0.05$) when comparing groups.

low. Indeed, these cells were absent in a large number of fields (data not shown). Although some aggregates were observed, the size of the overall immune compartment in AT samples in this study of male, middle-aged animals, and humans appeared to be extremely small. This observation emphasized that the adipocyte is the key cell in AT in lean healthy individuals, and that AT immune cells are scarce. The flow cytometry strategy used here to phenotype immune cells within the SVF after AT dissociation might overestimate the relative proportions of these cells in AT. The results of tissue section imaging suggested that direct interaction between immune cells and adipocytes probably predominates, given the high proportion of adipocytes.

Importantly, our immunohistofluorescence analyses provided an indirect estimate of the size of the adipocytes in VAT from all groups studied (**Figure 5B**). The mean ± SD adipocyte size differed significantly when comparing middle-aged male C57Bl/6 mice (57.4 ± 0.5 μm), cynomolgus macaques (59.0 ± 0.2 μm), and humans (81.0 ± 0.4 μm). These results show that the species' ATs differ with regard to both the SVF and the adipocyte compartment.

DISCUSSION

White AT is a complex tissue with regard to its (i) many metabolic, regenerative and immune functions, (ii) complex cellular interactions, and (iii) high plasticity (as evidenced by the metabolic and immune changes induced by metabolic insults). The renewed interest in the biology of AT has prompted fundamental studies in mice, clinical research in patients, and (in certain infectious contexts) studies of NHPs. However, the steady-state immune properties in each model have not been clearly established, and a review of the literature data suggests that there are major discrepancies between the models in this respect. To fully appraise the immune properties of AT in the respective models, we compared "healthy," middle-aged male rodents, NHPs and humans. Our results highlighted a number of differences in the AT between the various models. Before addressing the composition of the stromal vascular fraction of adipose tissue, we first aimed to analyze the density of SVF cells per gram of AT in the various models by counting SVF cells recovered following collagenase treatment. Although various exclusion criteria were used for the selection of "healthy" subjects, we still observed important heterogeneity in SVF cell recovery, precluding any definitive conclusion to be drawn between the three models. We then aimed to evaluate the density of CD45 expressing cells by immunohistofluorescence (that allowed to provide co-staining for adipocytes (FABP-4), CD45, or CD34 expressing cells). A potential bias is the heterogeneity in SVF cell density as a function of the location within AT (perivascular vs. deeper area of AT). Analyses (performed here on deep sections of AT) showed a limited density of SVF CD45+ cells, precluding any comparison. Lastly, microscopy analyses showed differences in adipocyte size and density as a function of the model. We were thus not even convinced that the comparison of SVF cell numbers per gram of AT would be appropriate. Due to these technical hurdles (heterogeneity, limited density, change in adipocyte size as a function of the model), we did not introduce any data on the number of SVF cells collected per gram of AT in the current manuscript. However, the comparison of the composition of AT in the various model is robust. A striking observation was that the Treg frequency was much higher in the C57Bl/6 model than in the other mouse strains or in primates. Based on the proportions of many immunosuppressive subsets, the AT's immunomodulatory profile appeared to be more "potent" in the C57Bl/6 model than in primates. The proportions of macrophages and ASCs were lower in human VAT than in murine VAT, whereas the overall proportion of T cells was higher. The differences in B cells, γδT cells, and ILCs are also suggestive of lower immunosuppressive potential in human AT.

These comparisons are obviously subject to debate. The criterion for "middle age" corresponded to 12 months of age in mice, 6 years of age in cynomolgus macaques, and 53 years of age in humans. Although this comparison appears to be reasonable, it still constitutes a study limitation. The same criticism can be raised with regard to the apparently "healthy" status of all the individuals. Although healthy mice and cynomolgus macaques can be maintained with relative ease, it is more difficult to consider that humans undergoing elective surgery are indeed "healthy." In the present study, and on the basis of our previous experiments (58), we selected male patients undergoing cholecystectomy because the latter condition is usually not related to immune or metabolic disorders. The exclusion criteria included the presence of inflammatory bowel diseases, ischemic colitis, cancer, HIV infection, obesity, metabolic disorders (such as diabetes), and the administration of medications with metabolic effects. Despite the application of these precautions, we nonetheless observed major discrepancies between the models. Lastly, one must also take into account the heterogeneity of the AT's immune composition as a function of its location in the body. Although we analyzed both SCAT and VAT, the latter corresponded to gonadal fat in mice and abdominal

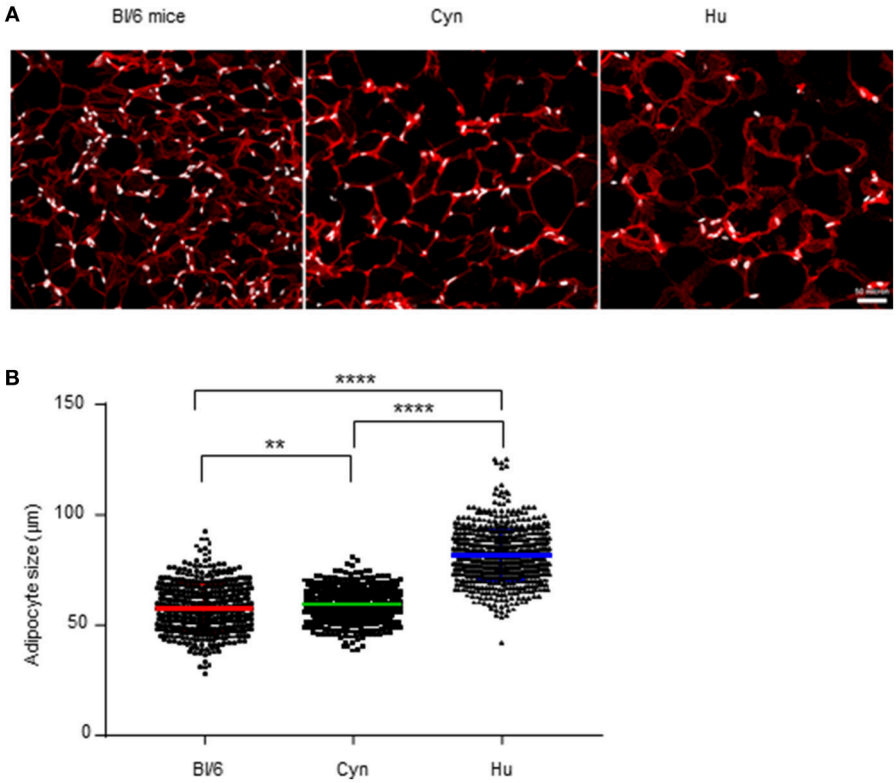

FIGURE 5 | Relative distributions of adipocytes and SVF cells. **(A)** Immunohistofluorescence image of adipocytes (FABP-4 staining, in red) and SVF nuclei (DAPI staining, in white) from VAT collected from non-obese male C57Bl/6 mice, cynomolgus macaques, and healthy human donors. **(B)** We measured the adipocyte size (using Image J software) on 5 images for each sample. Each point represents the size of one adipocyte. The mean ± SEM and the 95% confidence interval are shown. The graph represents the pool of the 3 samples tested for each specie. Statistical significance was determined in an unpaired *T*-test (**$p < 0.01$, ****$p < 0.0001$).

omental fat in cynomolgus macaques and humans. Although gonadal fat is widely considered to be a robust model of VAT (71) (notably due to the limited quantity of abdominal VAT in mice), this difference must be taken into account. In this respect, a comparison of the three murine strains was crucial because the CBA and Balb/c mice exhibited much the same profile as the NHP and humans but differed from C57Bl/6 mice—suggesting that the intrinsic properties of gonadal AT are not responsible for the observed differences.

The observed differences between C57Bl/6 mice on one hand and the NHP and humans on the other raise the question of which mechanisms might be responsible. Firstly, one could hypothesize that environmental factors (e.g., differences in the microbiota, the history of infection or even exposure to pollutants) have a role. Interestingly, the observed differences between the mouse strains suggest that a history of infection (which was limited in all three strains, given their housing conditions) does not drive the accumulation of Tregs in AT. However, this strain-dependent heterogeneity may reflect differences in both the microbiota (72, 73) and intrinsic metabolic factors. C57Bl/6 mice exhibit lower gut microbiota diversity, richness and Firmicutes/Bacteroidetes (F/B) ratio compared to Balb/C mice (73). These observation led to various points of discussion. Higher diversity and richness is assumed to be beneficial for host health (74) and may thus protect Balb/c animals from metabolic disorders and obesity. The F/B ratio is also considered a significant marker of gut microbiota composition and high F/B ratio is associated with obesity (75, 76). Although a more refined characterization of the bacteria of both phyla is required, this variation in the composition of microbiota may provide an important rationale for the different susceptibility of the mouse strain to obesity and/or adipose tissue cell composition. Composition of the microbiota has been shown to modulate Treg frequencies in healthy individuals (77, 78) although these studies did not analyze adipose tissue. Altogether, the variation in gut microbiota modulate the development of immune responses and may contribute to the higher sensitivity of the C57Bl/6 strain to obesity (79). It is obviously more difficult to determine which factors are responsible for differences in the baseline composition of "healthy" AT as a function of species. Regardless of the mechanisms involved, our present data suggest that the AT environment comprises less immunosuppressive cells in humans than in the C57Bl/6 reference murine model. However, the lack of functional assay data means that these results should be interpreted with a degree of caution. One can postulate that the immunosuppressive and/or effector properties displayed by a defined cell subset in mice may

not be recapitulated in NHP and humans. Alternately, the immunosuppressive properties may be carried on by different cell subsets in the various species. The difference in the frequencies of immunosuppressive cells will have to be taken into account for the development of suppressive assay that may require different stimulation to trigger the immunosuppressive function of the AT immunosuppressive cells. We were thus currently unable to provide a direct functional comparison of the immunosuppressive properties of AT between all species.

However, the persistence of multiple pathogens (HIV (55, 56, 80), *M. tuberculosis* (81), and trypanosomes (82) in human AT indirectly suggests that strong immunomodulation enables the persistence of pathogens in this tissue. It is tempting to speculate that immunosuppressive properties still develop in human AT but rely on mechanisms other than Tregs or other immunosuppressive cells. In a previous report, we described the high proportion of PD-1-expressing (and thus potentially exhausted) T cells in human AT (58) which may contribute to the low efficacy of immune responses. Additionally, our present study of fixed AT sections served as a useful reminder that the most prominent cell in AT (i.e., the main contributor to the AT's specific microenvironment) is the adipocyte—the role of which as an immune regulator remains to be characterized in detail. Indeed, the flow cytometry analysis performed after collagenase digestion induces bias in favor of the SVF at the expense of the adipocytes. Our immunohistochemical results emphasized the very low number of CD45+ cells detected per field, and suggest the adipocytes' predominant role as immune regulators within AT. This observation underlines the importance of studying direct interactions between adipocytes and immune cells (83). A second important observation relates to the difference in adipocyte size in the different models—suggesting that the adipocytes have different metabolic profile. It remains to be seen whether these metabolic properties of adipocytes influence the infiltrating immune cells. Finally, these differences in adipocyte size and density also suggest that transcriptomic analyses may be difficult to analyze due to the differences in adipocyte density either between species or between metabolic contexts. Evaluation of cytokine production in AT by SVF cells may be highly affected by the inflammatory status, but masked or minored by AT associated hyperplasia.

Lastly, our observations raise the question of which animal model should be used to study AT. Given that it is not easy to collect large quantities of AT from healthy subjects, the collection of AT from animal models is still an important means of evaluating this tissue's functional properties. Depending on the research question, each model has specific advantages: the mouse model provide access to various knock-out or knock-in system that might be useful for characterizing specific and possibly complex interactions between cell partners in AT. The choice of the B6 background may enable a more specific focus on the Treg compartment and the mechanisms that might modulate and/or preserve the compartment's activity. The striking differences in Treg proportions in the various mouse strains also questioned the physiological role of Tregs accumulation in AT on metabolic homeostasis and susceptibility to obesity. Considering the regenerative potential of AT, the proportion of ASCs differs from one model to another; the low proportion of ASCs in human samples suggest that the mouse or cynomolgus macaque model is more appropriate for studying these cells. However, the ASCs' functional properties need to be properly assessed in each model. The difference in the proportion of ASCs between humans (low) and the mouse and cynomolgus macaque models (high) was a rare example of a discrepancy between cynomolgus macaques and humans. This finding might indicate that ASCs are highly sensitive to environmental pollutants; whereas murine and cynomolgus macaque models are fairly well-protected from environmental pollutants, healthy humans are obviously exposed to various toxics that can accumulate in AT (84, 85). This hypothesis warrants further investigation. The influence of sex on ASCs should also be investigated further. Indeed, ASCs have been used to treat women (but not men) for systemic sclerosis in clinical trials (86)–suggesting that women have larger numbers of ASCs.

In summary, we compared the cell composition of AT in various experimental models (primarily the C57Bl/6 mouse, the cynomolgus macaque NHP, and humans). We observed marked differences in the proportions of immunosuppressive subset profiles (e.g., Tregs) in these models. Although the mechanisms responsible for this discrepancy are unclear, it is clear that the steady-state immune compartment in AT differed markedly in middle-aged male humans vs. mice. This observation has several implications: (i) the steady-state immune composition of human AT may have to be reassessed by considering age- and sex-matched healthy controls; (ii) the high incidence of obesity in humans may be due to the lower immunosuppressive activity of AT, which therefore protects less well against metabolic insults; and (iii) the adipocyte has a central role in the AT's functions, and there is a low degree of interaction between immune cells in non-obese male human AT.

AUTHOR CONTRIBUTIONS

AL, MLV, AD, and JG performed AT dissociation and FACS staining experiments. ST performed the immunohistofluorescence analysis. AL, OL, and SB ensured access to human samples. RH, NB, and RL ensured access to cynomolgus macaques samples. CC, BF, BV, RL, OL, and CB designed the experiments and supervised the analyses. AL and CB wrote the paper. OL and CB supervised the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00117/full#supplementary-material

Supplementary Figure 1 | The proportion of Tregs in AT, as a function of the age of Foxp3-GFP reporter mice. The frequencies of Foxp3+ cells among CD4 T cells in SVF from SCAT (open symbols) and VAT (closed symbols) in male Foxp3-GFP mice aged 2–3, 12, and 18–22 months. Bars represent median values. Statistical significance was determined in a non-parametric Wilcoxon test ($*p < 0.05$).

Supplementary Table 1 | Identification strategy for each subsets in the different species.

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REVIEW
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T Cells in Adipose Tissue in Aging

Antu Kalathookunnel Antony[1], *Zeqin Lian*[1] *and Huaizhu Wu*[1,2]*

[1] *Department of Medicine, Baylor College of Medicine, Houston, TX, United States,* [2] *Department of Pediatrics, Baylor College of Medicine, Houston, TX, United States*

Similar to obesity, aging is associated with visceral adiposity and insulin resistance. Inflammation in adipose tissue, mainly evidenced by increased accumulation and proinflammatory polarization of T cells and macrophages, has been well-documented in obesity and may contribute to the associated metabolic dysfunctions including insulin resistance. Studies show that increased inflammation, including inflammation in adipose tissue, also occurs in aging, so-called "inflamm-aging." Aging-associated inflammation in adipose tissue has some similarities but also differences compared to obesity-related inflammation. In particular, conventional T cells are elevated in adipose tissue in both obesity and aging and have been implicated in metabolic functions in obesity. However, the changes and also possibly functions of regulatory T cells (Treg) in adipose tissue are different in aging and obesity. In this review, we will summarize recent advances in research on the changes of these immune cells in adipose tissue with aging and obesity and discuss their possible contributions to metabolism and the potential of these immune cells as novel therapeutic targets for prevention and treatment of metabolic diseases associated with aging or obesity.

Keywords: adipose tissue, inflammation, T cells, insulin resistance, aging, obesity

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Kjetil Taskén,
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Wenxian Fu,
University of California, San Diego, United States
Meilian Liu,
University of New Mexico Health Sciences Center, United States

***Correspondence:**
Huaizhu Wu
hwu@bcm.edu

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OVERVIEW

The rapidly increasing elderly population worldwide can cause a wide range of implications in healthcare policies of all nations. In the United States, the population aged 65 and over is projected to be 83.7 million by 2050, almost double the estimate of 43.1 million in 2012 (1). Both aging and obesity are associated with low-grade, chronic inflammation that may be detrimental to health. While obesity is triggered by excessive nutrient intake and sedentary lifestyle, aging is caused by deteriorative changes in adult organisms with advancing age. In certain circumstances, aging is termed "inflamm-aging," whereas increased inflammation of adipose tissue in obesity is described as "meta-flammation" or metabolically activated inflammation (2, 3).

DEVELOPMENT OF "INFLAMM-AGING"

Aging is an intricate, dynamic, and physiologic process that adversely affects most body functions, including the development and maintenance of the immune system (4). Basic aging mechanisms such as cellular senescence and diminished number or dysfunction of immune progenitor cells are causative factors of development of low-grade inflammation (5). Immunosenescence is a term to describe the decline of immune function associated with aging, which can lead to increased susceptibility to infections, cancer, and metabolic and autoimmune disorders (6, 7).

During the state of infection or tissue damage in healthy young individuals, the innate immune system, including neutrophils, monocytes, and natural killer (NK) cells, responds quickly. In addition, the adaptive immune system is activated by the action of antigen-presenting cells (APCs), and effector T and B lymphocytes are developed and fight against the insult with a refined antigen-specific immune response. After the effective removal of the invading pathogen, the host immune response must be deactivated and return to a quiescent state to prevent further tissue damage. A subset of T lymphocytes called regulatory T cells (Treg) are responsible for suppressing the deleterious effects of immune response (6). In general, both innate and adaptive immune systems are affected by aging, but adaptive immunity, especially T lymphocytes, are most susceptible to the detrimental effects of aging. The thymus is the key organ orchestrating production of new T lymphocytes, but age-associated chronic involution of the thymus results in a reduced proportion of naïve to memory T cells in the periphery (6, 8). As the number of naïve CD8+ T cells declines with aging, the diversity of naïve and memory T cell receptors (TCRs) is also reduced significantly in mice and humans (9). In parallel, aging represents a striking decline in humoral and cell-mediated responses mainly caused by the senescence of T lymphocytes (10). Hence, gradual deterioration of the immune system over the course of time leads to a mismatch between proinflammatory and anti-inflammatory signals that may disrupt inflammatory homeostasis causing "inflamm-aging."

ADIPOSE TISSUE INFLAMMATION IN AGING

Aging is commonly accompanied by obesity, especially abdominal/visceral adiposity that leads to numerous health problems such as insulin resistance, metabolic syndrome, cardiovascular disease, and disability (11, 12). Adipose tissue functions as the connecting link among nutrition, metabolism, thermoregulation and proper immune system function in healthy individuals (13). Alterations in adipose tissue are major contributors to age-associated metabolic dysfunctions and other health issues (5, 14, 15).There are two types of adipose tissue depots: (1) brown adipose tissue, composed of brown adipocytes, which contain numerous mitochondria and lipid droplets and function as the site of adaptive thermogenesis (16); (2) white adipose tissue, which includes visceral adipose depots and subcutaneous adipose depots and acts as the prime location where metabolic energy is stored in the form of triglycerides during periods of nutritional excess (17). In healthy young individuals, subcutaneous depots act as a metabolic sink where all the excess calories are stored in adipocytes in the form of triglycerides (18). But after the middle age, the ability of subcutaneous fat depots to store lipids declines (19), leading to relocation of excess fat to visceral fat depots, causing visceral adiposity (20). This excessive lipid accumulation in visceral fat depots, along with the surrounding tissue microenvironment, may drive adipose tissue inflammation. Indeed, when compared to subcutaneous adipose tissue, visceral adipose tissue contains more immune cells and plays a more critical role in immunometabolic homeostasis (21). The main immune cell types in visceral adipose tissue include macrophages (ATMs) and T lymphocytes, and other immune cell types which may change in numbers and phenotypes in aging and obesity (22–29). In this review, we focus on changes in T lymphocytes in adipose tissue in aging and the potential roles of adipose tissue T cells in metabolic functions.

CHANGES IN ADIPOSE TISSUE T CELLS IN AGING

The changes in adipose tissue T cells in obesity have been well-documented in mice and humans (24, 30–33). In contrast, information on age-related changes in adipose tissue T cells is limited. Most studies showing the effects of aging on T cells has focused on lymphoid tissues and blood. It has been well-recognized that aging in humans and mice increases the proportion of memory T cells in blood and lymphoid organs (34, 35). Further, T cells in aging tend to polarize to a proinflammatory phenotype, secreting high levels of type 1 cytokines such as IFN-γ, TNF-α, and IL-6 (34, 36–39) and expressing elevated levels of chemokine receptors with enhanced chemotaxis to chemokines (40–42). In humans, one study showed that peripheral blood CD8+ T cells that are positive for IFN-γ, IL-2, and TNF-α are significantly increased with age among all three CD8+ subsets, i.e., naïve, effector/cytotoxic, and memory T cells (38). Another study revealed that intracellular TNF-α and IL-6 levels in blood T cells were significantly increased in the older age (37). This aging-associated elevation of proinflammatory cytokines could be one of the reasons for thymic involution and the reduced proportion of naïve to memory T cells (8). Thymic involution and immune system aging could result in alterations of T cell development, activation, homeostasis, and trafficking in peripheral tissues.

Limited numbers of studies have shown T cell changes in aging adipose tissue; data were generated mainly from mouse models at different ages [from 10 to 15 months ["middle age"] to >18 months ["old age"]]. Aging is commonly associated with visceral obesity and has some similarities but also differences in changes of adipose tissue immune cells, including T cells, when compared to diet-induced obesity (**Figure 1**).

Conventional CD4+ T Cells and CD8+ T Cells

Similar to obesity, aging is associated with significant increases in adipose tissue T cells. Compared to young mice, aged mice (18–22 months old) have ∼2-fold increases in CD3+ T cells in adipose tissue when normalized to tissue weight (43). Both conventional CD4+ T cells and CD8+ T cells are significantly increased, with a greater increase in CD8+ than CD4+ T cells, in visceral fat of aged mice compared to that of young mice (43), which is also similar to the change with obesity (31, 44). The increases in adipose tissue T cells, particularly CD8+ T cells, were also observed in 11- to 16-months-old middle-aged mice (23, 45) and appeared to be influenced by sex, with females having

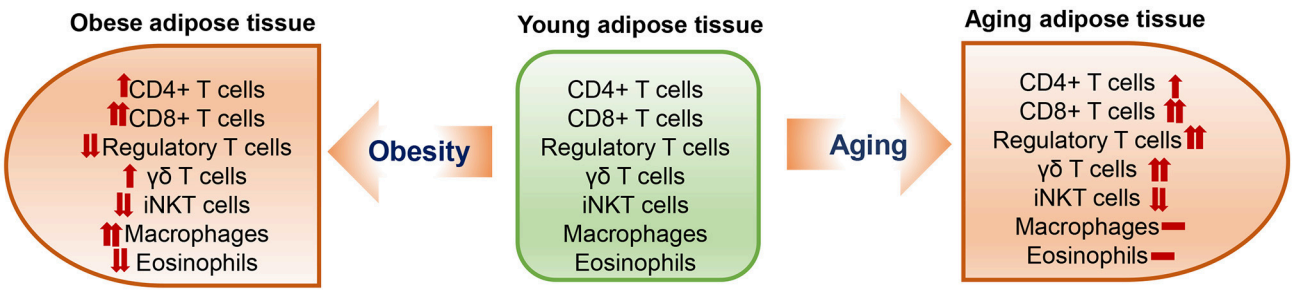

FIGURE 1 | Changes in adipose tissue T cells, macrophages and eosinophils in obesity and aging.

a higher percentage of CD8+ T cells than males (45), in contrast with obesity, in which a greater change in adipose tissue T cells occurred in males than females (24). Furthermore, CD69+-activated and IFN-γ-expressing CD8+ T cells and activated CD4+ T cells are increased in adipose tissue of middle-aged mice (23, 45). These changes are similar to those in obesity (31, 44). However, the age-associated changes in adipose tissue T cells appear to be independent of adiposity (46). The increases in adipose tissue T cells in aging are tissue specific and are not observed in blood or lymphoid organs (43, 45).

Regulatory T Cells (Treg)

Treg normally represent a small portion of CD4+ T cells, and regulate inflammation and prevent autoimmune response mainly by suppressing conventional T cell proliferation and activation (47–49). Compared to lymphoid tissues and blood, adipose tissue is highly enriched with Treg in normal conditions. When compared to their spleen and lymph node counterparts, adipose Treg are a unique population having a specific antigen repertoire and a different transcript profile (30), with overexpression of transcripts encoding transcription factors (e.g., peroxisome proliferator–activated receptor (Ppar)–γ, Gata-3), chemokines or their receptors (e.g., CCR1, CCR2), cytokines or their receptors (e.g., IL10, IL1rl1), and proteins important in lipid metabolism (e.g., Dgat1, Pcyt1a) (50). In diet-induced obesity, adipose tissue Treg are dramatically reduced (30), and this Treg reduction is accompanied by loss of the adipose Treg signature in the remaining Treg population (50). In contrast to obesity, in aging, Treg are elevated in adipose tissue and continuously rise from young age to adult, middle-age and old age in mice (23, 30, 43, 51). Compared to young mice, middle-aged to old mice have 7–11-fold increases in adipose tissue Treg, which account for >50% of total CD4+ T cells in adipose tissue (23, 43). When compared to those in young mice, Treg of aged mice (25 weeks) have substantial increases in a set of transcripts (Ppar-γ, Gata-3, Klrg1, Ccr2, and l1rl1), which continue to increase with aging and may result in local adaptation to the lipophilic, hypoxic adipose tissue environment (50).

Other Immune Cells

In addition to adaptive T lymphocytes, innate T lymphocytes such as γδT cells and invariant natural killer T (iNKT) cells are also located in adipose tissue. Both γδT cells and iNKT cells are resident cells in adipose tissue. While γδT cells tend to be increased (52), iNKT cells are decreased in adipose tissue in obesity (53–55). Similarly, a recent study showed that adipose tissue γδT cells also increased with age in mice from 5 to 28 weeks, whereas adipose tissue iNKT cells decreased significantly with age (51).

It is also worth notice that aging is different from obesity in changes of some other important immune cells in adipose tissue. For example, it is well recognized that macrophages are increased and eosinophils are decreased in obese adipose tissue (25–28, 56). However, available data indicate that the numbers of adipose tissue macrophages and eosinophils show no or only modest changes in aging (23, 43, 46). Nevertheless, adipose tissue macrophages appear to have proinflammatory phenotypes in old mice (43).

FUNCTIONS OF ADIPOSE TISSUE T CELLS

In healthy young humans and mice, various T cell subpopulations harboring in adipose tissue may play pivotal roles in homeostasis and maintenance of immune cells, energy metabolism, and thermogenesis. Changes in adipose T cells in aging and obesity may contribute to adipose tissue inflammation and associated metabolic dysfunctions (**Figure 2**).

Role of T Cells in Adipose Tissue Inflammation

Conventional T cells, including CD4+ Th1 cells and effector CD8+ T cells, are elevated in adipose tissue and may play important roles in adipose tissue inflammation in both aging and obesity (31, 43–46). Conventional T cells are important inflammatory components producing high levels of inflammatory molecules such as IFN-γ, thereby contributing to inflammation. In addition, altered T cells and related inflammatory molecules may contribute to aging- or obesity-related adipose inflammation by influencing other immune cells such as macrophages in adipose tissue (31, 43, 44, 46). Some reports showed that in obesity induced by high-fat diet (HFD), conventional T cell infiltration and accumulation are the primary events and play important roles in the initiation of adipose tissue inflammation and in ATM infiltration and activation (31, 57, 58).

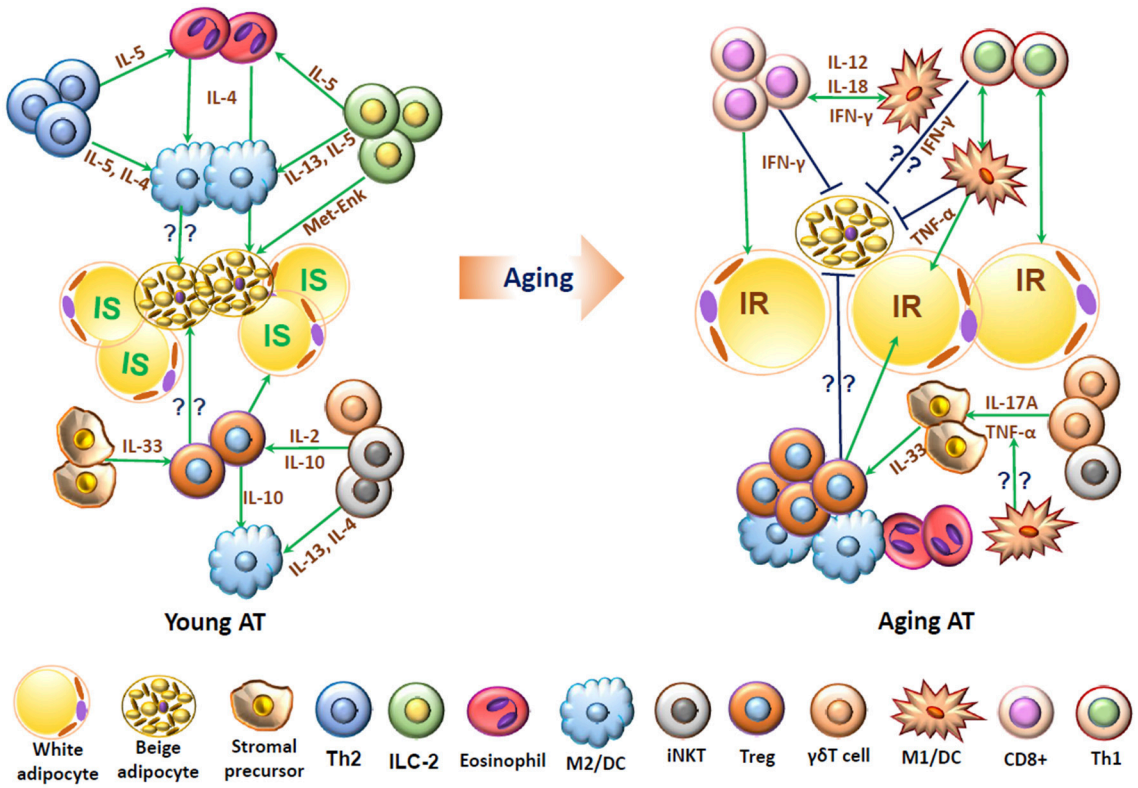

FIGURE 2 | Schematic representation of functions of T cells and associated immune cells in young and aging adipose tissue. In young adipose tissue, insulin-sensitive (IS) white adipocytes, beige adipocytes, and stromal cells are surrounded by "type 2" immune cells, including alternatively activated macrophages (M2), T helper type 2 (Th2) cells, eosinophils, innate lymphoid type 2 (ILC2) cells, regulatory T cells (Treg), and invariant natural killer T (iNKT) cells, which interact with each other and produce type 2 cytokines such as IL-4, IL-5, and IL-13 and may help maintain normal adipose functions, including adipocyte insulin sensitivity and beige fat thermogenesis. In aging, adipose tissue contains increased numbers of T cells including conventional CD4+ cells, CD8+ T cells and Treg and also proinflammatory M1-like macrophages/dendritic cells (DCs), which produce proinflammatory molecules such as IFN-γ and TNF-α and may contribute to adipose dysfunctions such as insulin resistance (IR) and impaired beige fat thermogenesis.

Indeed, combined CD4+ and CD8+ T cell deficiency in obese mice decreased ATMs and reduced adipose tissue inflammation (59). In addition, γδT cells, Vγ4, and Vγ6 subsets in particular, may also contribute to macrophage accumulation and inflammation in adipose tissue in obesity. Deletion of γδT cells or Vγ4/6 prevents obesity-induced macrophage accumulation and inflammation in mice (52). In contrast to conventional CD4+, CD8+ T cells, and γδT cells, Treg are dramatically decreased in adipose tissue in obesity, and expansion of Treg in obese mice protects against adipose tissue inflammation, with decreased ATMs and related inflammatory markers (30, 60). Conversely, depletion of Treg in young mice may increase adipose tissue levels of several inflammatory markers (30). These data suggest a protective role of Treg in obesity-induced adipose tissue inflammation. In contrast, Treg are increased in adipose tissue of aging mice, and depletion of adipose tissue Treg did not significantly enhance systemic and tissue inflammation in aging mice (23).

iNKT cells are enriched in adipose tissue and may play a role in adipose tissue Treg homeostasis by producing IL-2 in young mice (see section Regulatory T Cell Maintenance in Adipose Tissue). However, data are not consistent about the roles of iNKT in adipose tissue inflammation and insulin resistance associated with obesity, which were recently discussed in other review articles (61–63) and are not included in this review.

In addition to their crucial role in visceral adipose inflammation, T cells, conventional CD4+ and CD8+ T cells in particular, also infiltrate into skeletal muscle, mainly localized within intermyocellular and perimuscular adipose tissue, and play substantial roles in skeletal muscle inflammation in obesity (59, 64, 65). Their potential role in aging-related inflammation in skeletal muscle remains to be investigated.

Inflammation has been involved in adipose tissue remodeling (28). In particular, proinflammatory M1-like macrophages have been implicated in adipose tissue remodeling associated with obesity (66). Given the crucial roles of T cells, especially Th1 cells and cytokine IFN-γ, in macrophage M1 polarization(66, 67), T cells may also play a role in adipose tissue remodeling via regulation of macrophage phenotypes. However, a potential direct role of T cells in adipose tissue

remodeling, particularly in relation to aging, remains to be studied.

Roles of Conventional T Cells in Insulin Resistance

Inflammation in adipose tissue has been implicated in insulin resistance and metabolic dysfunctions associated with obesity. Depletion of CD8+ T cells ameliorated systemic insulin resistance, while adoptive transfer of CD8+ T cells aggravated insulin resistance in obese mice, demonstrating a crucial role of CD8+ T cells in systemic metabolic dysfunctions in obesity (31). CD4+ Th1 cells may have similar contributions to obesity-related insulin resistance; reductions in adipose tissue Th1 by ablation of major histocompatibility complex (MHC) class II molecule (MHCII) on adipocytes or ATMs were associated with improved insulin resistance in obese mice (58, 68, 69). Our study showed that combined deficiency of CD4+ and CD8+ T cells in obese mice, with reduced inflammatory status, improved insulin resistance systemically and in adipose tissue as well as in skeletal muscle (59, 65). The mechanisms underlying contributions of proinflammatory T cells (mainly CD4+ Th1 and effector CD8+ T cells) to insulin resistance may include direct adverse effects of these T cells or T cell cytokines such as IFN-γ on metabolic functions and insulin sensitivity in adipocytes or skeletal muscle through the JAK/STAT1 pathway (24, 65, 70) and T cell effects on other immune cells such as macrophages, which also play important roles in metabolic functions including insulin resistance. In contrast to Th1 and effector CD8+ T cells, Th2 cells, which produce type 2 cytokines such as IL-4 and IL-5, may protect against obesity and related insulin resistance; transfer of CD4+ T cells reverses weight gain and insulin resistance in HFD-fed lymphocyte-free mice, mainly through polarization into Th2 cells (32).

Aging is commonly associated with insulin resistance and increased prevalence of metabolic syndrome in most populations. Given the massive increases of CD8+ T cells and conventional CD4+ T cells in aging adipose tissue (43) and the discussed roles of T cells in metabolic functions, it is plausible that adipose tissue T cells may also contribute to age-related metabolic dysfunction and insulin resistance (5, 9). However, more elaborate studies are needed to unveil aging-related changes in the phenotypes of adipose tissue T cells and the exact roles of adipose tissue T cells in age-associated metabolic functions.

Role of Treg in Metabolic Function

Visceral adipose tissue of lean mice contains more Treg cells than that of obese counterparts (30). Possible functions of Treg in lean adipose include monitoring the activity of conventional T cells and regulating proper functioning of neighboring macrophages and adipocytes (30). Gain-of-function and loss-of-function approaches demonstrated that Treg play a protective role in insulin sensitivity and energy homeostasis in obesity (30, 60). Treg may improve insulin sensitivity through the release of anti-inflammatory molecules such as IL-10 and TGF-β that may counteract the proinflammatory signals in both humans and rodents (71, 72). Consistent with these findings, Deng et al. also showed that maintenance of adipose Treg in obese mice with adipocyte-specific deletion of MHCII was associated with improved insulin resistance (69). In contrast to obesity, in aging adipose tissue, Treg undergoes significant expansion. Depletion of adipose Treg was found to be protective against age-associated metabolic dysregulation. Aging mice with depletion of adipose Treg exhibited increased insulin sensitivity compared to control mice (23), suggesting that Treg in adipose tissue may play a detrimental role in age-associated insulin resistance. The inflammatory status of mice with adipose Treg depletion did not change significantly compared to control mice. Although the mechanisms whereby adipose Treg contribute to age-related insulin resistance remain to be investigated, it seems likely that the pathophysiological mechanisms that regulate age-associated insulin resistance and obesity-induced insulin resistance may be different (23, 30).

Roles of T Cells in Thermogenesis

Adipose tissue is one of the key organs responsible for whole body energy homeostasis via energy storage/dissipation depending on nutrient intake and external temperature fluctuations (73). Beige adipocytes, which may develop within white adipose depots, particularly in subcutaneous adipose depots, have a similar energy dissipation function as that of brown adipocytes, which is mainly induced by cold and beta-adrenergic activation (74–76). Innate and adaptive immune system components are reported to contribute to and regulate the energy storage/dissipation functions of adipose tissue (77–79). Recent reports suggest that along with macrophages, T cells may play a significant role in the regulation and maintenance of thermogenesis and overall adipose tissue energy homeostasis.

Th2 cells and associated type 2 immune cell populations such as innate lymphoid type 2 (ILC2) cells and eosinophils in young lean adipose tissue have a significant role in defining a favorable adipose niche for beige adipocyte development and thermogenesis (80). The major cytokines produced by Th2 cells, eosinophils, and ILC2 cells during type 2 immune response are IL-4, IL-5, and IL-13 (81, 82), which may promote the proliferation and differentiation of PDGFRα+ adipose stromal precursor cells to thermogenic beige adipocytes and therefore help to maintain thermogenesis in young lean conditions (83). In addition, type 2 cytokines and eosinophils are essential factors for the differentiation and propagation of alternatively activated M2 macrophages (56), which may play a role in inducing adipose thermogenesis by producing catecholamine (77). However, a more recent study by Fischer et al did not show a role of M2 macrophages in inducing adipose thermogenesis (84).

In addition to Th2 cells, PLZF+ γδT cells, and iNKT cells may also contribute to induction of adipose thermogenic function (51, 85, 86). Adipose-residing γδT cells are important for the preservation of body temperature and thermogenic function, possibly by producing IL-17A. Under cold challenge, mice deficient in either γδT cells or IL17A have reduced UCP-1 expression and are unable to survive (51). In obesity, activation of iNKT cells induces adipose thermogenesis, leading to weight loss, in mice, likely through induction of FGF21 (86). The action

of FGF21 in white adipose tissue is implicated by the activation of PGC1α along with induction of adiponectin, resulting in improved energy expenditure (87, 88).

In aging, the functional beige adipocytes decline, as the number of fully active beige adipocytes in human and mice depend on the whole body metabolic fitness (89–91). How changes in adipose tissue immune cells contribute to age-related decline in adipose thermogenic functions remain largely unknown. Depletion of adipose Treg in mice reduced age-associated weight gain and adiposity, with enhanced energy expenditure (23), indicating a role of adipose Treg in age-related energy metabolism. Recently, Moysidou et al. demonstrated an inhibitory effect of CD8+ T cells on adipose thermogenesis in mice, possibly by secreting IFN-γ, which may have direct effects on thermogenesis or interfere with the effects of other immune cells, such as eosinophils and ILC2 cells, on thermogenesis (79). It is possible that conventional CD4+ T cells, particularly Th1 cells, have similar functions in adipose thermogenesis because of IFN-γ expression (79). Based on the elevations in CD8+ and conventional CD4+ T cells in aging adipose tissue, it is reasonable to hypothesize that these immune cells may play a role in age-associated decline in adipose thermogenic functions and energy expenditure. However, this hypothesis remains to be tested.

MECHANISMS FOR CHANGES IN ADIPOSE TISSUE T CELLS

Regulatory T Cell Maintenance in Adipose Tissue

Treg are resident cells in adipose tissue (30). These adipose Treg in mice are seeded from the thymus during an early stage of life and expand within adipose with aging (23, 43, 92). Recently, Li et al. showed that immature Treg from the thymus undergo a priming step in the spleen prior to infiltration into adipose tissue, which may permit them to leave lymphoid organs and to survive in non-lymphoid organs, including adipose tissue (93). While iNKT cells, a type of lipid-sensing innate T cells, may assist in regulating adipose tissue Treg number and function in young mice by producing IL-2 (85), two factors, i.e., interaction of TCR-antigen-MHCII on APCs and cytokines such as IL-33, may be the main drivers of visceral adipose Treg accumulation in aging (92). Treg in aging adipose tissue express high levels of ST2, a receptor for IL-33, and IL-33 efficiently induces Treg differentiation and expansion in aging visceral adipose tissue (23, 93, 94). Recently, a subpopulation of γδT cells termed PLZF+ γδT cells was demonstrated to play a considerable role in age-related adipose Treg accumulation via producing IL-17A, which induces stromal cell production of IL-33 in adipose tissue (51). Two subpopulations of APCs have been identified in aging mouse visceral adipose tissue—MHCII+CD11b+CD11c+ macrophages and MHCII+CD11b-CD11c+ dendritic cells—both of which were colocalized with Tregs and may play important roles in Treg maintenance within adipose tissue in aging, possibly via the TCR-antigen-MHCII interaction (92). Importantly, PPAR-γ, the master regulator of adipocyte differentiation, has been shown to be a crucial molecular driver for Treg cell accumulation and function in visceral adipose tissue (95). In obesity, Th1 inflammation mediated by obese adipocyte- or macrophage-expressed MHCII may contribute to the reduction in adipose Treg in diet-induced obesity via producing IFN-γ, which blocks the effects of IL-33 on Treg proliferation. Adipocyte-specific deletion of MHCII prevents diet-induced adipose inflammation and Treg reduction (69). In addition, influx of inflammatory macrophages, release of inflammatory cytokines and imbalance of adipokines in obesity may restrict the survival of adipose tissue Treg by modulation of the adipose tissue microenvironment (30, 67, 96).

Conventional T Cell Infiltration Into Adipose Tissue

Infiltration or migration of T cells into lymphoid organs or peripheral tissues is tightly and specifically regulated by collective effects of various adhesion molecules and chemokines/receptors (97–99). Several reports explained the mechanisms for infiltration of conventional T cells into adipose tissue and the roles of adhesion molecules and chemokines/receptors under obese conditions, but few reports are available for those related to aging. Using mouse models of obesity, our group observed that CD11a, a β2 integrin that is highly expressed on T cells, is upregulated in obesity and plays a crucial role in CD8+ T cell infiltration in adipose tissue in obese mice (44). In addition, dysfunctional, damaged or necrotic adipocytes and immune cells, including T cells, can secrete chemokines that may accelerate lymphocyte homing into adipose tissue (100). In our earlier study, we observed that RANTES, a CC chemokine (CCL5), and its receptor, CCR5, were upregulated in adipose tissue of obese mice and humans and that RANTES was colocalized with T cells within mouse adipose tissue. Our *ex vivo*/*in vitro* studies indicated that RANTES is an adipokine that can be produced by adipocytes and plays an important role in T cell migration, suggesting a potential role of the RANTES/CCR5 axis in adipose T cell accumulation in obesity (24). Another report showed that the preadipocyte- and endothelial cell-derived stromal-derived factor-1α (CXCL12), mediated early infiltration of CD4+ T lymphocytes in obesity, which preceded the increase of macrophages in adipose tissue of mice on HFD (101). In obese humans, adipocyte-secreted CCL20 may contribute to the accumulation of CD4+ helper and CD8+ cytotoxic T lymphocytes within adipose tissue, possibly via interaction with CCR6 that was upregulated on T cells in obese adipose tissue (100). However, the key molecules that mediate T cell infiltration into adipose tissue in aging remain to be identified.

Activation of Conventional T Cells in Adipose Tissue

CD4+ T Cell Activation

TCRs identify the presence of a specific antigen by binding to short peptide sequences from the antigen that is displayed on APCs. These short peptide sequences from the antigen are usually presented on the cell surface of APCs with the help of

MHCII molecules, which are crucial for activation of CD4+ T cells (102). Classically, naïve CD4+ T cells become activated and differentiated to effector T cells by three signals: signal 1, interaction of TCR with a peptide antigen-MHCII complex carried by APCs; signal 2, costimulatory signals such as CD28 and cytotoxic T lymphocyte antigen (CTLA) expressed on T lymphocytes and their ligands CD80 and CD86 expressed on APCs; and signal 3, cytokines such as IL-12, TGF-β, and IL-10 secreted by APCs and Treg (29, 58). Deng et al. reported that both visceral and subcutaneous adipocytes from obese humans and mice expressed all MHCII components required for antigen presentation and increased levels of CD80 and CD86, and may therefore function as APCs. Indeed, the primary adipocytes isolated from obese mice could induce antigen-specific CD4+ T cell activation (58). Xiao et al. further described that mostly large adipocytes from obese adipose tissue exhibited an elevated expression level of MHCII molecules and acted as APCs to activate CD4+ T cells to secrete IFN-γ (103). In the early stage of obesity induced by HFD, elevated free fatty acids may be the initial stimulus for adipocyte hypertrophy and MHCII-related gene upregulation, possibly via activation of JNK and STAT1, which may further activate CIITA, a prime regulator of MHCII expression (103, 104). As obesity progresses, free fatty acids may act synergistically with IFN-γ to upregulate MHCII on adipocytes. Studies by Morris and Cho et al. indicated that ATMs colocalized with T cells in lymphoid clusters within adipose tissue and may act as APCs, which express high levels of MHCII and also costimulatory molecules and process and present antigens to induce CD4+ T-cell proliferation and activation in adipose tissue of obese mice (29, 68, 105). Taken together, one important mechanism for obese adipose CD4+ T cell activation may be mediated through MHCII expressed on ATMs and adipocytes. However, its role in aging-related adipose tissue CD4+ T cell activation remains to be investigated.

CD8+ T Cell Activation

Compared to CD4+ T cells, CD8+ T cells show a greater increase in adipose tissue in obesity and in aging (31, 43, 106). Similar to CD4+ T cells, CD8+ T cells exhibit effector memory or effector phenotypes expressing elevated levels of IFN-γ in obese adipose tissue (31, 44). The mechanism for CD8+ T cell activation in adipose tissue is not fully understood. Nishimura et al. showed that adipose tissue from obese mice induced proliferation of splenic CD8+ T cells, indicating a CD8+ T cell-activating environment in obese adipose tissue (31). In addition to a role in adaptive immunity, memory CD8+ T cells are involved in innate immunity, being able to become activated and to proliferate under cytokine stimulation (107, 108). Indeed, CD8+ T cells from mouse adipose tissue respond to cytokines and become activated and proliferate under stimulation of IL-12 and IL-18, which are mainly produced by APCs and are elevated in obese adipose tissue (44). Results from a CD11a-knockout mouse model revealed that CD11a also plays a pivotal role in adipose CD8+ T cell trafficking, proliferation, accumulation and activation (44).

In parallel to the changes in adipose CD8+ T cells in obesity, aging is reported to accelerate accumulation of CD8+ T cells in adipose tissue, which may contribute to increased adipose inflammation. However, the mechanisms for the aging-related changes in adipose tissue CD8+ T cells remain unknown. From the above discussion regarding the impact of immune system aging on T cell homeostasis and phenotypes in lymphoid organs and peripheral blood, it is reasonable to hypothesize that immune system aging may contribute to the changes in adipose tissue T cells and inflammation associated with age.

CONCLUSIONS AND PERSPECTIVES

Similar to obesity, aging is associated with visceral adiposity and metabolic dysfunctions, including insulin resistance. Numerous studies have investigated the potential mechanisms and functions of various subpopulations of adipose T cells in obesity and related metabolic complications. Limited reports have also shown expansion of T cells, including conventional T cells and Treg, in adipose tissue in aging. However, little is known about the mechanisms of adipose T cell accumulation and their role in metabolic diseases associated with aging. Hence, future studies will need to address mechanisms and functions of adipose T cell populations in aging. In particular, some key questions need to be addressed. First, do the changes in adipose tissue T cells observed in aging mice also occur in humans? Second, what are the major factors that drive accumulation and phenotypes of various types of T cells in adipose tissue in aging? Third, why do adipose Treg function differently in age- and obesity-associated insulin resistance? Fourth, how do other T cell subpopulations, conventional T cell populations in particular, contribute to age-related metabolic disease? Finally, and most importantly, will targeting immune cells and inflammation be practical and beneficial in preventing and treating age-related metabolic disease?

In recent years, some clinical trials have illustrated the potential of targeting inflammation with pharmacological agents to treat metabolic diseases. Improvements of glucose metabolism and β-cell function and reduction of HbA1c were reported in diabetic patients after treatment with anakinra, a recombinant analog for IL-1Ra that blocks the action of the inflammatory cytokine IL-1β (109, 110). In another study, a selective JAK1/JAK2 inhibitor, baricitinib, was found to be effective in treating diabetic kidney disease and also lowering HbA1c in patients with type 2 diabetes and diabetic nephropathy (111, 112). Cenicriviroc, an oral dual chemokine receptor CCR2/CCR5 antagonist, was recently shown to ameliorate insulin resistance, hepatic inflammation and fibrosis in obese humans and mice with non-alcoholic steatohepatitis (113, 114). However, to date, inflammation-targeting therapies have not been very successful in treating metabolic diseases, particularly in humans. Further, because of the chronic nature of most metabolic diseases, the potential side effects (vs. benefits) of long-term use of inflammation-targeting drugs need to be evaluated. Nevertheless, further advances in our understanding

of the roles and mechanisms of inflammation in metabolic diseases may open up novel avenues for the discovery of newer classes of pharmacological targets/agents for diabetes treatment, which may also provide novel opportunities for prevention and treatment of age-associated metaboli disease.

AUTHOR CONTRIBUTIONS

HW contributed to manuscript initiation and revision. AK and ZL contributed to manuscript writing.

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REVIEW
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Adipose Tissue T Cells in HIV/SIV Infection

Celestine N. Wanjalla[1,2], *Wyatt J. McDonnell*[1,2,3] *and John R. Koethe*[1,2*]

[1] Division of Infectious Diseases, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, United States, [2] Center for Translational Immunology and Infectious Disease, Vanderbilt University Medical Center, Nashville, TN, United States, [3] Department of Pathology, Microbiology, and Immunology, Vanderbilt University, Nashville, TN, United States

Adipose tissue comprises one of the largest organs in the body and performs diverse functions including energy storage and release, regulation of appetite and other neuroendocrine signaling, and modulation of immuity, among others. Adipocytes reside in a complex compartment where antigen, antigen presenting cells, innate immune cells, and adaptive immune cells interact locally and exert systemic effects on inflammation, circulating immune cell profiles, and metabolic homeostasis. T lymphocytes are a major component of the adipose tissue milieu which are altered in disease states such as obesity and human immunodeficiency virus (HIV) infection. While obesity, HIV infection, and simian immunodeficiency virus (SIV; a non-human primate virus similar to HIV) infection are accompanied by enrichment of $CD8^+$ T cells in the adipose tissue, major phenotypic differences in $CD4^+$ T cells and other immune cell populations distinguish HIV/SIV infection from obesity. Furthermore, DNA and RNA species of HIV and SIV can be detected in the stromal vascular fraction of visceral and subcutaneous adipose tissue, and replication-competent HIV resides in local $CD4^+$ T cells. Here, we review studies of adipose tissue $CD4^+$ and $CD8^+$ T cell populations in HIV and SIV, and contrast the findings with those reported in obesity.

Keywords: HIV—human immunodeficiency virus, SIV–simian immunodeficiency virus, obesity, adipose tissue, immunology, inflammation, stromal vascular fraction

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University of Texas Health Science Center at Houston, United States

***Correspondence:**
John R. Koethe
john.r.koethe@vumc.org

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INTRODUCTION

The adipose tissue stromal vascular fraction (SVF) contains a diverse range of innate and adaptive immune cells, which interact in a complex paracrine signaling environment that affects adipocyte energy storage and other functions. T lymphocytes, or T cells, are a major component of the adipose tissue environment recruited from the circulatory and lymphatic systems, and these T cell populations undergo marked changes with progressive adiposity in overweight and obese persons. More recently, a handful of studies have identified substantial changes in adipose tissue T cell density and cellular characteristics in human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV; a non-human primate virus similar to HIV) infection, including shifts in total $CD4^+$ and $CD8^+$ T cells and subsets, cytokine production, antigen specificity, interactions with adipocytes and other SVF cells, and capacity for latent infection with HIV or SIV proviruses (1–6). These studies, and their principal findings, are described in **Table 1**.

In general, mammals have predominantly white adipose tissue in their viscera and subcutaneous fat depots. While the accumulation of visceral adipose tissue (VAT) generally has greater detrimental effects on metabolic health, T cell subsets in subcutaneous adipose tissue (SAT) and VAT appear similar from an immune perspective. Paired SAT

and VAT samples from SIV-infected macaques showed no statistically significant differences in the percentage of $CD4^+$ T cells, activated HLA-DR^+ T cells, or $CD69^+$ memory T cells (2). Furthermore, paired SAT and VAT samples from 47 surgical patients showed close correlation between the percentage of $CD8^+$ cells ($r = 0.90$, $p < 0.01$), $CD4^+$ cells ($r = 0.90$, $p < 0.01$), T_H17 cells ($r = 0.75$, $p = 0.01$), and T_H1 cells ($r = 0.67$, $p < 0.04$) (8).

In contrast to SAT and VAT, brown fat is mainly supraclavicular, paravertebral and suprarenal (9–11). While white adipose tissue primarily functions as an energy store, brown adipocytes have more mitochondria and are involved in energy expenditure and thermogenesis. The latter may replace white adipocytes after thermogenic stimulation (12). Beige adipocytes are a third group that demonstrate a functional resemblance to brown adipocytes. They contain high levels of mitochondria and may be derived from white adipocytes (13, 14). Obese persons have less brown adipose tissue compared to their lean counterparts, and brown adipose tissue generally contains fewer immune cells compared to white adipose tissue. These distinctions of function and location are important to contextualize studies on the role of the immune system in adipose tissue. At present, the majority of studies of adipose tissue T cells in HIV and SIV are representative of white adipose tissue physiology from the SAT and VAT compartments.

An enrichment of adipose tissue $CD8^+$ T cells and an increase in the CD8:CD4 ratio accompanies HIV and SIV infection, which is a phenomenon also observed in obesity. However, adipose tissue changes in HIV should not be considered "equivalent" to obesity, as marked differences in $CD4^+$ T cell and macrophage profiles are present in the two conditions. It is thought that several mechanisms drive both $CD8^+$ T cell enrichment and the shifts in T cell distribution in obesity. Several chemokines are detected in obese adipose tissue, including CXCL10, CXCL8, CCL5, and CCL2 (15–17). At present, there is a paucity of data on chemokine receptor expression on adipose tissue T cells, though these T cells can infiltrate inflamed adipose tissue via chemotactic recruitment by CCL5/RANTES and interaction with CXCR4 and CCR5 (18). Notably, CCL20 expression by human adipocytes is higher in obese individuals (19). Finally, when discussing adipose tissue immunology in HIV infection, it is paramount to consider the impact of HIV DNA and RNA in the local environment on T cell subset profiles and cellular function.

ADIPOSE TISSUE T CELL CHANGES IN HIV/SIV

Increase in the Adipose Tissue CD8:CD4 T Cell Ratio in HIV and SIV

One of the first studies of T cells in the SAT and VAT of persons living with HIV (PLWH), by Couturier et al., identified major differences in $CD4^+$ and $CD8^+$ T cell populations compared to HIV-negative controls (1). Similar findings were subsequently reported in other HIV and SIV studies (2, 4, 6). Adipose tissue was collected from 3 living and 2 deceased PLWH, and 4 healthy controls. Cells within the SVF were isolated by collagenase digestion, separated by Ficoll gradient, and analyzed by flow cytometry. The adipose tissue SVF $CD3^+$ T cells were predominantly memory $CD4^+$ $CD45RO^+$ T cells (61%) in the HIV-negative controls, with fewer memory $CD8^+$ T cells (15%). Furthermore, the proportion of memory $CD4^+$ T cells in adipose tissue of healthy controls was ∼50% higher compared to blood (1). In contrast, this distribution was reversed in PLWH, with more adipose tissue memory $CD8^+$ T cells (46%) than memory $CD4^+$ T cells (35%), which represented an ∼50% enrichment in memory $CD8^+$ T cells over the blood and could not be attributed to depletion of circulating $CD4^+$ T cells. **Figure 1** summarizes the major differences in $CD8^+$ and $CD4^+$ T cell profiles in adipose tissue and blood from HIV/SIV-infected subjects vs. HIV/SIV-negative controls (described in further detail below).

A study of 10 PLWH on the same antiretroviral therapy (ART) regimen (efavirenz, tenofovir, emtricitabine) with long-term virologic suppression by Koethe et al. found a higher proportion of total $CD8^+$ T cells in SAT compared to blood (6). This study was limited by a lack of a HIV-negative comparison group. SAT samples were obtained by liposuction biopsies and processed by collagenase digestion and Ficoll separation similar to Couturier et al. (1). SAT was enriched in $CD8^+$ T cells (61%) over $CD4^+$ (33%), as compared to 52 and 43% in the blood, respectively. Memory T cell proportions, defined as $CD45RO^+$ $CD8^+$ and $CD4^+$ T cells, were not significantly different between SAT and blood.

As observed in PLWH, cynomolgus macaques with SIV infection have a higher percentage of $CD8^+$ T cells, and lower $CD4^+$ T cells, in both the SAT and VAT compared to non-infected animals (2, 3). In a study by Damouche et al. the proportion of $CD8^+$ T cells was significantly higher in the SAT and VAT of SIV-infected macaques (2). Correspondingly, $CD4^+$ T cell percentages were significantly lower (18% in SAT and 20% in VAT) compared to non-infected animals (40% in both SAT and VAT). Notably, this study demonstrated that the change in the CD8:CD4 ratio was not driven by a reduction in the total number of $CD4^+$ T cells in infected animals. Rather, SIV-infected animals had significantly higher density of $CD8^+$ T cells in VAT and a somewhat higher density in SAT (2). For both non-infected healthy and SIV-infected monkeys, nearly all of the adipose tissue $CD4^+$ and $CD8^+$ T cells were memory T cells (>94% $CD95^+$), with a large fraction of activated cells marked by expression of $CD69^+$ (62–84%) and $CD25^+$ (3–13%). This seemingly CD8-mediated inversion of the typical $CD4^+$ and $CD8^+$ T cell ratio was also noted in PLWH in a separate study by Damouche et al. (4).

In a second study of macaques, a higher adipose tissue CD8:CD4 ratio was observed in animals infected with SIV 4-weeks prior (ratio 1.51) compared to non-infected animals (ratio 1.38) (3). Interestingly, the CD8:CD4 ratio was similarly elevated (1.59) in non-infected animals with chronic enterocolitis, suggesting chronic illness or inflammation may affect adipose $CD8^+$ and $CD4^+$ T cell trafficking independently of viral infection. Gene expression profiling on the adipocyte enriched floating fraction from SIV-infected animals showed higher levels of CCL19, interleukin (IL)-2, and IL-7, which may contribute to T cell homing and survival (3). Additional studies are needed

TABLE 1 | Summary of studies on adipose tissue T cells and the viral reservoir in HIV and SIV.

References	HIV/SIV	Subjects	Principal T cell findings	Principal viral reservoir findings
Couturier et al. (1)	HIV	4 ART-treated HIV+ humans (3 alive, 1 cadaver) 1 ART-naïve HIV+ cadaver	• Higher CD8:CD4 ratio in AT compared to blood • AT $CD4^+$ and $CD8^+$ T cells predominantly $CD69^+$ memory cells	• *Gag* and *Env* proviral DNA detected by nested PCR • Co-culture of latently HIV-infected $CD4^+$ T cells with adipocytes and IL-2, IL-7, or IL-15 increased T cell activation and HIV production
Damouche et al. (2)	HIV and SIV	13 ART-treated, VL suppressed HIV+ humans 23 SIV+ cynomolgus macaques 21 SIV-negative cynomolgus macaques	HIV+: • none- study primarily assessed viral reservoir SIV^+: • Higher adipose tissue CD8:CD4 ratio in SIV+ vs. SIV-negative • Higher $CD4^+$ and $CD8^+$ T cell HLA-DR (activation) expression in SIV+ vs. SIV-negative • $CD4^+$ and $CD8^+$ predominantly $CD95^+$ memory cells in AT • $CD4^+$ predominantly $CD69^+$ in AT (although co-expression of CD69 on memory T cells was not assessed) • Similar memory T cell subset distribution in SIV+ and SIV-negative • Higher inflammatory macrophages in SIV+ vs. SIV-negative	HIV: • HIV DNA detected in total SVF cells and sorted adipose tissue $CD4^+$ T cells • Level of HIV DNA in $CD4^+$ T cells similar to blood • HIV RNA detected by *in situ* tissue hybridization and after *in vitro* reactivation of $CD4^+$ T cells SIV: • SIV DNA present in SVF and in sorted $CD4^+$ T cells and macrophages • SIV RNA detected by *in situ* tissue hybridization and in $CD4^+$ T cells and macrophages
Couturier et al. (3)	SIV and SHIV	8 SHIV-SF162p3-infected rhesus macaques (acute) 8 SIVmac251-infected macaques (chronic) 7 non-infected macaques	• Higher adipose tissue CD8:CD4 ratio in SHIV+ vs. SHIV-negative • $CD4^+$ and $CD8^+$ predominantly $CD95^+$ $CD69^+$ memory cells • Similar levels of NKT cells in SHIV+ compared to SHIV-negative	• Infectious SIV inducible from SVF CD4 T cells;
Damouche et al. (4)	HIV	11 ART-treated HIV+ humans 19 HIV-negative humans	• Higher adipose tissue CD8:CD4 ratio in SAT, but not VAT, from HIV+ vs. HIV-negative • Higher proportion of Treg cells in SAT from HIV+ vs. HIV-negative • No difference in $CD4^+$ Th1, Th2 and Th17 cell proportions in HIV+ vs. HIV-negative • No difference in $CD4^+$ expression of Ki-67 or HLA-DR in HIV+ vs. HIV-negative • Higher $CD4^+$ and $CD8^+$ expression of PD-1 in AT vs. blood from HIV+ subjects • AT $CD4^+$ and $CD8^+$ T cells predominantly $CD69^+$ cells	• None—study primarily assessed $CD4^+$ T cell subsets
Hsu et al. (5)	SHIV	6 SHIV+ rhesus macaques	• Increase in adipose tissue $CD4^+$ cells after SHIV infection ($CD8^+$ not reported)	• Lower SHIV RNA in SAT compared to rectum and lymph node • SHIV RNA levels similar in SAT and VAT
Koethe et al. (6)	HIV	10 ART-treated, VL suppressed HIV+ humans	• Higher CD8:CD4 ratio in adipose tissue vs. blood • Higher $CD8^+$ expression of HLA-DR and CD57 in adipose tissue • Higher $CD8^+$ TCRβ clonality in adipose tissue; distinct TCRβ V and J gene usage • AT $CD4^+$ and $CD8^+$ T cells exhibited limited CD69 expression	• Level of HIV DNA in adipose tissue $CD4^+$ T cells similar to blood
Couturier et al. (7)	HIV	8 HIV-infected AT specimens	• HIV-infected $CD4^+$ T cells cultured with adipocytes and ART had suppressed viral replication and increased survival • After 7 days $CD4^+$ $p24^+$ T cells cultured with adipocytes had higher CD69 expression (~8–12%) compared to <2% $CD69^+$ $p24^+$ T cells if cultured in media alone	• $CD4^+$ T cells from AT-SVF of 2/3 individuals produced infectious virus in an outgrowth assay • NNRTIs were less detectable in AT cells likely because they are hydrophilic. Dolutegravir was found in higher intracellular concentrations in AT

ART, antiretroviral therapy; AT, adipose tissue; HIV, human immunodeficiency virus; SAT, subcutaneous adipose tissue; SHIV, simian-human immunodeficiency virus; SIV, simian immunodeficiency virus; SVF, stromal vascular fraction; TCR, T cell receptor; VAT, visceral adipose tissue.

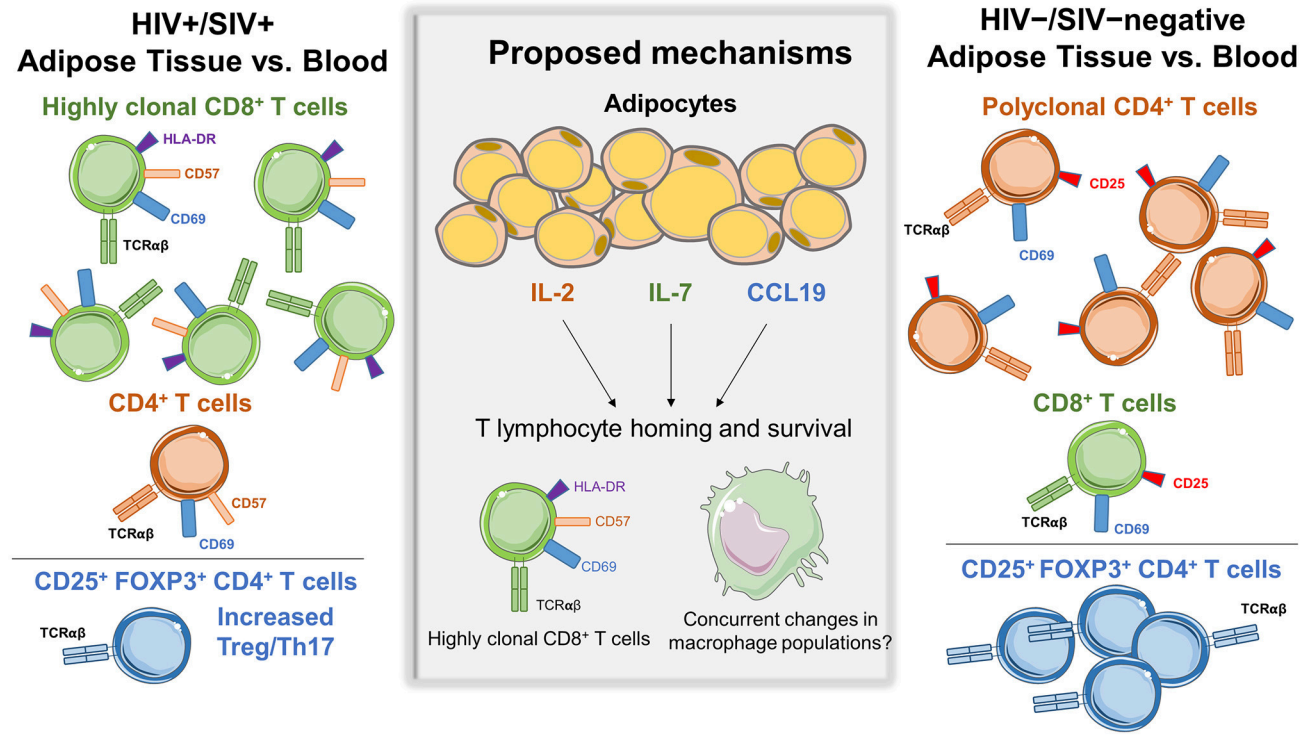

FIGURE 1 | Differences in adipose tissue and blood CD4$^+$ and CD8$^+$ subsets in HIV/SIV+ vs. HIV/SIV-negative subjects. Adipose tissue in HIV/SIV+ subjects is enriched in CD8$^+$ T cells compared to blood, while HIV/SIV-negative adipose tissue is enriched in CD4$^+$ T cells. A proposed mechanism is the preferential recruitment of cells from circulatory/lymphatic systems via differences in cytokine expression by adipocytes and adipose tissue stromal vascular fraction cells in HIV/SIV+ including IL-2, IL-6, IL-7, IL-8, CCL19, IL-12p40, and MIP-1α, eotaxin and interferon gamma. While a study in HIV+ subjects found increased CD8$^+$ T cell clonality in adipose tissue, there is currently not compelling evidence of *in situ* CD4$^+$ or CD8$^+$ T cell proliferation within adipose tissue.

to replicate these recent reports and elucidate a mechanism for enrichment of CD8$^+$ T cells in adipose tissue of PLWH and SIV-infected macaques.

CD57, CD69, HLA-DR and PD-1 Expression on Adipose Tissue T Cells

Recent studies have assessed CD57, CD69, HLA-DR, and PD-1 expression on T cells in the blood and adipose tissue of PLWH and SIV-infected macaques, and as compared to non-infected controls. Koethe et al. examined expression of CD57 on adipose tissue T cells from PLWH on long-term ART, as CD4$^+$ and CD8$^+$ T cell CD57 expression in blood is higher in PLWH compared to HIV-negative persons (20–22). CD57 is a terminally sulfated glycan carbohydrate epitope found on T cells and natural killer (NK) cells. CD57 is a marker of late differentiation, though there is limited consensus as to whether CD57 expression implies reduced replicative capacity, an inability to proliferate in response to antigen stimulation, or increased susceptibility to activation-induced apoptosis (20, 23, 24). CD8$^+$ T cells expressing CD57 produce more interferon-γ and tumor necrosis factor-α (TNF-α) after T cell receptor (TCR) stimulation compared to CD8$^+$CD57$^-$ T cells, and demonstrate a distinct gene expression profile characterized by greater cytotoxic effector potential (e.g., production of perforin, granzymes, and granulysin) (25, 26). A higher percentage of CD57$^+$ T cells is also implicated in several inflammatory diseases (27–29).The proportion of CD8$^+$ T cells expressing CD57 was significantly higher in SAT compared to blood (37 vs. 23%, respectively) from PLWH, and activated CD8$^+$ T cells expressing HLA-DR were over six-fold higher in SAT compared to blood (5.5 vs. 0.9%, respectively).

A notable difference between the Couturier and Koethe studies was the reported expression of CD69 on adipose tissue CD8$^+$ and CD4$^+$ T cells. CD69 is an inducible, early-activation indicator which serves as a putative tissue-resident marker on memory T cells in human mucosal and lymphoid tissues (30–32), but is largely absent on circulating blood memory T cells (31). In a study by Couturier et al., surface expression of the CD69 activation marker on adipose tissue memory CD4$^+$ and CD8$^+$ T cells was 67 and 60%, respectively, in the PLWH and 72 and 61%, respectively, in the HIV-negative, compared to <5% in the blood. In contrast, Koethe et al. found overall expression of CD69 was low (<5%) on both adipose tissue CD8$^+$ and CD4$^+$ T cells (though still higher than in blood), but among adipose tissue memory CD4$^+$ T cells CD69 expression was 20-fold higher compared to blood (5.9 vs. 0.3%). The reason for this disparity is unclear and could include differences in CD69 antibody binding, time-related increases in CD69 expression in adipose tissue samples collected after death or by surgical resection as opposed to rapid T cell extraction from liposuction

aspirates, an artifact introduced by collagen digestion or other aspects of processing, or greater residual contamination of liposuction aspirates by peripheral blood. Additional studies are needed to assess CD69 expression with different experimental methods, though a study comparing activation markers on adipose tissue T cells in lean, overweight and obese individuals using aspiration via a 14 G needle found ∼5–10% CD69 expression on $CD4^+$ T cells and ∼25% expression on $CD8^+$ T cells (33).

In a study by Damouche et al., expression of the HLA-DR activation marker on $CD8^+$ and $CD4^+$ T cells was significantly higher in SAT, VAT, and blood from SIV-infected macaques compared to non-infected animals (2). However, the authors did not observe a significant difference in the proportion of Ki-67-expressing SAT and VAT $CD4^+$ or $CD8^+$ T cells in SIV-infected animals compared to controls. Cycling and recently divided T cells express Ki-67, while resting cells do not. This suggests that differences in the activation profile, and the higher density of $CD8^+$ T cells, in adipose tissue may not result from recent *in situ* proliferation. Another significant contribution of this study was the visualization of T cells in adipose tissue. Histology showed that $CD4^+$ T cells were primarily located among adipocytes far from the capillaries, while $CD8^+$ T cells clustered in the pericapillary area (2). The positioning of $CD4^+$ T cells deeper in adipose tissue could serve to protect latently-infected $CD4^+$ cells against cytotoxic killing by $CD8^+$ T cells.

PD-1 (CD279) expression on $CD4^+$ T cells is a negative co-stimulator that can inhibit TCR signaling (34, 35) and is important for mediating tissue tolerance of potentially autoreactive cells (36). In the context of HIV, cells expressing PD-1 could hinder clearance of chronic viruses by allowing tolerance due to their exhausted phenotype (37). Murine $CD4^+$ memory T cells expressing CD44 and PD-1 were increased in VAT of high fat diet fed (HFD) mice (55%) compared to mice fed a normal diet (33%), and these cells expressed less IL-2 and IFN-γ compared to PD-1^- $CD44^{hi}$ $CD4^+$ T cells (38). Notably, the PD-1^+ $CD44^{hi}$ $CD4^+$ T cells activated via the TCR also expressed large amounts of osteopontin (OPN), a negatively charged, N-glycosylated secreted phosphoprotein produced by numerous cell types, and higher levels of OPN were present in the serum of HFD mice. A fraction of the PD-1^+ $CD44^{hi}$ $CD4^+$ T cells also expressed CD153 (CD30L), a type II membrane glycoprotein that is present primarily on activated $CD4^+$ T cells, and the $CD153^+$ PD-1^+ $CD44^{hi}$ $CD4^+$ T cells were found to express genes linked to senescence (38). Adoptive transfer of these $CD153^+$ PD-1^+ $CD44^{hi}$ $CD4^+$ T cells into lean mice on a normal diet induced inflammation in VAT, as well as insulin resistance. This study suggests a role for PD-1 expressing T cells in metabolic disease.

Damouche et al. found high expression of PD-1 on $CD4^+$ T cells in SAT (45%) and VAT (62%) compared to in peripheral blood (3%) (4). However, there were no significant differences in PD-1 expression between PLWH and HIV-negative individuals. The authors further demonstrated that PD-1 expression was higher in $CD69^+$ $CD4^+$ T cells compared to $CD69^-$ $CD4^+$ T cells within SAT and VAT of HIV-negative individuals (a similar analysis was not done in PLWH due to limited sample number). Additionally, this study found a higher proportion of TIGIT/PD-1^+ $CD4^+$ T cells in SAT compared to VAT, which the authors postulated may be important for HIV-1 persistence. This finding suggests differences between the two fat depots, and evaluation of TIGIT/PD-1^+ cells in the context of the viral reservoir in PLWH is an area for future research.

Increased $CD8^+$ T Cell Receptor Clonality in Adipose Tissue From PLWH

The higher CD8:CD4 ratio in adipose tissue from SIV-infected macaques was attributed to an increase in $CD8^+$ T cells as opposed to depletion of $CD4^+$ T cells in one study (2), though additional data are needed in humans. As $CD8^+$ T cell density increases, key questions include: (1) whether recruitment of primarily activated and memory $CD8^+$ T cells from the circulation is stochastic; (2) whether $CD8^+$ T cells with select antigen specificities and corresponding TCR characteristics are preferentially migrating to adipose tissue; (3) whether $CD8^+$ T cells are clonally expanding *in situ* in response to TCR stimulation; and (4) whether T cells that infiltrate adipose tissue re-enter circulation. The Koethe et al. study of PLWH with long-term virologic suppression found increased $CD8^+$ TCR clonality in adipose tissue compared to blood using bulk TCRβ CDR3 deep sequencing, where bias-controlled V and J gene primers are used to amplify rearranged V(D)J segments (6, 39, 40). In all five subjects with adequate DNA for adipose tissue $CD8^+$ TCR sequencing, the 10 most prevalent TCRβ clones comprised a significantly larger percentage of total clones in SAT (25%) compared to paired blood (16%), and the Shannon's Entropy index, a measure of overall repertoire diversity, was lower in adipose tissue compared to blood (4.39 vs. 4.46, respectively). Notably, the same TCRβ sequences, also referred to as public clonotypes (i.e., specific rearrangements observed in multiple individuals), were not observed to occur at >0.9% frequency in the adipose tissue among any two study subjects. Gene usage and V-J gene pairing differed between blood and adipose tissue, but these differences were not statistically significant, potentially due to the low number of subjects.

The finding of increased adipose tissue $CD8^+$ T cell clonality in PLWH has correlates in the obesity literature (41–43). A notable finding from *in vitro* studies is that obese fat can independently activate $CD8^+$ T cells and induce proliferation, while lean fat has little effect (41). In mice, Yang et al. observed shifts in the clonality of both the adipose tissue $CD4^+$ and $CD8^+$ TCR repertoires in lean vs. obese animals using PCR-based spectratyping, which was based on shifts in gene family expression from a normally Gaussian frequency distribution of CDR3 length (43). Winer et al. examined TCR Vα diversity and observed clonal expansion in SAT of $CD4^+$ T cells of mice on a HFD (42). A major limitation of these studies from the obesity literature was the use of spectratyping, which can only provide indirect evidence of clonal expansion, cannot detect individual TCR sequences, and cannot distinguish between T cell clones sharing the same V gene region but distinct junctional regions. The introduction of

TCR sequencing makes it possible to examine immune receptor repertoires with fine specificity, and to examine the genetic and biochemical properties of both individual TCRs and the broader repertoire.

McDonnell et al. recently evaluated liver and adipose tissue $CD4^+$ and $CD8^+$ TCR repertoires in mice fed a HFD or low fat diet. They performed high-throughput TCR sequencing, which allowed distinction of T cell clones and quantification of clonal expansion (44). The authors found that mice on a HFD had reduced $CD8^+$ TCR diversity similar to previous studies (6, 39, 40). Moreover, they identified public TCR clonotypes and a predominance of positively-charged CDR3 regions with less polar amino acids. Notably, adipose tissue from mice on a HFD expressed elevated isolevuglandins (isoLG; a group of negatively charged reactive gamma-ketoaldehydes generated by free radical oxidation) in M2-polarized macrophages. Co-culture of these isoLG-bearing M2 macrophages with $CD8^+$ T cells isolated from the spleens of obese mice on a HFD led to an increase in T cell proliferation and activation *in vitro*, and these T cells showed increased expression of CD69, a marker suggestive of TCR-linked activation. This was the first study to indicate macrophages presenting isoLG can promote $CD8^+$ T cell activation, and may reflect a contribution of modified proteins to the propagation of inflammation within the adipose tissue of obese mice and humans.

The finding of a more clonal $CD8^+$ T cell population in the adipose tissue of PLWH, and elevated $CD4^+$ and $CD8^+$ clonality in obesity, raises the question of where and how antigen might be presented to drive TCR clonal expansion. Several published reports indicate that antigen presentation occurs directly in adipose tissue, with a focus on $CD4^+$ T cells. Several distinct MHC-II-expressing antigen presenting cells (APCs) in adipose tissue can present antigens to $CD4^+$ T cells; these include B cells (45), dendritic cells (46), macrophages (47), and even adipocytes (48). Mice with global deficiency of MHC-II demonstrate protection from adipose tissue inflammation and systemic insulin resistance when placed on a HFD (47, 48); the accumulation of $CD4^+$ T cells and reduction in $CD11c^+$ macrophages may be responsible for the reduced adipose tissue inflammation in these animals (47). Cells in the adipose tissue also secrete adipokines such as leptin, adiponectin, and retinol binding protein 4 that can directly activate these APCs (49–51).

Adipose Tissue $CD4^+$ T Cell Subsets in HIV and SIV

While HIV and SIV infection are characterized by an increased density of adipose tissue $CD8^+$ T cells, the limited studies on $CD4^+$ T cell subsets show less clear differences between PLWH and HIV-negative persons. A study by Damouche et al. focused on the $CD4^+$ T cell compartment in adipose tissue from ART-treated PLWH and HIV-negative controls (4). As observed in the earlier study by Couturier et al. (1), HIV-negative subjects had a predominance of $CD4^+$ T cells over $CD8^+$ T cells in SAT (55 vs. 25%, respectively), but this ratio was inverted, to a degree, in the SAT of those with HIV (40 $CD4^+$ vs. 50% $CD8^+$ T cells). Interestingly, the difference in CD8:CD4 ratio according to HIV status was not observed in VAT. $CD4^+$ and $CD8^+$ T cell subsets were equally represented (43 and 41%, respectively) in VAT from HIV-negative subjects, and this relative proportion did not differ in the PLWH. This finding differs from a study of SIV-infected macaques, which found a significantly higher CD8:CD4 ratio in VAT from SIV-infected animals compared to controls (2).

Among $CD4^+$ T cell subsets, Damouche et al. observed a significantly *higher* proportion of SAT regulatory T cells (Tregs; defined as $CD25^+$ $FOXP3^+$ $CD4^+$ T cells) in the PLWH compared to HIV-negative subjects, but no major differences in T_H1 and T_H17 pro-inflammatory subsets (4). Treg cells, a subset that can be difficult to evaluate due to the technical aspects of staining for markers such as Foxp3, accounted for 7.2% of SAT $CD4^+$ T cells in the PLWH, compared to 1.7% in the HIV-negative. A similar but non-significant difference was observed in VAT Treg cells. Obesity is commonly associated with *reduced* T regs in adipose tissue of obese humans and mice, and loss of these cells is postulated to facilitate the influx of pro-inflammatory T cells and macrophages (52). Furthermore, the authors did not observe a significant difference in T_H17 cells, identified by expression of ROR-γ transcription factors, according to HIV status. The proportion of T_H1 $CD4^+$ T cells (cells with intermediate or high levels of T-bet expression) also did not differ according to HIV status, while T_H2 $CD4^+$ T cells (expressing GATA-3) were barely detected in the adipose tissue from both PLWH and HIV-negative subjects. Taken together, a unifying finding of these experiments is a higher Treg/T_H17 ratio in the SAT of PLWH, potentially reflecting a compensatory, anti-inflammatory response (4).

In the same study, no significant differences were observed in the proportion of adipose tissue $CD4^+$ or $CD8^+$ T cells expressing either Ki-67 or HLA-DR between PLWH vs. HIV-negative controls (4). The median percentage of Ki-67^+ cells was $<2\%$ in both SAT and VAT from PLWH and controls, potentially indicating limited T cell proliferation within the tissue. The expression of HLA-DR on $CD4^+$ cells in SAT (24%) and VAT (21%) was higher compared to blood in the HIV-negative, but again similar values were observed in the PLWH. Finally, the authors observed far higher PD-1 expression on adipose tissue $CD4^+$ T cells (45% expression in SAT and 62% in VAT) compared to peripheral blood (3%), but again there were no significant differences according to HIV status (4). Notably, PD-1 expression was primarily present on $CD69^+$ $CD4^+$ T cells, suggesting those cells thought to represent a "tissue resident" phenotype are also more likely to demonstrate the features of quiescent cells. The authors postulated that the high degree of exhaustion marker expression is indicative of limited T cell proliferation and activation, as evidenced by the low Ki-67 expression, thus reducing downstream macrophage activation and tissue inflammation. However, while the enrichment of PD-1^+ $CD4^+$ T cells could have a protective role in limiting inflammation, high PD-1 expression on adipose tissue T cells may be of particular importance in the context of HIV as these cells are thought to constitute a large part of the latent HIV reservoir (53–55). Studies to date have not yet measured Bcl-2, which will be important to clarify how susceptible these populations are to apoptosis.

COMPARISON OF ADIPOSE TISSUE CELL CHANGES IN HIV/SIV AND OBESITY

T Cells in HIV/SIV and Obesity

The finding that adipose tissue from PLWH and SIV-infected macaques is enriched in $CD8^+$ T cells was particularly intriguing as similar changes are a hallmark of obesity. Studies of obese humans and obese animal models show a striking increase in adipose tissue $CD8^+$ T cells and $CD4^+$ T_H1 cells, a decrease in Treg cells, and an increase in M1-phenotype (TNF-α, IL-6, IL-12, IL-23-producing) pro-inflammatory macrophages compared to non-obese controls (41, 42, 45, 52). A landmark 2009 paper by Nishimura et al. showed the recruitment of M1-phenotype macrophages into adipose tissue in mice was dependent on the prior infiltration of $CD8^+$ T cells (41).

$CD4^+$ Treg cells protect against tissue inflammation and insulin resistance, and are less frequent in obese adipose tissue compared to lean. Adipose tissue Tregs in obese mice have a unique TCR profile compared to Tregs from the spleen and lymph nodes, as evidenced by single cell sequencing from each of these compartments (52). An important caveat of this study was that the "limited" Ltd transgenic mice used can only generate a highly restricted Vα repertoire that pairs with a single TCRβ chain (56). Experimental models demonstrate that the adoptive transfer of $CD4^+$ T cells into lymphocyte-free $Rag1^{-/-}$ mice reverses weight gain and insulin resistance, while depletion of $CD8^+$ T cells in adipose tissue reduces macrophage density and improves insulin sensitivity (41, 42).

Adoptive transfer models utilizing invariant natural killer T cells (iNKT) have demonstrated a role for these cells in modulating adipose tissue inflammation. NKT cells are lymphocytes that express NK cell surface markers and recognize CD1d (a non-polymorphic MHC I-like molecule) in its lipid-containing conformation (57). They are an important link between the innate and adaptive immune system. Type I NKT (iNKT) cells express a highly restricted (invariant) TCRα-chain (Vα24-Jα18 in humans paired with Vβ11 and Vα14-Jα18 paired with Vβ8.2, Vβ7, or Vβ2 in mice). Type II NKT cells on the other hand have diverse TCRs (58). Notably, the different TCRs expressed on type II NKT cells can recognize the same lipid antigens. In humans, type II NKT cells are more frequent than type I NKT cells, but the opposite is the case in mice (59, 60). Type II NKT cells have been shown to prevent high fat-induced obesity in some studies (61, 62), though they were shown to exacerbate diet-induced obesity in another (63). iNKT cells are highly plastic and can express both anti-inflammatory (61) and pro-inflammatory (64) cytokines depending on the activating stimuli. They recognize alpha-galactosylceramide and exert anti-inflammatory effects controlling the presence of Treg and macrophage populations in the adipose tissue in an IL-2- and IFN-γ-dependent manner (61, 65–68). Adipocytes can also serve as non-professional antigen presenting cells for iNKT cells with high levels of CD1d expression (69).

$CD4^+$ iNKT cells express CXCR4 and CCR5 chemokine receptors and can be infected by HIV (70–72). Infection of $CD4^+$ iNKT cells in humans and non-human primates leads to decline of $CD4^+$ T cells. Couturier et al. compared NKT cells ($CD3^+/CD16^+/CD27^+/CD56^+$) in VAT of SHIV-SF162p3- infected macaques and healthy controls during chronic and acute infection, and found no differences in the percentage of these cells between groups (3). At present, there are no studies that assessed adipose tissue NKT cells in PLWH, and further studies are warranted to explore the role of NKT cells in tissue inflammation and metabolic disease (73).

Macrophages in HIV/SIV and Obesity

Progressive weight gain is accompanied by adipose tissue macrophage infiltration and inflammation (18, 41, 74–79). In obesity, hypertrophied adipocytes express increased macrophage inflammatory protein 1α (MIP-1α) and macrophage chemotactic protein 1 (MCP-1), which promote macrophage infiltration, and IL-8, which promotes neutrophil chemotaxis (80–82). Adipose tissue from obese individuals and animal models contains a greater density of macrophages with a pro-inflammatory M1 cytokine phenotype (characterized by high TNF-α, IL-6, IL-12, IL-23, and inducible nitric oxide synthase production). An environment high in these cytokines inhibits adipocyte insulin signaling by reducing expression of insulin receptor substrate-1 (IRS-1), phosphoinositide 3-kinase p85α, and glucose transporter type 4 (GLUT4) via cell surface receptors and other mechanisms (75, 83–86). The contribution of adipose tissue macrophages to metabolic disease is also supported by the higher levels of inflammatory cytokine and other protein expression in SAT from insulin resistant persons compared to insulin sensitive, including higher MCP-1, CD68, scavenger receptor A, visfatin, and oxidized LDL receptor 1 (87).

Studies of PLWH report conflicting results on adipose tissue macrophage changes compared to HIV-negative controls, but, in general, studies of PLWH have not demonstrated the marked enrichment in pro-inflammatory M1 macrophages observed in obesity. Peripheral lipoatrophy was accompanied by increased SAT macrophage density in one comparative study, in addition to increased fibrosis, apoptosis, and vessel density (88). In contrast, a study of gluteal fold adipose tissue (GFAT) found minimal differences in macrophage content (measured as cells/hpf) between PLWH with and without clinically-evident lipoatrophy, and HIV-negative controls, but higher IL-6, IL-8, IL-12p40, and MIP-1α, and lower interferon gamma and eotaxin levels, in adipose tissue supernatant from the PLWH (89). While the density of adipose tissue macrophages did not differ by lipoatrophy status in the PLWH, patients exposed to zidovudine and stavudine (older thymidine analog antiretroviral medications) had marginally higher density. Furthermore, median HIV DNA in circulating $CD14^+CD16^+$ monocytes, a subset more apt to be infected by HIV, was higher in the PLWH with clinically-evident lipoatrophy compared to those without lipoatrophy (89). HIV infection of $CD14^+CD16^+$ monocytes increases constitutive expression of pro-inflammatory cytokines, impairs phagocytic capacity, and increases antigen-stimulated cytokine expression (90–94). These findings suggest a role for adipose tissue macrophages in the development of lipoatrophy

in PLWH, though the effect may stem from qualitative changes (e.g., greater cytokine expression) rather than greater density.

Adipose tissue $CD3^-/CD14^+/HLA\text{-}DR^+$ macrophages represented a small proportion (<1–2%) of the total SVF cells in SIV-negative macaques, but were ∼50% lower in acutely (4 weeks post-infection) SIV-infected macaques (3). In contrast, a study of macaques infected with SIV for a median of 15 months found a significantly higher proportion of macrophages among CD45+ cells in SAT, but not VAT, from SIV-infected animals compared to controls (2). Furthermore, the frequency of $CD206^+CD163^-$ macrophages (selected by the authors as markers of "M2" anti-inflammatory cells) was significantly lower in SIV-infected animals compared to non-infected animals in both SAT and VAT, while the proportion of $CD206^-CD163^-$ macrophages (thought to correspond to pro-inflammatory cells) was elevated in SIV-infected animals.

The relationship between $CD8^+$ T cell enrichment and the accumulation of macrophages in adipose tissue in PLWH is an area for additional study, which will inform both our understanding of metabolic changes in HIV and the pathogenesis of adipose tissue inflammation more broadly. In the 2009 study by Nishimura et al. $CD8^+$ T cell infiltration into adipose tissue in obese mice preceded the recruitment of M1-phenotype macrophages (41). The experimental depletion of $CD8^+$ T cells in obese mice, and diet-induced obesity in $Cd8^-/Cd8^-$ knockout mice, was accompanied by reduced adipose tissue macrophage content and local inflammatory mediators, suggesting $CD8^+$ T cells are both necessary and sufficient to promote macrophage recruitment in obesity. If HIV infection is characterized by adipose tissue $CD8^+$ T cell enrichment without pronounced macrophage recruitment, this may reflect the need for a costimulatory factor in obese adipose tissue to recruit macrophages in the presence of $CD8^+$ T cells, may indicate that cytokine expression profiles of adipose tissue $CD8^+$ T cells differ in obesity and HIV infection, or reflect another as yet unknown mechanism.

Studies in Humanized Mice

Several groups have used humanized mice to study HIV-1 tissue and cellular replication as well as immune responses during acute and chronic infections. Arainga et al. infected humanized mice with HIV-1 and examined tissue sites for viral infection. The authors assessed the blood, brain, gut, kidney, lungs, liver, lymph nodes, and spleen, and were able to detect HIV-1 integrated DNA and multi-spliced and unspliced RNA in different cell types, though adipose tissue was not evaluated (95). This study suggests that humanized mice infected with HIV and treated with ART might serve as a model to study the effects of chronic, treated HIV infection on metabolic status. A study by Cheng et al. evaluated humanized mice engrafted with hematopoietic stem cells (HSC), reconstituted with human lymphoid and myeloid lineages, and infected with HIV-1 before treatment with ART (96). The study showed a reduction of HIV replication to undetectable levels with treatment, but the persistence of HIV-1 in reservoirs and a viral rebound after stopping. At present, to our knowledge, no group has used humanized mice infected with HIV to explore adipose tissue biology.

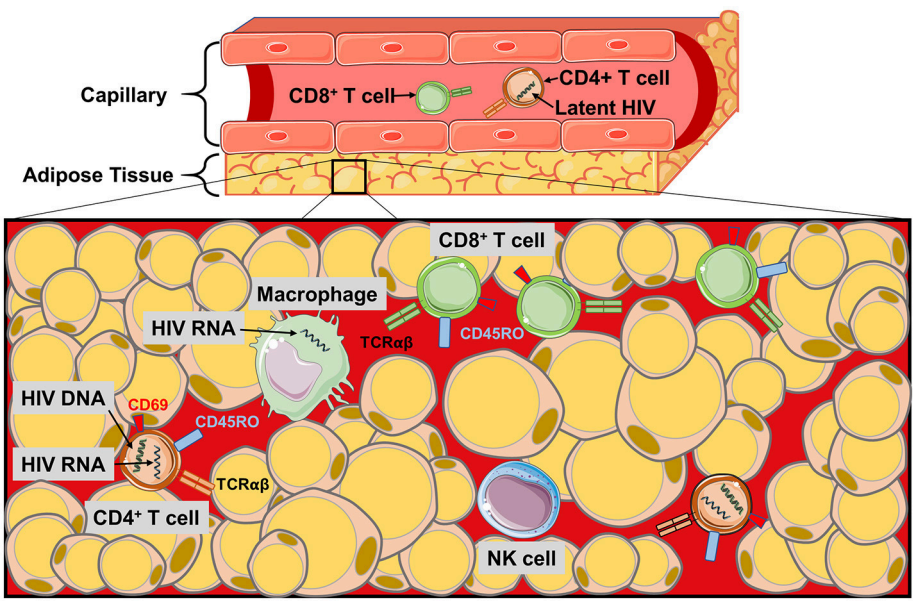

FIGURE 2 | The HIV/SIV viral reservoir in adipose tissue. Schematic of the immune cell populations in PLWH and SIV-infected primates where latent and replicating HIV and SIV have been identified. HIV RNA and proviral DNA has been detected in the $CD4^+$ T cells of the SVF, and also in macrophages during SIV infection. Poor perpetration of some antiretroviral agents into adipose tissue and the distance of $CD4^+$ T cells from the pericapillary area may make targeting the latent reservoir of adipose $CD4^+$ T cells difficult. Similarly, the localization of the $CD8^+$ T cells to the pericapillary area may prevent cytotoxic killing of HIV-infected $CD4^+$ T cells in the adipose tissue.

ADIPOSE TISSUE: A VIRAL RESERVOIR

HIV and SIV

$CD4^+$ T cells in adipose tissue are predominantly an activated memory phenotype ($CD45RO^+CD69^+$), which may serve as a reservoir for HIV persistence (1, 2, 19, 33, 97). Replication-competent HIV has been detected in sorted and *ex vivo* $CD4^+$ T cells from the adipose tissue of aviremic, ART-treated patients, and in adipose-resident $CD4^+$ T cells the median copy number of latent HIV proviral DNA (i.e., integrated into the host cell DNA) in adipose tissue $CD4^+$ T cells was equivalent to circulating $CD4^+$ T cells (2). Similarly, SVF cells from several adipose depots (visceral, subcutaneous, and deep neck) contained detectable HIV DNA at comparable frequencies to memory $CD4^+$ T cells purified from peripheral blood, mesenteric lymph nodes, and thymus (1). Furthermore, SIV DNA and RNA were detected in SVF from SAT and VAT, sorted blood $CD4^+$ T cells, and $CD14^+$ macrophages in macaques (2, 3, 5). These findings indicate adipose tissue may serve as a HIV and SIV DNA reservoir, and a site for actively replicating (RNA-producing) virus. Viral persistence in adipose tissue may be partly due to inadequate distribution of ART. *In vitro* experiments using HIV-1 infected $CD4^+$ T cells and primary human adipocytes revealed a reduced drug efficacy due to lower penetration of nucleoside/nucleotide reverse transcriptase inhibitors. Integrase inhibitors, on the other hand, penetrate adipose tissue (7). **Figure 2** summarizes the major findings regarding HIV and SIV persistence in adipose tissue.

Adipose tissue latently-infected $CD4^+$ T cells likely promote a local inflammatory environment. While the co-culture of preadipocytes or adipocytes with HIV-infected memory $CD4^+$ T cells did not promote T cell activation or virus production, the addition of IL-2, IL-7, or IL-15, three cytokines present in adipose tissue (98–100), increased CD69 activation marker expression and p24 HIV antigen production by two-fold or more (1). Furthermore, preadipocyte IL-6 expression increased nearly three-fold in the presence of latently-infected $CD4^+$ T cells (1). These findings may reflect a cycle in which an inflammatory adipose tissue environment promotes virus production and activation of latently-infected $CD4^+$ T cells, and these $CD4^+$ T cells in turn increase preadipocyte pro-inflammatory cytokine production (101–104).

Finally, if adipose tissue serves as a reservoir for latently-infected $CD4^+$ T cells, these cells may be more protected from cytotoxic $CD8^+$ T cells than those in circulation. This possibility is supported by studies in macaques showing $CD4^+$ T cells are present deep in the adipose tissue while $CD8^+$ T cells are generally localized to the pericapillary area (2). The development of therapies for eradication of the latent HIV reservoir will need to consider the potential challenges posed by adipose tissue $CD4^+$ T cells.

Non-retroviral Reservoir

The presence of HIV and SIV proviral DNA and free RNA virus in adipose tissue is not unique to these two pathogens, and studies over the past 70 years have documented the infiltration of adipose tissue by a number of viruses spanning multiple Baltimore groupings. These pathogens are presumably engaged to varying degrees by the local immune system, resulting in changes in immune cell populations, expression of cytokines and other immune mediators, and effects on adipocyte energy storage and metabolic fitness.

Studies in the 1950s described the *in vivo* infection of adipose tissue and the adipotropism of Coxsackie virus (105), rabies (106), and polioviruses (107, 108). Later work in murine and primate models in the 1980s and 1990s described the infection of adipose tissue and subsequent metabolic and pathologic changes introduced by cytomegalovirus (109–111) and Ebola-Reston virus (112). More recent work has re-established adipose tissue as a key reservoir for influenza (113), the murid herpesviruses MHV68 and MHV4 (114, 115), and cytomegalovirus (116, 117). Epidemiologic studies have also linked untreated viral infections to changes in adipose tissue mass and metabolism, including childhood obesity associated with cytomegalovirus and total herpesvirus burden (118), and obesity and insulin resistance in chronic hepatitis C infection in adults (119).

CONCLUSION

The multifaceted roles of adipose tissue as an energy storage depot, metabolic regulator, and endocrine organ occur against the backdrop of a complex mix of innate and adaptive immune cells interacting in an environment prone to infiltration by a range of viral pathogens. T cells are a major component of the adipose tissue milieu, and while the enrichment of $CD8^+$ T cells appears broadly similar in obesity and HIV infection, differences in $CD4^+$ T cells and, likely, macrophage populations suggest that the stimuli driving $CD8^+$ cell enrichment and the interactions of $CD8^+$ cells with other immune cells differs between the two conditions.

At this time, studies of T cell populations in HIV/SIV-infected subjects have only utilized surface marker phenotyping, and additional data on transcriptional profiles is needed to understand whether adipose tissue T cells in HIV/SIV and obesity are behaving in a similar manner, and whether adipose tissue T cells from PLWH have similar functional properties to T cells in the peripheral blood. Finally, HIV and SIV proviral DNA, and free RNA virus, can be detected in the SVF of VAT and SAT, and replication-competent HIV is found in $CD4^+$ T cells. Evidence that adipose tissue is a viral reservoir for HIV and SIV suggests that some of the T cells within the adipose tissue may be virus-specific. Future studies to identify TCRs expressed by T cells within adipose tissue and their corresponding epitopes will help answer this question, and is currently being explored by our group. Finally, HIV-infected $CD4^+$ T cells within adipose tissue may serve as a relatively protected viral compartment, which should be considered in future studies of interventions to deplete the HIV reservoir.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Innate Lymphocytes in Adipose Tissue Homeostasis and Their Alterations in Obesity and Colorectal Cancer

Manuela Del Cornò, Lucia Conti and Sandra Gessani*

Center for Gender-Specific Medicine, Istituto Superiore di Sanità, Rome, Italy

Colorectal cancer (CRC) is the third most common cancer worldwide and a leading cause of death, with burden expected to increase in the coming years. Enhanced adiposity, particularly visceral fat, is associated with increased cancer incidence representing an important indicator of survival, prognosis, recurrence rates, and response to therapy for several tumors including CRC. Compelling evidence has been achieved that the low-grade chronic inflammation characterizing obesity represents a main factor that can favor carcinogenesis. Adipocytes and adipose tissue (AT) infiltrating immune cells contribute to obesity-related inflammation by releasing soluble factors affecting, both locally and systemically, the function of several cell types, including immune and cancer cells. The unbalanced production of immune mediators as well as the profound changes in the repertoire and activation state of immune cells in AT of obese subjects represent key events in the processes that set the basis for a pro-tumorigenic microenvironment. AT harbors a unique profile of immune cells of different origin that play an important role in tissue homeostasis. Among these, tissue-resident innate lymphocytes are emerging as important AT components whose depletion/aberrant activation occurring in obesity could have an impact on inflammation and immune-surveillance against tumors. However, a direct link between obesity-induced dysfunction and cancer development has not been demonstrated yet. In this review, we provide an overview of human obesity- and CRC-induced alterations of blood and adipose tissue-associated innate lymphocytes, and discuss how the adipose tissue microenvironment in obesity might influence the development of CRC.

Keywords: obesity, colorectal cancer, adipose tissue, immune profile, innate lymphocytes

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Melissa Jane Conroy,
Trinity College, Dublin, Ireland

****Correspondence:***
Lucia Conti
lucia.conti@iss.it

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INTRODUCTION

White adipose tissue (AT) is a complex immunocompetent organ, enriched with adipocytes and immune cells that contribute to metabolic, endocrine, and immune activities (1). AT is a dynamic organ making up a substantial proportion of the body that, in severe obesity, can account for 50% of body mass (2). This tissue has remained a largely unexplored and unappreciated immune site and only recently emerged that AT harbors a unique profile of immune cells, with either pro-inflammatory (M1 macrophages, dendritic and mast cells, neutrophils, Th1 CD4 and CD8 T cells, B lymphocytes) or anti-inflammatory (M2 macrophages, eosinophils, T_{reg} and Th2 CD4 T cells) activity, playing a key role in immune homeostasis and metabolic regulation of

AT (1). Studies carried out in the last decade led to the characterization of non-recirculating lymphocyte populations residing in non-lymphoid tissues and organs such as AT (3). These include unconventional T cells—like invariant natural killer T (iNKT) cells, mucosal-associated invariant T (MAIT) cells, γδ T lymphocytes—the family of innate lymphoid cells (ILC) and tissue-resident memory T cells (3). AT immune cells are key to maintain tissue and immune homeostasis and their profile and function are profoundly altered in obesity (4). The obesity-associated low-grade chronic inflammation represents a major risk factor for related morbidities including some types of cancer. The observation that anti-inflammatory drug use reduces colorectal cancer (CRC) risk and retards intestinal tumors in ulcerative colitis patients has provided compelling evidence for a link among inflammation, obesity, and cancer (5). Growing evidence highlighted the importance of adipocyte microenvironment in modulating the function of immune and cancer cells. The nature of AT/cancer interplay is still largely unknown, but investigations in the field over the last 10 years indicate that this tissue occupies a central place in tumor pathogenesis and progression (6). Systemic and local alterations occurring as a consequence of obesity not only increase the likelihood of tumor development/progression but also set the basis for unfavorable responses to therapy (6).

This review will provide an overview of alterations in blood and AT populations of innate lymphocytes in obesity and CRC. The interplay between AT and cancer cells as a potential mechanism linking obesity to CRC development is discussed.

TABLE 1 | Adipose tissue vs. blood distribution of innate lymphocytes and alterations in their frequencies in obesity and colorectal cancer.

Cell type	Lean	Obese		CRC		References
	AT vs. PB	*AT*	*PB*	*AT*	*PB*	
iNKT	AT^ > PB					(11, 12)
		↓	↓*			(11, 13–17)
		↓	=			(18)
				↓		(11)
NKT-like	VAT > PB					(11, 19)
	VAT = PB					(20)
	VAT > SAT					(21)
		=	=			(11, 19, 20, 22)
				↑	=	(19)
MAIT	VAT > PB	↓	↓↑#			(23)
		=				(15)
					↓	(24, 25)
γδ T	VAT = PB					(19)
		=	=	=	=	(15, 19, 26)
Vγ9Vδ2 T			↓			(27)
Vγ9Vδ2/γδ T			↓		↓	(19)
ILC1 (NK)		=	=	=	=	(19, 28)
			↓			(29)
$CD56^{br}$	AT^ > PB					
$CD56^{dim}$	PB > AT^					(19)
$CD56^{br}$	VAT = PB					
$CD56^{dim}$	VAT = PB					(20)
$CD56^{br}$		↑				(30)
ILC2		↓				(31)

*PB, peripheral blood; VAT, visceral AT; SAT, subcutaneous AT; *in both adult and childhood obesity; #opposite trend in childhood vs. adult obesity; ^ in both VAT and SAT; =, ↑, ↓: similar, higher or lower frequency as compared to healthy lean controls; >: greater than in the comparison between AT and PB.*

INNATE LYMPHOCYTE PROFILES IN OBESITY AND COLORECTAL CANCER

AT exhibits differential profiles in distinct body depots and fat distribution, particularly subcutaneous vs. visceral, has important implications for health (7). The visceral or omental AT (VAT) is immunologically more dynamic than the subcutaneous AT (SAT) and abdominal adiposity is strongly associated with the risk of metabolic disorders, cardiovascular diseases, and cancer (7).

Recent studies clearly showed that the balance between homeostasis and inflammation in AT is mainly controlled by the stromal vascular fraction, that contains, among other immune cells, different populations of innate lymphocytes exhibiting, in homeostatic conditions, regulatory/anti-inflammatory properties (8). Obesity is associated with profound changes in AT infiltrates and systemic profiles of innate lymphocytes, contributing to the dysregulated soluble mediator release and to skewing the balance toward a pro-inflammatory status (8). In addition to act as major regulators of AT homeostasis, innate T cell populations actively contribute to the early defense against cancer (9, 10).

A summary of the main alterations of AT and blood innate lymphocytes in obesity and CRC is provided in **Table 1**.

Natural Killer T Cells

Among innate lymphocytes populating AT are Natural Killer T (NKT) cells, evolutionarily conserved lipid-sensing T cells that strongly influence inflammatory responses (32). They include iNKT, non-invariant NKT and NKT-like cells with the lipid antigen-reactive iNKT cells making up a large proportion (about 10%) of total AT lymphocytes (11, 13, 14, 33). The frequency of iNKT cells in VAT is higher than in any other organ, likely due to lipid antigen abundance in this body compartment and to the antigen presentation capacity of AT-resident myeloid cells including adipocytes (11, 13, 14, 33). Notably, iNKT cell enrichment results from blood recruitment (12) and their frequency correlates with the number of cells expressing the MHC-like glycoprotein CD1d (11).

AT-associated iNKT cells are unusually poised to readily produce anti-inflammatory/regulatory cytokines. They acquire a Th2-like phenotype in response to lipid antigens and have the ability to produce IL-10. Invariant NKT cells also act as regulators of other immune cells thus bridging innate to adaptive immunity (2, 34). In obesity, a reduced iNKT cell frequency was observed in both blood and VAT (11, 14, 15), and in the latter, it parallels a decreased CD1d-expressing cell number (11, 14). Furthermore, iNKT cell frequency inversely correlates with obesity degree, and with insulin resistance and fasting glucose, suggesting that AT resident iNKT cells exert a regulatory role against obesity-associated metabolic disorders (13, 14). Circulating iNKT cell frequency decreases in adult and childhood obesity (16, 17), although other investigators described a reduction only in VAT of adult obese subjects (18). This reduction parallels an increased

proportion of early activated iNKT cells in the circulation (18). Interestingly, weight loss induced by gastric bypass surgery in severely obese subjects partially rescues circulating iNKT cell numbers (35) and reverts their activation state (18).

As iNKT cells play a key role in the regulation of inflammation and in cancer surveillance, their contraction in obesity could have important implications for the control of tumor growth. Notably, iNKT cell frequency and CD1d-expressing cell number decline in VAT from CRC affected patients (11).

Like iNKT lymphocytes, the CD1d-independent NKT-like cells are poorly represented in blood of lean individuals and preferentially accumulate in VAT rather than in SAT (11, 19, 21). However, no differences in VAT vs. blood distribution were found in other studies (20). Although not modified in obese subjects (11, 19, 20, 22), these cells are selectively enriched in VAT from CRC patients as compared to healthy individuals, irrespective of BMI (19).

Mucosal-Associated Invariant T Cells

MAIT cells are emerging as important players in chronic inflammatory disorders including obesity. They express an invariant Vα7.2 TCR chain and high levels of the NK cell-associated receptor CD161, recognize bacterial-derived metabolites presented by the MHC-like molecule MR-1 and are mainly involved in the control of bacterial infections (36). MAIT cells are relatively abundant in the blood and are present in both VAT and SAT (15, 23). Like iNKT cells, they selectively accumulate in lean individual AT, where, unlike their blood counterpart, they produce IL-10 upon stimulation, thus contributing to tissue homeostasis (23).

Obesity profoundly affects MAIT cell number and function. A decreased frequency of circulating MAIT cells was reported in obese individuals. The reduction was found to be stronger in subjects with insulin resistance or with severe obesity, and was counteracted by surgery-induced weight loss (15). In parallel, a preferential activation of MAIT cells, displaying high proliferation and a striking IL-17 profile, was observed in VAT from obese individuals (15). Carolan and co-authors confirmed obesity-related depletion of circulating MAIT cells, but reported a reduced accumulation in VAT (23). An opposite situation was found in obese children, whose circulating MAIT cell frequency is increased and positively correlates with BMI. In both adults and children, however, obesity results in a skewed differentiation of MAIT cells toward an IL-17^{+} phenotype (23).

The role of MAIT cells in cancer is still under debate. Whether they promote malignancy through the production of IL-17, or contribute to anticancer immunity by expressing cytotoxic effector molecules still needs to be elucidated (36). Although alterations of MAIT cell distribution and function have never been studied in the AT of cancer patients, evidence has been provided that the percentages and absolute numbers of circulating MAIT cells decrease in gastric and colon tumors (24, 25). This depletion only occurs in patients affected by mucosal-associated cancers and is associated with an increased number of tumor infiltrating MAIT cells, especially in subjects with advanced CRC, thus reflecting the degree of cancer progression (24, 25).

γδ T Lymphocytes

γδ T cells sense self and non-self danger signals expressed in stressed, infected or tumor cells by recognizing non-peptidic phosphorylated antigens in a MHC-independent manner (37). They exert their effector functions either directly by cytotoxic activity or indirectly through the production of immune mediators and activation of other immune cell populations (37). In humans, three major subsets have been identified: Vδ1 T cells, predominantly present in the thymus and peripheral tissues; Vδ2 T cells, always expressing the Vγ9 chain, which constitute the majority of blood γδ T lymphocytes and can be recruited in inflamed tissues in pathologic conditions (38); Vδ3 T cells, poorly represented in the blood and enriched in the liver.

Despite studies demonstrating a role of IL-17-producing γδ T cells in murine obesity, the involvement of γδ T cells in human obesity-associated chronic inflammation has been only poorly investigated. We recently reported comparable frequencies of blood and VAT-associated total γδ T lymphocytes in obese and CRC-affected subjects as compared to healthy lean individuals (19). Accordingly, no alterations in circulating γδ T cells were reported in obese (15) and CRC-affected subjects (26). However, when the analysis was extended to the Vγ9Vδ2 subset, we observed a systemic reduction of the Vγ9Vδ2/γδ T cell ratio, inversely correlating with BMI, in both obese subjects and CRC patients (19). Likewise, a reduced number of blood Vγ9Vδ2 T cells was reported in obese individuals, accompanied by a skewed maturation phenotype and impaired IFN-γ production, mainly attributed to IL-2 deprivation in obesity (27). The low frequency of Vγ9Vδ2 T cells with reduced activation potential in obesity could negatively impact cancer immune-surveillance.

Innate Lymphoid Cells

ILC, a heterogeneous immune cell population not expressing T- or B-cell receptor, include ILC1, ILC2, and ILC3 groups, characterized by the ability to produce Th1-, Th2-, and Th17-like cytokines, respectively (39). They contribute to homeostasis in both barrier and non-barrier tissues and have been implicated in infection, chronic inflammation, and metabolic disorders (39). Among ILC, NK cells, belonging to ILC1 group, and ILC2 have been described in human AT where they contribute to inflammation or to maintain tissue homeostasis, respectively.

A selective distribution of NK cell subsets in VAT and blood was recently reported in lean individuals, with CD56^{bright} cells preferentially enriched in VAT and CD56^{dim} cells more represented in the blood (19). Conversely, NK cell subsets were found to be equally distributed in VAT/SAT and blood compartments in previous studies (20). The VAT/blood distribution was not substantially changed in obesity (20, 28), although a slight increase in VAT CD56^{bright} and a parallel decrease in CD56^{dim} NK cell frequency was detected (20). A reduced number of NK cells, associated to an expansion in inhibitory-receptor (CD158b, NKB1) bearing cells, was found in the peripheral blood of obese individuals, particularly in metabolically unhealthy subjects (29). Obese subject circulating NK cells were also reported to express higher levels of activation markers despite an impaired cytotoxic activity against tumor cells (40, 41). Furthermore, a selective accumulation of a distinct IL-6

receptor-expressing subset, correlating with systemic low-grade inflammation markers, was recently reported in the circulation of obese subjects (28). Conversely, Dovio and colleagues failed to detect any obesity-associated NK cell dysfunction (42). In addition, O'Rourke and colleagues reported an activated phenotype also for AT resident NK cells that, in obesity, upregulate the NKG2D receptor (20). Although the total NK cell number is similar in blood and AT of lean and obese subjects, affected or not by CRC, NK cells residing in SAT of obese individuals display a poor cytotoxicity against target tumor cells (19, 28, 30). Moreover, reduced NK cell survival and expansion of IL-10-producing NK cells have been described in other obesity-associated cancers (43). Altogether, these data suggest that NK cell chronic stimulation could occur in obesity leading to their exhaustion. This could contribute to the greater susceptibility of obese individuals to develop cancers or infectious diseases.

ILC2 play an important role in mounting protective innate responses against parasites and helminthes and in maintaining intestinal epithelium integrity (44). Due to their capacity to secrete anti-inflammatory cytokines, they may act as anti-obesity immune regulators. However, despite the evidence obtained in mouse models, studies investigating ILC2 abundance in human AT and their role in metabolic homeostasis are scarce. Cells expressing GATA binding protein 3 and IL-33 receptor, consistent with ILC2 described in other human tissues, have been recently identified in lean individual SAT and their frequency decreases in obese subjects, as already described for murine obesity (31).

INFLUENCE OF ADIPOSE TISSUE MICROENVIRONMENT ON IMMUNE AND COLORECTAL CANCER CELLS

AT Modulation of Innate Lymphocyte Function

Growing evidence points to the existence of a cross-talk between AT and immune cells with the latter able to influence adipocyte metabolic function (45). Less is instead known about the influence of AT on cells of the immune system, particularly innate lymphocytes. Although a variety of mediators (cytokine, chemokines, lipids and their metabolites, hormones and growth factors) released by adipocytes have the potential to modulate innate lymphocyte functions and recruitment (46), the studies describing adipocyte/innate T cell interplay failed to characterize the specific factors involved. In particular, evidence was provided that MAIT cell function is influenced by AT released soluble factors. It was reported that the frequency of IL-17-producing MAIT cells increases in the presence of AT conditioned media (CM) from obese subjects, while that of IL-10-producing MAIT cells is increased by CM from lean subjects (23).

Likewise, CM from obese individuals strongly reduce the expression of the survival molecule Bcl-2 while increasing the percentage of Ki-67-expressing MAIT cells (15). These observations suggest that soluble factors released by AT in obesity might lead to the death of MAIT cells or skew their differentiation toward a pathogenic phenotype thus contributing to the establishment/perpetuation of chronic inflammation.

We also provided evidence that, in obesity and CRC conditions, adipocyte microenvironment delivers immunosuppressive signals to differentiating dendritic cells (DC), as assessed by their enhanced expression of inhibitory molecules and reduced IL-12/IL-10 ratio (47). Likewise, DC generated in adipocyte CM from obese and CRC subjects fail to induce the activation of Vγ9Vδ2 T lymphocytes. Of note, adipocytes from obese and CRC subjects release higher amounts of pro-inflammatory/immunoregulatory cytokines/chemokines (IL-6, CXCL8, CCL2), and exhibit a higher content of pro-inflammatory ω6 polyunsaturated fatty acids (FA), with respect to lean subjects (47).

An intimate interaction between adipocytes and iNKT cells has been reported in mouse models, where CD1d-expressing adipocytes contribute to lipid antigen presentation and activation of iNKT cells (14). Although an interaction between adipocytes and iNKT cells has not been demonstrated yet in human AT, the correlation between CD1d expression and iNKT cell frequency in lean subject AT, and their concomitant reduction in obesity (11, 13, 14, 33), point to the existence of a dynamic cross-talk between these cell populations. Depending on the lipid antigens produced in response to nutritional environment, iNKT cells might be primed toward either anti- or pro-inflammatory phenotypes, thus contributing to AT homeostasis or inflammation, respectively. In this regard, it is of interest that adipocyte secreted lipids are potent inhibitors of LPS-induced IL-12p40 secretion in macrophages. This effect is AT depot-independent and its extent correlates with BMI (48). Likewise, adipocyte-derived lipids, particularly free FA, exert a potent immunomodulatory effect by stimulating $CD4^+$ T cell proliferation in an AT depot-independent manner (49).

All together these observations provide evidence for a cross-talk between AT and innate T cells, at least in part promoted by soluble mediators (lipids or cytokines/chemokines or still unknown factors), that is dysregulated in obese and cancer affected subjects thus leading to altered tissue recruitment and function of innate immunity cell subsets.

AT Cross-Talk With Colorectal Cancer Cells

The altered systemic and AT environment occurring in obesity not only increases the likelihood of tumor development/progression but also sets the basis for unfavorable responses to therapeutic regimens (9, 10). Several studies suggest that, within the constitutively active pro-inflammatory AT microenvironment characterizing obesity, the bidirectional cross-talk between AT and cancer cells may play an important role (50). Such interplay occurs via deregulated expression of mediators of different nature: cytokines (i.e., IL-1, IL-6, TNFα), chemokines (i.e., CCL2, CXCL8), lipids and their metabolites (i.e., free FA, PGE_2), hormones (estrogens), growth factors (i.e., IGF-1, VEGF), that are potentially linked to increased risk of cancer development/progression (21, 47–49, 51, 52). Growing evidence suggests that non-cancer cell types in the tumor microenvironment, such as adipocytes and

infiltrating immune cells, interact to enhance inflammation, reprogram cancer cell metabolism, and affect processes involved in invasion, metastasis, and immune clearance, all of which can support tumor progression and impact patient outcome (53).

In this regard, Amor et al. (51) reported enhanced expression of pro-inflammatory and angiogenic factors (i.e., nitrite/nitrate, IL-6, TNF-α, VEGF) by peritumoral AT of CRC affected obese patients with respect to lean subjects. Likewise, Catalan and colleagues showed increased release of pro-inflammatory and angiogenesis-related factors (lipocalin-2, chitinase 3-like 1, TNF-α, osteopontin, HIF-1α, VEGF), as well as increased MMP9 enzymatic activity in VAT of overweight subjects affected by CRC (52). On the other side, lipoprotein lipase and FA synthase activity were found to be reduced in peritumoral AT suggesting a tumor-induced impairment of lipid storing capacity of adipocytes in CRC patients (54).

Only few studies investigated the direct interaction between AT and cancer cells in *in vitro* models. Lysaght and colleagues reported higher levels of VEGF in serum and VAT CM from centrally obese as compared to non-obese CRC patients (21). Interestingly, CM from obese subjects increase the proliferation of colon cancer cells in a VEGF-mediated manner. By comparing SAT and VAT depots, the latter was shown to contain higher levels of VEGF and IL-6 (21). Furthermore, adipocyte secreted factors from obese subjects decrease oxygen consumption rate and expression of mitochondrial respiratory chain complexes in colon cancer cells. The observed inhibition of mitochondrial respiration and function is characteristic of the metabolic reprogramming of malignant transformation and is partially mediated by leptin (55).

Evidence on the existence of a direct relationship between AT-associated innate lymphocyte dysfunctions and CRC development is still lacking. We propose that the contribution of innate lymphocytes to obesity-associated tumor development/progression relies on: (i) alteration of cytokine profile that contributes to inflammation and ultimately affects intestinal mucosa homeostasis, and (ii) impaired direct antitumor activity that favors a shift from immune-surveillance toward tumor escape.

INNATE LYMPHOCYTE ALTERATIONS IN OBESITY AND THEIR IMPACT ON CRC

Obesity is associated with profound changes in AT and systemic profiles of innate lymphocytes, contributing to dysregulated soluble mediator release and to skewing the balance toward a pro-inflammatory status. In addition, the cytotoxic activity of innate lymphocyte populations and their capacity to directly orchestrate the early defense against cancer are impaired in obese individuals.

A number of studies have documented the critical role of these cells in tumor immunity and highlighted the therapeutic potential of their targeting in different cancer types including CRC (9, 10). Activated innate lymphocytes produce cytokines and cytotoxic factors and are capable of directly lysing tumors. Furthermore, they can drive the activation of other immune cell populations, such as DC, and are able to target tumor-associated macrophages, myeloid-derived suppressor cells and IL-10-secreting neutrophils which generally characterize the immunosuppressive tumor microenvironment, as well as cancer stem cells (9, 10). Thus, alterations of AT and blood innate

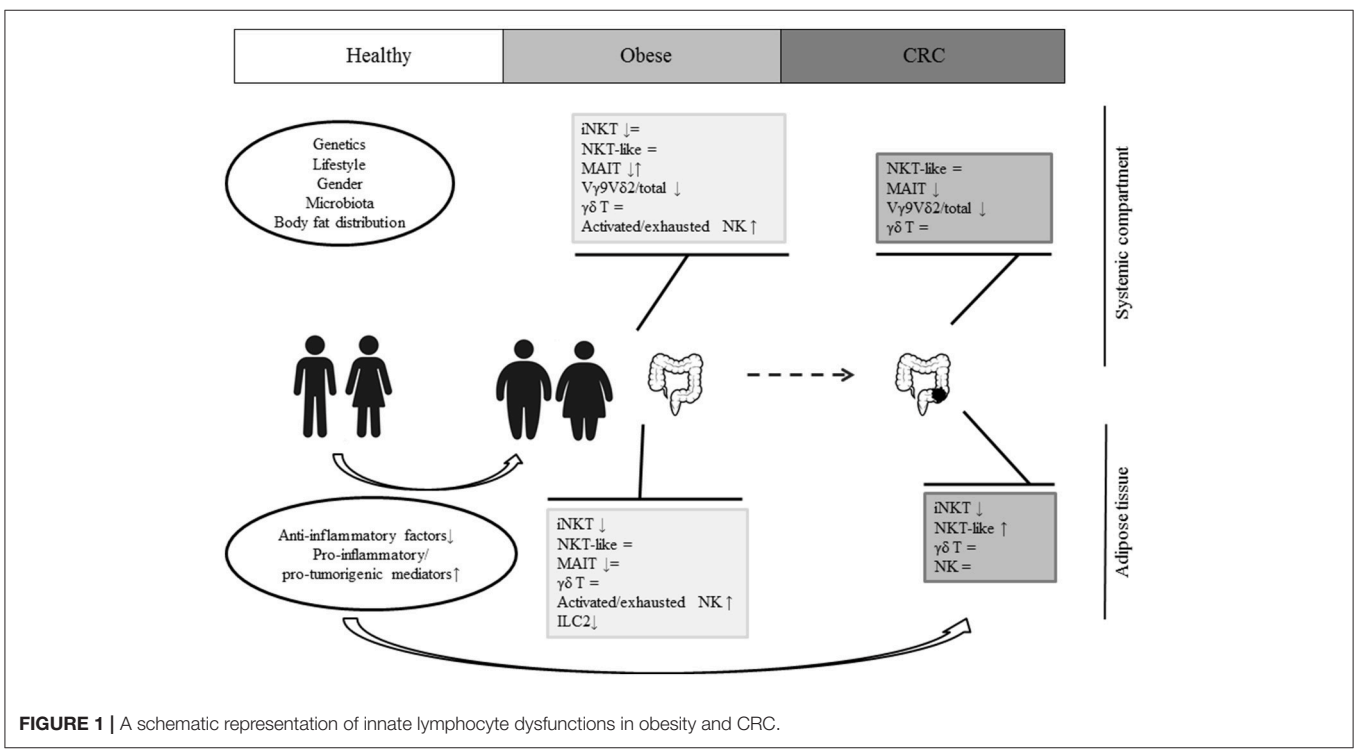

FIGURE 1 | A schematic representation of innate lymphocyte dysfunctions in obesity and CRC.

lymphocyte frequencies and functions occurring in obesity are expected to have an impact on both inflammation and surveillance against tumors. In particular, MAIT cells have the potential to either promote malignancy through the production of IL-17, or contribute to anticancer immunity by expressing cytotoxic effector molecules (36). The skewed MAIT cell differentiation toward an IL-17 producing phenotype occurring in obese subjects could at least in part explain the detrimental role of AT inflammation in the generation of a pro-tumorigenic environment. Of note, aberrant activation of the IL-23/IL-17 axis has been linked to tumorigenesis and adverse prognosis in CRC (56), suggesting that the hyper-production of IL-17 occurring in AT could be of relevance for obesity-associated CRC pathogenesis. Likewise, the exhausted phenotype of NK cells, the aberrant activation of iNKT cells and the contraction of the Vγ9Vδ2 T lymphocyte subset observed in obese subjects could contribute to the greater susceptibility of these individuals to develop cancer or infectious diseases. Indeed subjects with low circulating NK cell activity have a 10-fold higher risk of developing CRC compared to subjects with high NK cell activity, and the HNK1 NKG2D genotype, associated with high NK cell activity, exerts a protective effect against CRC (57, 58). In support of this, alterations of some of these innate lymphocyte subsets have also been observed in blood or AT of CRC patients, suggesting a direct link between obesity-induced dysfunctions and cancer.

CONCLUDING REMARKS

Obesity results from a complex interplay of different factors including genetics, gender, lifestyle, microbiota, and body fat distribution. The shift from a lean to an obese condition is associated with profound changes in the balance between anti- and pro-inflammatory mediators, release of pro-tumorigenic factors and alteration of local and systemic immune profiles. As schematically depicted in **Figure 1**, a selective obesity-associated depletion and/or reduced activity of different innate lymphocyte populations has been recently highlighted, pointing to a key role of these cells in the control of inflammation. However, although it is likely that obesity-associated inflamed/immunosuppressive environment can ultimately alter intestinal mucosa homeostasis and support tumor development/progression, the role of innate lymphocytes in this process has not been fully elucidated yet. Moreover, whether their capacity to home to the tumor site and to perform their effector functions is impaired in obesity remains to be defined. If this knowledge is acquired, it will provide further rationale to the promising approach of boosting the effector functions of innate lymphocytes, in addition to those of conventional T cells, to improve cancer immunotherapy.

Obesity is a major risk factor for different cancer types, including CRC, with the latter known to be highly modifiable through lifestyle. Further studies aimed at dissecting the influence of a healthy/unhealthy lifestyle on the mechanisms linking obesity to CRC will allow a better exploitation of lifestyle-based interventions in controlling tissue homeostasis and immune-surveillance.

AUTHOR CONTRIBUTIONS

MD, LC, and SG contributed to the conception, writing, and editing of this manuscript. All authors read and approved the final manuscript.

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REVIEW
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T Cells in Adipose Tissue: Critical Players in Immunometabolism

Qun Wang[1]* *and Huaizhu Wu*[2]*

[1] Key Laboratory of Infection and Immunity of Shandong Province, Department of Immunology, School of Basic Medical Sciences, Shandong University, Jinan, China, [2] Department of Medicine and Department of Pediatrics, Baylor College of Medicine, Houston, TX, United States

Adipose tissue performs immunoregulatory functions in addition to fat storage. Various T cells in different fat depots either help maintain metabolic homeostasis under healthy conditions or contribute to metabolic disorders in pathological conditions such as obesity, diabetes, cardiovascular diseases, or even cancer. These T cells play critical roles in immunometabolism, which refers to the intersection of immunity and metabolism. Numerous studies have examined the presence and changes of different T cell subsets, including helper T cells, regulatory T cells, cytotoxic T cells, and natural killer T cells, in adipose depots in health and diseases. In this review, we will discuss the adipose tissue niches that influence the patterns and functions of T cell subsets and in turn the impact of these T cells on cell- or body-based immunometabolism accounting for health and obesity.

Keywords: T cell, adipose tissue, immunometabolism, obesity, insulin resistance

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John J. Miles,
James Cook University, Australia

***Correspondence:**
Qun Wang
wangqun@sdu.edu.cn
Huaizhu Wu
hwu@bcm.edu

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INTRODUCTION

Immunometabolism focuses on the interaction of immunity and metabolism, from metabolic patterns of immune cells to metabolic homeostasis or disorders dominated by immune cells. The former, as reviewed by Man et al. (1), is considered *cellular immunometabolism,* which includes the intracellular metabolism of different immune cells or immune cells in different conditions, such as macrophages or T cells during activation, polarization, proliferation, and differentiation. The latter is *tissue immunometabolism,* which explores the impacts of immune cells on tissue and systemic metabolism in various microenvironments (1–4). The immune system, which is influenced by the metabolic status of the body, in turn produces substantial impacts on local and systemic metabolic homeostasis or disorders. In recent years, the effects of immune responses on metabolic abnormalities such as obesity, diabetes, and fatty liver disease have drawn great interest from researchers, and emerging evidence has suggested that adipose tissue serves as an important intersection linking immunity with metabolism (4, 5). Besides its traditional function in fat storage, adipose tissue is currently recognized as an endocrine organ (6–8). Importantly, accumulating data have shown that adipose tissue contains a large number of immune cells, including macrophages, eosinophils, innate lymphoid cells (ILCs), T cells, and B cells, which regulate immune homeostasis and inflammation, subsequently influencing metabolism of adipose tissue and the whole body (4, 5, 9–11). As the core component of adaptive immunity, T cells play indispensable roles in tissue immunometabolism. Here, we review the distinct profiles of T cell subsets in specific adipose tissue microenvironments and their influences on metabolic homeostasis or disorders.

ADIPOSE TISSUE PROVIDES A STAGE FOR THE INTERPLAY BETWEEN IMMUNITY AND METABOLISM

There are two types of adipose tissue in mammals, white adipose tissue (WAT) for energy storage in the form of fat (triglyceride) and brown adipose tissue (BAT) for energy dissipation through thermogenesis. WAT is widely distributed throughout the body including subcutaneous adipose tissue (SAT) underneath the skin and intra-abdominal fat depots known as visceral adipose tissue (VAT) (12–14). Human SAT predominantly exists in the areas of abdomen, leg, and buttock, whereas VAT is mainly around omentum, mesenterium, and perirenal areas (12, 14). The initial link between WAT and immune function was demonstrated in several studies that revealed an association of obesity with WAT inflammation (15). Hotamisligil and colleagues demonstrated that the proinflammatory cytokine tumor necrosis factor (TNF)-α is elevated in VAT in obese animals compared with their lean counterparts and plays critical roles in obesity-induced insulin resistance (15, 16). In humans, the level of TNF-α is also increased in fat tissues from obese individuals and positively correlated with hyperinsulinemia (16). In addition, the proinflammatory cytokine interleukin (IL)-6 from adipose tissue also contributes to obesity-induced insulin resistance in both humans and mice (17, 18). Adipocytes are able to produce numerous inflammatory molecules, including TNF-α and IL-6, whereas macrophages appear to be the major source of these proinflammatory cytokines in adipose tissue *in vivo* as demonstrated in a mouse model (19). Indeed, macrophages were first reported to be increased and to polarize into classically activated M1-like phenotype in adipose tissues in obese humans and animals (6, 19–22). The chemokine monocyte chemoattractant protein-1 (MCP-1) is elevated and may contribute to the infiltration of macrophages in obese WAT and subsequently to obesity-induced insulin resistance (21, 23–25). Subsequently, T cells were found to be elevated in adipose tissue in obese mice and humans (26), and effector T cells, including $CD4^+$ helper T (Th) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), may serve as active players in obesity-associated WAT inflammation (27–30). In addition, several other immune cell populations or subsets mainly associated with type 2 immune response, such as type 2 innate lymphoid cells (ILC2), alternatively activated M2 macrophages, eosinophils, invariant natural killer T (iNKT) cells, and regulatory T or B cells, reside in adipose tissue under normal conditions but are reduced in obesity (31–35). These type 2 immune cells may be involved in maintenance of both immune and metabolic homeostasis under normal conditions. Energy excess or obesity can cause the disruption of this homeostasis and induce a new immune cell profile in adipose tissue that drives adipose tissue inflammation, insulin resistance, and related metabolic disorders.

VARIOUS T CELL SUBSETS IN DIFFERENT ADIPOSE TISSUE NICHES

Based on the composition of T-cell antigen receptors (TCR), T cells can be classified into two populations, αβT cells and γδT cells, both of which perform critical immune functions. While αβT cells serve in adaptive immunity, γδT cells act mainly in innate immunity. According to the cell surface markers, αβT cells can be further divided into two subsets: $CD4^+$ T cells and $CD8^+$ T cells. After activation by antigen stimulation, T cells can proliferate and differentiate into effector T cells. $CD4^+$ T cells differentiate into effector Th cells and $CD8^+$ T cells differentiate into CTLs, thus exerting distinct effects. An important regulatory subset among $CD4^+$ T cells is regulatory T (Treg) cells, which have a specific molecular signature as $CD4^+$ $CD25^+$ $Foxp3^+$. Treg cells inhibit the activation of T cells and the functions of effector T cells as well as B cells and NK cells, participating in the maintenance of tissue homeostasis and self-tolerance, or in the pathogenesis of some morbidities through negatively regulating immune responses (36).

The implication of T cells in obesity-induced inflammation was first indicated by the increased T cell accumulation in VAT in obese mice and humans as compared with their lean counterparts (26). The chemokine CCL5 (also known as regulated on activation, normal T cell expressed and secreted [RANTES]) is upregulated in VAT in obesity and may account for the recruitment of T cells into obese VAT (26, 37, 38). Importantly, T cells are increased early, likely preceding the infiltration of macrophages, in VAT in mice on high-fat diet (HFD), and play important roles in macrophage recruitment and VAT inflammation (30, 39, 40). While different effector T cell subsets are implicated in adipose tissue inflammation, regulatory T cell subsets are involved in healthy or normal adipose tissue homeostasis (31). Given the heterogeneity of T cells, we will discuss in this section the various patterns and functions of different T cell subtypes in adipose tissue niches.

TREG CELLS SERVE TO MAINTAIN ADIPOSE TISSUE HOMEOSTASIS

The first finding regarding adipose-resident Treg cells was from Feuerer and colleagues, who reported an enrichment of $CD4^+$ $Foxp3^+$ Treg cells in VAT from lean mice (31). Besides the canonical gene signature such as Foxp3, CD25, glucocorticoid-induced tumor necrosis factor receptor (GITR), cytotoxic T lymphocyte antigen-4 (CTLA-4), and OX40, these Treg cells in VAT possess a phenotype different from those residing in lymphoid tissues, with distinct expression patterns of many Treg signature genes such as CD103 and G protein–coupled receptor−83 (31). Treg cells are markedly reduced in VAT of mice with diet-induced obesity. In addition, depleting Treg cells in lean mice induces the gene expression of inflammatory mediators (such as TNF-α, IL-6, and CCL5) and impairs the metabolic signal pathway in VAT, whereas expanding Treg cells in HFD-fed obese mice improves metabolic parameters, possibly through the regulation of adipose tissue inflammation, suggesting that Treg cells play crucial roles in the maintenance of immune and metabolic homeostasis of adipose tissue and may have beneficial effects on systemic metabolic abnormalities associated with obesity (31, 41).

The mechanisms for the enrichment and function of Treg cells in lean VAT have not been fully defined. To date, several factors are considered to be critical for the maintenance of Treg cells in VAT. First, peroxisome proliferator-activated receptor-γ (PPAR-γ) expressed by Treg cells is necessary for the accumulation, phenotype, and function of VAT Treg cells in lean mice through collaborating with Foxp3 to induce a distinct Treg signature; while obesity induces the disappearance of this VAT Treg signature by phosphorylation of PPAR-γ at position Ser273 (42–44). These findings established a foundation for adipose Treg cells and opened a new area of research to elucidate the precise mechanisms by which PPAR-γ regulates VAT Treg signature. Second, the IL-33/suppression of tumorigenicity 2 (ST-2) axis plays an essential role in the amplification of Treg cells in VAT (45–47). IL-33 is a cytokine of the IL-1 family and can be produced by human adipocytes or mice stromal cells in VAT; ST-2, the receptor for IL-33, is highly expressed on VAT Treg cells in both humans and mice (45, 46, 48, 49). IL-33 can promote the development and proliferation of Treg cells and then restore their numbers in VAT, with attenuation in VAT inflammation and improvements in the metabolic parameters in obese mice (45–47). More recently, Kohlgruber and colleagues reported that adipose-resident γδT cells positive for the Broad-complex, Tramtrack, and Bric-à-brac/poxvirus and zinc finger (BTB-POZ) transcription factor PLZF produce IL-17, which induces IL-33 expression from adipose stromal cells, thereby contributing to age-dependent Treg cell accumulation in adipose tissue (49). The same research group also found that *i*NKT cells, a unique regulatory population residing in adipose tissue, help sustain the immune homeostasis of adipose tissue through regulating the number and function of Treg cells (50) (see discussion below). In addition, IL-33 can active ILC2, which promotes Treg cell accumulation in VAT through the ligation of ICOSL on ILC2 with ICOS on Treg cells. This process can be suppressed by interferon (IFN)-γ, which elicits VAT inflammation and metabolic disorders (51). Thus, it is acknowledged that the accumulation of Treg cells in VAT is a multifactorial process, which is further substantiated by a recent report showing the collaboration of TCR, Foxp3, and ST2 in this process (52). Some other factors such as leptin, IL-21, and autophagy-related atg7 have been reported to influence Treg cells in VAT and systemic insulin sensitivity (53–55). However, the direct link and underlying mechanisms remain to be established. Taken together, the available data indicate that various cells and molecules form specific adipose tissue niches that contribute to the pool and function of adipose Treg cells and maintain the homeostasis of systemic metabolism (**Figure 1**).

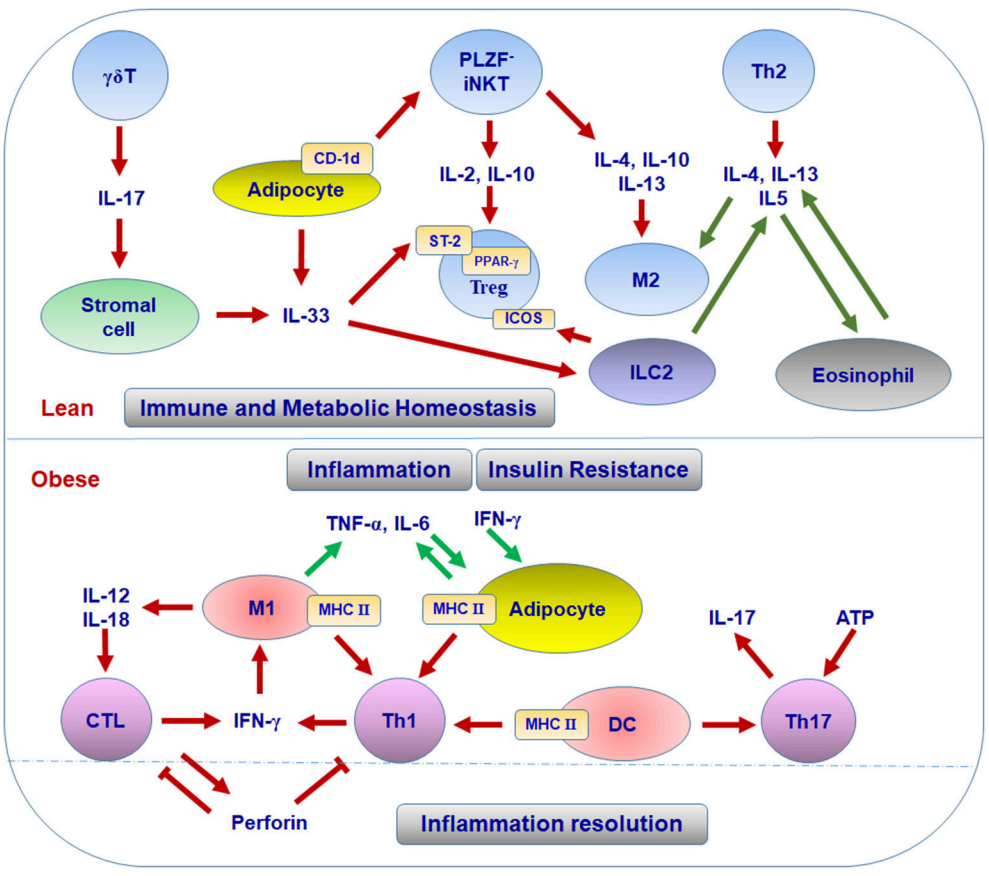

FIGURE 1 | T cell subsets in different adipose niches in lean and obese conditions.

TH CELLS MEDIATE DIVERGENT IMMUNE AND METABOLIC EFFECTS ON ADIPOSE TISSUE THROUGH DIFFERENT SUBTYPES

Based on the stimuli, Th cells can polarize into different phenotypes that express distinct cytokine profiles and exert different effector functions. Th1 cells produce IFN-γ, which promotes the polarization of classically activated M1 macrophages, and participate in elimination of intracellular microbes. Th2 cells produce IL-4, IL-5, and IL-13 to promote the polarization of alternatively activated M2 macrophages, help B cell responses, and participate in the immune responses against helminths. Th17 cells produce IL-17 to induce neutrophil inflammation and participate in the pathogenesis of several autoimmune diseases (36).

Th1 Subset

The first Th subset identified in adipose tissue was Th1 cells. The infiltration of $CD4^+$ T cells was initially identified in human VAT and showed significant correlation with the body mass index (39). Furthermore, waist circumference correlated with IFN-γ mRNA in SAT from patients with type 2 diabetes, suggesting an association of adipose tissue Th1 cells with obesity (39). $CD4^+$ T cells are also increased in VAT of obese mice compared with lean controls. Importantly, $CD4^+$ T cells from obese VAT produce higher amounts of IFN-γ than those from lean VAT, indicating Th1 polarization in obese VAT (29, 40, 56–58). Furthermore, deficiency of T cells, including $CD4^+$ T cells or IFN-γ significantly reduces adipose tissue inflammation and improves insulin sensitivity in obese mice, suggesting the substantial contribution of Th1 cells to adipose inflammation and metabolic dysfunctions associated with obesity (29, 59). Th1 cells and IFN-γ, the major Th1 and CTL cytokine, can directly interrupt insulin signaling, leading to insulin resistance in adipocytes and skeletal muscle myocytes, which may contribute to systemic insulin resistance in obesity (29, 58, 60). As to the mechanisms for Th1 polarization in obese VAT, obesity increases the levels of class II major histocompatibility complex (MHC II) and costimulatory molecules on VAT macrophages/dendritic cells (DCs) and also on adipocytes. These adipocytes and macrophages/DCs can function as antigen-presenting cells (APCs) to promote adipose tissue $CD4^+$ T cell proliferation and production of IFN-γ, which further activate adipocytes and macrophages/DCs with elevated MHC II, thereby forming a positive loop to amplify Th1 cell-mediated inflammation in adipose tissue (**Figure 1**) (39, 40, 57, 59, 61, 62). Importantly, adipocyte- or macrophage-specific depletion of MHC II in obese mice reduces $CD4^+$ T cell numbers (especially the effector/memory $CD4^+$ T cells) and IFN-γ production in VAT and improves insulin sensitivity, indicating critical roles of MHC II on adipocytes or macrophages in adipose tissue Th1 polarization, which contributes to obesity-induced adipose tissue inflammation and insulin resistance (40, 56, 63).

On the other hand, several pivotal costimulatory receptors on T cells such as 4-1BB and CD28 are reported to be implicated in obesity-induced adipose tissue inflammation and metabolic disorders, as evidenced by the fact that 4-1BB or CD28 deficiency in mice improves related metabolic parameters and reduces T cell infiltration in VAT (64, 65). Considering the crucial roles of costimulatory signals 4-1BB−4-1BBL and CD28–B7 in T cell activation and proliferation and Th1 polarization (66–68), it is reasonable to deduce the potential contribution of 4-1BB and CD28 to Th1 polarization in obese VAT; however, future studies are needed for confirmation.

ICOS is another inducible costimulatory receptor that can be broadly expressed by various T cells and implicated in the expansion and function of Th2 and Treg cells, and even Th1, Th17, and NKT cells (51, 69–73). The expression patterns of ICOS on adipose tissue T cells and its potential complex functions in lean and obese states are intriguing and warrant investigation. Furthermore, as exosomes from macrophages/DCs can carry MHC II or costimulatory molecules that help the activation and proliferation of T cells (74–76), it is very likely that APCs residing in VAT secrete exosomes to promote activation and further Th1 polarization of $CD4^+$ T cells, thereby driving adipose tissue inflammation and insulin resistance. This is a promising field of research that deserves further exploration and may help to delineate the mechanisms for Th1 polarization in obese VAT.

Th17 Subset

A few earlier studies reported the association of obesity with IL-17 or Th17 cells (77–79). However, these studies lacked conclusive evidence for the presence of Th17 cells in adipose tissue. First, besides Th17 cells, γδT cells are another important source of IL-17. Therefore, the role of IL-17 in obesity cannot be completely attributed to Th17 cells and needs to be further elaborated. Second, the increase of Th17 subset in lymphoid tissues, including spleen and lymphoid nodes, in obesity, or in concert with other medical conditions like experimental allergic encephalomyelitis or trinitrobenzene sulfonic acid colitis may be distinguished from the presence of Th17 subset in adipose tissue, and additional studies are needed to explain fully the influence of Th17 cells on immunometabolism (77–79). Nevertheless, Bertola and colleagues found that IL-17–producing T cells are dramatically increased in SAT from overweight or obese individuals compared with that from lean subjects and further demonstrated that DCs from obese subjects may drive the differentiation of Th17 cells, which produce high amounts of IL-17, indicating that Th17 cells may directly participate in adipose tissue inflammation and insulin resistance in obesity (80). In addition, several more studies confirmed the elevation of Th17 cells in adipose tissue, especially in VAT, in patients with adiposity (81–83). More recently, a study showed that ATP drives the Th17 responses via P2X7 receptor in human VAT, pointing to a possibility that adipose tissue niches facilitate the differentiation of Th17 cells (84). However, more studies are needed to verify the profile, functions, and underlying mechanisms of Th17 cells in adipose tissue and the direct contribution of Th17 cells to adipose tissue inflammation and insulin resistance.

In addition to Th17, γδT cells that reside in adipose tissue and are increased in obesity (85), also express IL-17. As mentioned above, adipose γδT cells can contribute to the regulation of age-dependent adipose tissue Treg homeostasis through IL-17–induced IL-33 production from adipose stromal cells (49). Thus,

based on the increases of both Th17 and γδT cells in obese adipose tissue and the effect of IL-17 on IL-33 production, these two IL-17–producing cell subsets may also have the potential to initiate downregulation of the immune inflammatory reaction in adipose tissue during obesity, although this remains to be demonstrated.

Th2 Subset

Th2 cells produce type 2 cytokines, including IL-4, IL-5, and IL-13, which play important roles in macrophage polarization into M2 phenotypes. Several cell types, including eosinophils and ILC2, have been identified in lean adipose tissue to produce type 2 cytokines and may contribute to M2 polarization, inflammation resolution, and metabolic homeostasis in WAT under normal conditions (34, 35). However, data are limited on Th2 cells in adipose tissue. A study showed that the percentage of Th2 cells in human SAT and VAT negatively correlates with systemic inflammation and insulin resistance, indicating a protective role of Th2 cells in inflammation and metabolic dysfunctions (83). Another study revealed that after adoptive transfer into obese Rag1-null mice, $CD4^+$ T cells gained a Th2 profile, indicated by the production of IL-4 and IL-13, which was associated with reversal of enhanced weight gain and insulin resistance in recipient obese Rag1-null mice. Consistently, transfer of $CD4^+$ T cells deficient in signal transducer and activator of transcription 6 (STAT6), a transcription factor important for Th2 polarization, into obese Rag1-null mice resulted in the reduction in Th2 cells in VAT and the loss of protective effects on obesity-related metabolic parameters in recipient Rag1-null mice (86).

CTLS PERFORM BIDIRECTIONAL FUNCTION IN ADIPOSE TISSUE INFLAMMATION AND HOMEOSTASIS

Similar to $CD4^+$ T cells, $CD8^+$ T cells are significantly increased in adipose tissue in obesity in both humans and mice (28, 30, 59, 87, 88). Along with macrophages, $CD8^+$ T cells participate in formation of crown-like structures (CLSs) surrounding dying/dead adipocytes in adipose tissue of mice (30). Increased infiltration as well as IL-12– and IL-18–mediated proliferation and activation may contribute to the increase and activation of $CD8^+$ T cells in adipose tissue in obese mice (28). The increase in adipose tissue $CD8^+$ T cells appears to precede and contributes to the accumulation of adipose tissue macrophages and metabolic dysfunctions in obesity. In support of this, depletion of $CD8^+$ T cells in obese mice dramatically decreases numbers of M1 macrophages and CLSs in adipose tissue, accompanied by an improvement of insulin sensitivity, whereas adoptive transfer of $CD8^+$ T cells into CD8-deficient mice fed with HFD increases numbers of adipose tissue macrophages and CLSs, with elevated levels of proinflammatory cytokines and aggravated insulin resistance (30). The increase of IFN-γ-expressing $CD8^+$ T cells in VAT in obesity further substantiates the contribution of $CD8^+$ T cells to macrophage activation through the action of IFN-γ (28, 30, 89, 90).

Besides their contribution to adipose tissue inflammation, effector CTLs may also function in restricting T cell expansion and activation in inflamed WAT through perforin. Perforin-dependent cytotoxicity is not only an important way to attack target cells, but also a critical regulator to limit abnormal T cell activation in a physiological context (91, 92). In mice fed HFD, depletion of perforin causes aggravated adiposity and insulin resistance, together with upregulation of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells as well as M1 macrophages in VAT. Perforin-deficient $CD8^+$ T cells from VAT show increased proliferation but impaired early apoptosis. Transfer of perforin-deficient $CD8^+$ T cells into CD8-deficient mice exacerbates the metabolic parameters more than wild-type $CD8^+$ T cells (93). These findings suggest that CTLs in fat tissue not only mediate adipose tissue inflammation in obesity, but may also contribute, at least partially, to the resolution of T cell–mediated inflammation through perforin-dependent cytotoxicity (**Figure 1**).

NKT CELLS MAINTAIN THE IMMUNE AND METABOLIC HOMEOSTASIS IN ADIPOSE TISSUE

NKT cells are a unique subset of T cells that express both NK cell markers (such as NK1.1 or CD56) and T cell marker αβTCR. The main function of NKT cells is to recognize glycolipid antigen presented by MHC-class-I-like molecule CD1d. Based on the expression of an invariant TCRα chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans), CD1d-dependent NKT cells can be classified into type I and type II NKT cells, both of which can produce IFN-γ, the Th1 cytokine, and IL-4, a Th2 cytokine, and participate in the regulation of innate and adaptive immunity. Type I NKT cells express the invariant TCRα chain in combination with certain TCRβ chains (Vβ8.2,7,2 in mice, Vβ11 in humans) and are also called iNKT cells, whereas type II NKT cells do not express this invariant TCRα chain (94–96).

Compared with other organs, adipose tissue in both humans and mice is enriched with iNKT cells under normal conditions, whereas obesity dramatically decreases iNKT cells in adipose tissue (97, 98). Accordingly, weight loss restores adipose tissue iNKT cells in murine models and peripheral iNKT cells in obese humans (97, 98). Huh and colleagues reported that the maintenance of iNKT cell numbers and activation in adipose tissue relies on their interaction with CD1d expressed on adipocytes. Adipocytes with high expression of CD1d under normal conditions function as APCs to present lipid antigens to maintain iNKT cells in adipose tissue and stimulate their activation, whereas obesity reduces CD1d expression in human and mouse adipose tissue, leading to the reduction of adipose tissue iNKT cells (99–101).

Depletion of iNKT cells (deficient in Jα18) or deficiency of CD1d in mice exacerbates HFD-induced weight gain, adipocyte hypertrophy, fatty liver, and insulin resistance as compared with wild type controls, whereas adoptive transfer of iNKT cells into obese Jα18-deficient mice or activating iNKT cells by lipid ligand α-galactocylceramidee (αGalCer) in obese wild-type mice reverses HFD-induced phenotypes, with reduced

weight gain and adipocyte hypertrophy, and alleviated fatty liver and insulin resistance. These effects indicate a protective role of iNKT cells in HFD-induced weight gain and metabolic dysfunctions. Adipose tissue–resident iNKT cells express the transcription factor E4BP4, but not the BTB-POZ transcription factor PLZF. Under normal conditions, these adipose tissue iNKT cells produce high levels of IL-2 and type 2 cytokines such as IL-4, IL-10, and IL-13, but low level of IFN-γ, as compared to iNKT cells from the spleen. Indeed, the type 2 cytokines are downregulated in VAT of mice with CD1d deficiency and upregulated by αGalCe treatment, which is consistent with changes in the numbers of iNKT cells in VAT, suggesting that iNKT cells make substantial contributions to the levels of regulatory type 2 cytokines in VAT. These type 2 cytokines may inhibit the infiltration and activation of proinflammatory M1-like macrophages, but enhance polarization of M2 macrophages as well as expansion and suppressive function of Treg cells, thereby maintaining immune homeostasis and alleviating inflammation in adipose tissue (50, 98, 102). Adipocyte-specific deficiency of CD1d in obese mice attenuates the responses of iNKT cells to αGalCer, leading to reduced expression of IL-4 and IL-2 in iNKT cells, subsequent impairment of the anti-inflammatory responses mediated by M2 macrophages and Treg cells, and aggravation of adipose tissue inflammation and insulin resistance (99–101). All these findings support an important role of iNKT cells in maintaining adipose tissue homeostasis under normal conditions and in protecting against adipose tissue inflammation and metabolic dysfunctions associated with obesity, possibly through producing type 2 cytokines (**Figure 1**).

In addition, iNKT activation–mediated weight loss and improvement of insulin sensitivity in obese mice may also be attributable to β-oxidation–mediated energy expenditure and thermogenesis. Mechanistically, activation of iNKT with αGalCer treatment strongly induces the expression and production of FGF21 in both BAT and inguinal SAT, which drives the activation of BAT and browning of WAT to burn fat through β-oxidation. These findings point to another potential mechanism for the beneficial roles of iNKT cells in adipose tissue to maintain metabolic homeostasis through thermogenesis (103). However, a potential connection between iNKT effects on thermogenesis and inflammation remains to be clarified.

Taken together, these observations indicate that adipose tissue iNKT cells, as a unique regulatory immune cell subset, play important roles in both immune regulation and lipid metabolism to maintain the homeostasis of immunometabolism.

QUESTIONS AND PERSPECTIVES

T cells reside in the network of adipose tissue, in which different types of cells interact with each other through the action of various cytokines, adipokines and membrane receptors. Beyond the information that is already known, additional components of the network may influence the profile and functions of T cells in adipose tissue. For examples, it remains an open question whether unknown or newly-discovered T cell subsets such as Th9 and Th22 cells exist and function in adipose tissue. The distinct signatures and regulatory mechanisms of well-recognized adipose T cell subsets, including CTL, Th1, and Th17 cells, need to be elaborated. Given the direct participation of iNKT cells in thermogenesis and of Treg cells in lipid uptake (42, 103), precise elucidation of metabolic functions of various T cells in physiopathological adipose tissue may provide new insight for the direct contribution of T cells to metabolism beyond immunity. Moreover, the interactions of T cells with other, non-immune cells in the adipose stromal vascular fraction, such as stem cells or endothelial cells, may also be crucial events that impact the profile and functions of T cells. It has been demonstrated that adipose-derived stem cells from lean mice regulate macrophage polarization, thereby reducing adipose tissue inflammation, whereas those from obese subjects induce Th17 cells and activate monocytes, thus promoting inflammation

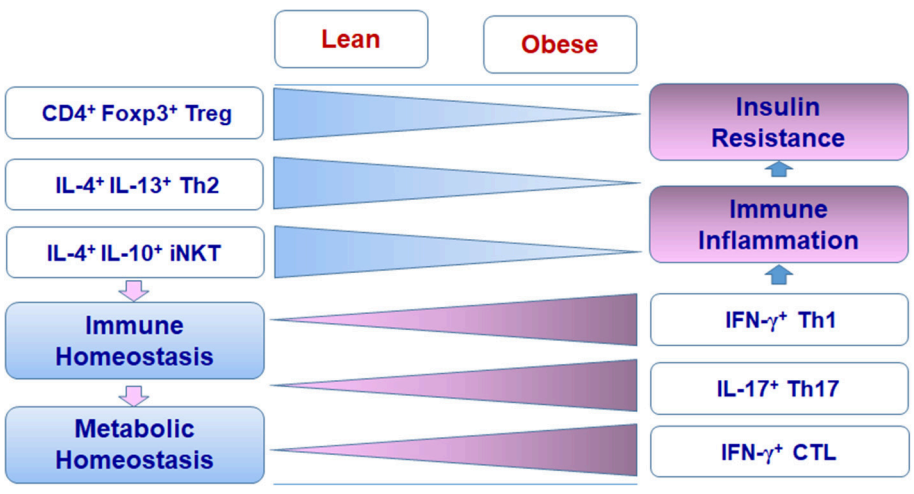

FIGURE 2 | Changes of T cell subsets in adipose tissue in obesity.

(104–106). Therefore, it is important to examine the effects of these cells on different adipose T cell populations, which may link immunity with metabolism in adipose tissue in a different manner. Finally, given the discussed roles of various types of T cells in obese adipose tissue, mainly observed in animal models and tissue culture, it is important to explore the feasibility of targeting these immune cells as new therapies for obesity-related metabolic disease in humans.

CONCLUDING REMARKS

Adipose tissue performs complex functions related to metabolism, immune responses, and endocrine effects. Besides adipocytes and preadipocytes, adipose tissue includes various immune cells that compose special adipose niches under different physiological or pathological conditions. T cells function as critical players in adipose tissue and influence the balance and functions of various populations of immune cells, exerting beneficial or detrimental effects on immunometabolism. In the healthy state, Treg cells, Th2 cells, and iNKT cells work with other regulatory immune cells such as M2 macrophages, ILC2, and eosinophils to maintain the immune and metabolic homeostasis of adipose tissue, providing a steady environment to retain normal systemic metabolism. When obesity develops, Th1 cells, Th17 cells, and CTLs accumulate in adipose tissue and, along with other proinflammatory immune cells such as M1 macrophages, disrupt the immune homeostasis, causing adipose tissue inflammation and systemic insulin resistance (**Figures 1**, **2**). The diversity of T cell pools in adipose tissue, either as friend or foe, may result from the change of metabolism and in turn influence metabolism in various ways.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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A Unique Population: Adipose-Resident Regulatory T Cells

Qin Zeng[1†], Xiaoxiao Sun[1,2†], Liuling Xiao[3], Zhiguo Xie[1], Maria Bettini[4] and Tuo Deng[1,2,3]*

[1] Department of Metabolism and Endocrinology, The Second Xiangya Hospital, Central South University, Changsha, China, [2] Key Laboratory of Diabetes Immunology, Central South University, Ministry of Education, Changsha, China, [3] Center for Bioenergetics, Weill Cornell Medical College, Houston Methodist Research Institute, Houston, TX, United States, [4] Section of Diabetes and Endocrinology, Department of Pediatrics, Baylor College of Medicine, McNair Medical Institute, Texas Children's Hospital, Houston, TX, United States

Regulatory T (Treg) cell is well known for its anti-inflammatory function in a variety of tissues in health and disease. Accordingly, Treg cells that reside in adipose tissue exhibit specific phenotypes. Their numbers are regulated by age, gender and environmental factors, such as diet and cold stimulation. Adipose-resident Treg cells have been suggested to be critical regulators of immune and metabolic microenvironment in adipose tissue, as well as involved in pathogenesis of obesity-related metabolic disorders. This review surveys existing information on adipose-resident Treg cells. We first describe the origin, phenotype and function of adipose-resident Treg cells. We then describe the major regulators of adipose-resident Treg cells, and discuss how the adipose-resident Treg cells are regulated in lean and obese conditions, especially in humans. Finally, we highlight their therapeutic potential in obesity-related disorders.

Keywords: adipose tissue, inflammation, regulatory T cell, obesity, metabolic disease

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Huaizhu Wu,
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Reviewed by:
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University of New Mexico Health Sciences Center, United States
Wenxian Fu,
University of California, San Diego,
United States

***Correspondence:**
Tuo Deng
tdeng@houstonmethodist.org

† These authors have contributed equally to this work

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INTRODUCTION

Adipose tissue inflammation is implicated in associations between obesity and its multiple complications (1, 2). Obese adipose tissue is laden with a variety of pro-inflammatory immune cells, including classically activated macrophages, natural killer (NK) cells, mast cells, neutrophils, dendritic cells (DCs), B cells, cytotoxic T cells and Th1 cells (3, 4). These cells can stimulate adipocytes and release pro-inflammatory factors, such as IFNγ, TNFα, IL-1β, and IL-6, leading to the development of local and systemic inflammation, insulin resistance, and type 2 diabetes. In contrast, lean adipose tissue contains significantly lower numbers of immune cells, the majority of which are anti-inflammatory cells including Treg cells, alternatively activated macrophages, eosinophils, and group 2 innate lymphoid cells (ILC2s). These cells actively maintain metabolic homeostasis in adipose tissue by secretion of anti-inflammatory mediators, such as IL-4 and IL-13.

An important anti-inflammatory immune cell population in adipose tissue is Treg cell. Treg cells play critical roles in the regulation of autoimmunity (5), allergy (6), microbial infection (7), and tumor development (8). Besides the classical Treg cells circulating through lymphoid organs, Treg cells residing in parenchymal tissue, so-called tissue Treg cells, are also crucial for the maintenance of organismal homeostasis (9). Adipose-resident Treg is one of the best-characterized tissue Treg cells. Dr. Diane Mathis's group published pioneering work in the field of adipose-resident Treg cells. In 2009, they reported that the abdominal fat of lean mice was enriched in Treg cells with a unique phenotype, and these adipose-resident Treg cells played an important role in maintaining

insulin sensitivity through limiting adipose tissue inflammation. Importantly, these cells decreased in fat tissue of obese animals (10). Three years later, the same group reported the molecular basis of this unique population of visceral adipose tissue (VAT) Treg cells by identifying peroxisome-proliferator-activated receptor γ (PPARγ), the master regulator of adipocyte differentiation, as the major driver behind development, tissue accumulation, and functional phenotype of these cells (11). Since then, the development, regulation and function of adipose-resident Treg cells have been extensively investigated. Accumulating evidence shows that adipose-resident Treg cells have unique properties, distinct from lymphoid organ Treg cells, and respond to different environmental challenges to regulate immune and metabolic states in normal and disease conditions.

In this review, we summarize current understanding of the characteristics, regulation and function of adipose-resident Treg cells, as well as discuss their metabolic impact and therapeutic potential. Elucidating the interactions underlying adipose-resident Treg cells and adipose inflammation is of significant interest and may provide new insights to overcome the harmful side effects of an obese state.

CHARACTERISTICS OF ADIPOSE-RESIDENT TREG CELLS

Adipose tissue types can be subdivided based on their distinct developmental origins, anatomical locations and functions. These include VAT, subcutaneous adipose tissue (SAT) and brown adipose tissue (BAT). VAT and SAT are composed primarily of white adipocytes with their major function being energy storage, while BAT consists of brown adipocytes with a primary function of thermogenesis. In lean adult male mice, high enrichment of Treg cells (∼50% of $CD4^+$ T cells) is observed only in VAT, but not in other fat depots (10). Therefore, initial studies were focused on VAT adipose-resident Treg cells. Recently, it has been shown that SAT and BAT also harbor distinct subsets of Treg cells characterized by unique gene signatures (12, 13). It is clear that Treg cells in different types of adipose tissue have distinct properties that are critical for the regulation of local immune and metabolic environment. In this section, we review the origin, phenotypes and functions of the Treg cells from different fat depots.

Origin

In 10-week-old mice VAT and SAT have relatively low frequencies of Treg cells (10–15% of $CD4^+$ T cells), comparable to what is observed in lymphoid tissues. However, only VAT Treg cells accumulate overtime reaching 40–50% at around 25 weeks of age (10, 14). The striking enrichment of Treg cells in VAT has attracted significant interest to investigate the origin and development of VAT Treg cells. Classical Treg cells are generated in the thymus or converted from conventional $CD4^+$ T cells in the periphery (15, 16). Because the T cell receptor (TCR) repertoire of VAT Treg cells is distinct from VAT conventional T (Tconv) cells, it is unlikely that the $Foxp3^+$ Treg cells in the VAT are differentiated from local Tconv cells (10). A recent study further excluded the possibility of conversion of VAT Treg cells from Tconv cells, and also showed little contribution of circulating Treg cells to the VAT Treg cell accumulation (17). This study revealed that VAT Treg cells were mostly seeded from the thymus in the early weeks of life and proliferated in response to antigen(s) presented by major histocompatibility complex class II (MHCII) and IL-33 beyond 10 weeks of age (17). More recent work has further demonstrated the accumulation of Treg cells in VAT was divided into two steps. Firstly, thymus-derived Treg cells were initially induced to express low level of PPARγ and weak VAT-Treg signature within the spleen; secondly, these $PPAR\gamma^{low}$ Treg cells were installed in VAT, where complete VAT-Treg signature was induced by TCR:MHCII interaction, IL-33, and Foxp3 expression. The combination of these consecutive cues was necessary for complete differentiation into the $PPAR\gamma^{high}$ VAT Treg lineage (18).

Origin of Treg cells in SAT and BAT have not been well studied. Since both of these tissues have normal frequencies of Treg cells (about 10% of $CD4^+$ T cells) and naïve $CD4^+$ T cells from these two tissues produce more induced Treg cells than VAT (13), the SAT and BAT Treg cells probably have different origin from VAT Treg cells.

Phenotype

The majority of studies addressing the phenotype of adipose-resident Treg cells were performed in VAT. Microarray analysis comparing Treg cells from VAT and lymphoid tissues revealed notable distinction among Treg cells from different tissue sites and showed a unique transcriptional profile in VAT Treg cells. VAT Treg cells displayed a distinct chemokine/chemokine receptor pattern, overexpression of Gm1960 (an IL-10-inducible Cxcr2 ligand), Ccr1, Ccr2, Ccr9, Ccl6, integrin αV, activated leukocyte cell adhesion molecule (Alcam), Cxcl2 and Cxcl10 and reduced expression of Ccl5 and Cxcr3, which might be responsible for their specific local accumulation (10). VAT Treg cells also produced significantly higher levels of IL-10 and showed enhanced IL-10 signaling compared with Treg cells from lymphoid tissue (10). Most VAT Treg cell signature genes were regulated directly or indirectly by PPARγ, a nuclear receptor overexpressed in Treg cells specifically from VAT but not from other tissues (11). Genetic ablation of PPARγ in Treg cells reduced the number and changed the transcriptional signature specifically in VAT Treg cells, indicating that PPARγ is a master regulator defining VAT Treg cell phenotype (11). In an effort to identify the cytokine receptors preferentially expressed by VAT Treg cells, Vasanthakumar et al. found that ST2, the receptor of IL-33, was highly expressed in Treg cells from VAT and was activated by IL-33 to provide a signal that was essential for the development and maintenance of VAT Treg cells (19). Interestingly, in spite of their unique gene expression signatures, VAT Treg cells were similar in their suppressive capacity as spleen Treg cells based on a standard *in-vitro* suppression assay (10). However, it is not clear if there is a difference in *in-vivo* activity between Treg cells from VAT and other tissues.

In addition to the transcriptional signatures that encompass chemokine receptor, transcription factor, and cytokine receptor phenotypes, VAT Treg cells express tissue-antigen specific

TCR repertoire. According to results from complementarity-determining region 3α (CDR3α) sequences, VAT Treg cells have a highly restricted distribution of sequences and exhibit distinguishable TCR repertoires from that of their counterparts in the spleen and lymph nodes (10). Furthermore, in Vα2-Vβ4 VAT-Treg TCR transgenic mice frequency and number of total Treg cells are specifically elevated in VAT, but not in the spleen (18). Moreover, VAT Treg cells depend on recognition of antigen(s) presented by MHCII on antigen-presenting cells (APCs) for their retention/accumulation in VAT (17). However, the special antigen(s) recognized by VAT Treg cells remain undiscovered.

Microarray gene expression profiling of BAT Treg cells from C57BL/6 female mice revealed a shared group of signature genes with VAT Treg cells, including PPARγ and IL-10, but also identified a specific BAT Treg gene signature, suggesting a unique subset of Treg cells in BAT (12). Cold exposure changed expression of a very small group of genes in BAT Treg cells, but the majority of genes remained unchanged (12). It is worth noting that this study compared the gene signature of BAT Treg cells from female mice to the previously published gene signature of VAT Treg cells from male mice. The reported BAT Treg-specific gene signature in this study may have been affected by the gender difference. More recently, it has been reported that in young 3-6-week-old mice BAT and SAT harbor higher $Foxp3^+$ Treg cell percentages than VAT, and Treg cells in BAT and SAT are more efficiently induced by cold exposure compared to VAT Treg cells (13).

In summary, Treg cells residing in different types of adipose tissue have distinct features, implying their specialized functions in regulation of immune and metabolic homeostasis in and beyond adipose tissue.

Function

Metabolic disorders are associated with and mediated by inflammatory processes (20, 21). As one of the most potent anti-inflammatory cell types, Treg cells have been proposed to play a protective role in insulin resistance and other metabolic disorders by several gain-of-function experiments (10, 22, 23). In both high-fed diet (HFD)-induced obese mice and mice heterozygous for the yellow spontaneous mutation (Ay/a), injection of IL-2 in complex with IL-2 antibody (mAb) increased the fraction of Treg cells in VAT and spleen, and reduced insulin resistance (10). Oral administration of anti-CD3 antibody and β-glucosylceramide (GC) in leptin-deficient ob/ob mice effectively induced Treg cells and alleviated the metabolic abnormalities, including pancreatic islet cell hyperplasia, fatty liver, adipose tissue inflammation and high blood glucose (23). In addition, adoptive transfer of $CD4^+Foxp3^+GFP^+$ Treg cells into db/db diabetic mice led to an increase in Foxp3 expression and a decrease in $CD8^+$ effector T cells in VAT, as well as a decrease of urinary albumin-to-creatinine ratio and glomerular diameter (22). These observations indicate that Treg cells can not only ameliorate insulin resistance, but also prevent diabetic nephropathy. The above studies used approaches that resulted in global increases of Treg cells, which were not limited to adipose tissue. Therefore, these studies failed to fully clarify the specific contribution of local adipose tissue resident Treg cells to the improvement of metabolic disorders. Unfortunately, an attempt to enhance Treg cells specifically in VAT by transfer of fat-resident Treg cells into obese mice failed due to the lability and low recoverable numbers of VAT Treg cells (10). However, in our recent study, genetic deletion of MHCII in adipocytes of obese mice substantially increased Treg cell fraction specifically in VAT and reduced adipose tissue inflammation and insulin resistance (24). Interestingly, these beneficial effects were dependent on the specific induction of VAT Treg cells, suggesting tissue specific function of VAT Treg cells against obesity-induced adipose inflammation and insulin resistance (24). In line with our observations, VAT-Treg TCR transgenic mice showed significantly improved insulin sensitivity and glucose tolerance (18). Besides adipose inflammation, Hashimoto's Thyroiditis (HT) is also associated with abnormal insulin sensitivity. A recent study focused on HT showed a drop in VAT Treg cells as well as insulin sensitivity in a mouse model of HT. The impaired insulin sensitivity was effectively reversed by the adoptive transfer of $CD25^+Foxp3^+$ Treg cells from peripheral blood; however, subsequent anti-CD25 antibodies administration had no effect on insulin sensitivity. Since anti-CD25 treatment selectively depletes $CD25^+Foxp3^+$ Treg cells in peripheral blood but not in VAT, the finding supports a potential specific role for VAT Treg cells in improving insulin resistance in HT (25).

The contradictory results from loss-of-function experiments are somewhat puzzling. Short term depletion of Treg cells by administration of diphtheria toxin in mice that express diphtheria toxin receptor (DTR) under the control of the Foxp3 promoter (Foxp3-DTR) significantly increased insulin sensitivity in VAT and liver, and decreased fasting insulin levels, but showed marginal *in-vivo* metabolic changes (10). Transgenic mice expressing the Vα5/Vβ8.2 TCR, which was found at a high frequency among obese adipose tissue T cells, displayed a tissue specific decrease of Treg cells in VAT, but did not show any metabolic phenotype in both lean and obese states (26). Similarly, VAT-specific loss of Treg cells by knockout of PPARγ in $Foxp3^+$ Treg cells did not affect insulin sensitivity in young, lean and obese mice (11, 27). However, these negatives do not rule out the potential critical role for VAT Treg cells in regulation of metabolism under certain circumstances. In lean condition, a decrease of VAT Treg cells may not change the inflammatory state because of a lack of inflammation in adipose tissue. While in obese condition, where the VAT Treg cells are already dramatically down-regulated and exhibit loss of function, further decrease of VAT Treg cells may not lead to changes in metabolic phenotypes. Interestingly, loss of VAT Treg cells improves glucose homeostasis in aged mice, suggesting its critical role in aging-induced insulin resistance (27). The detailed mechanism underlying the insulin-desensitizing effects of VAT Treg cells in aged mice is unclear and requires further investigation.

SAT and BAT, two important tissues responsible for cold-induced thermogenesis, also harbor tissue resident Treg cells. Cold exposure and beta-adrenergic stimulation enhanced the

accumulation of Treg cells in BAT and SAT, suggesting a potential role for adipose-resident Treg cells in cold-induced thermogenesis. Indeed, depletion of Treg cells blunted beta3-adrenergic receptor (ADRB3) agonist-induced expression of thermogenic and lipolytic genes, while adoptive transfer of Treg cells or expansion of Treg cells by IL-2/mAb injection showed opposite effects (13). These results suggested an important role for Treg cells in the regulation of thermogenesis in SAT and BAT. Since specific depletion or expansion of Treg cells in SAT and BAT is currently not possible, it is hard to determine the specific contribution of Treg cells to regulation of metabolic homeostasis, such as body weight and insulin sensitivity, in these tissues.

While additional studies are necessary to clarify the timing and location of the specific functions of adipose-resident Treg cells, it is clear that these cells are important contributors to maintaining immune and metabolic homeostasis, in part through regulation of adipose inflammation, insulin sensitivity, lipolysis and thermogenesis.

REGULATORS OF ADIPOSE-RESIDENT TREG CELLS

PPARγ

PPARγ is a member of the PPAR subfamily of nuclear hormone receptors. The name PPAR is derived from the first identified member of the family, PPARα, which has the ability to respond to various compounds that induce peroxisome proliferation (28). However, other members of the PPAR family do not show this function; instead, they play important roles in lipid metabolism and metabolic control, especially PPARγ.

PPARγ was first identified by homology cloning in Xenopus, which was reported to stimulate the peroxisomal degradation of fatty acids (29). There are two isoforms of PPARγ, PPARγ1 and PPARγ2, and the latter has additional 30 amino acids at its extreme N terminus in mice (30). Generally, PPARγ is accepted as a master regulator of adipogenesis and transcription of lipogenic genes (31, 32). It is also known as a suppressor of inflammatory signaling and its ability to exert anti-inflammatory effects in both acute and chronic inflammatory diseases (33, 34). PPARγ was reported to be expressed in Treg cells and protected autoimmunity by enhancement of Treg cells (35, 36). Recently, PPARγ was highlighted as a crucial molecular orchestrator of VAT Treg cell accumulation, phenotype and function (11).

Comparing the gene-expression profiles of VAT and lymphoid-organ Treg cells, Cipolletta et al. found that the level of transcripts encoding the nuclear receptor PPARγ was highly expressed in VAT Treg cells (11). Expression of genes that were positively or negatively correlated with PPARγ transcript levels occupied the majority of strongly up- or down-regulated genes in VAT Treg cells compared to lymphoid-tissue Treg cells (11). VAT Treg cells expressed both PPARγ1 and PPARγ2, with a predominance of the former. The evaluation of each isoform's ability to collaborate with Foxp3 to promote the VAT Treg cell gene signature showed that PPARγ1 and PPARγ2 could both induce most of the genes upregulated in VAT Treg cells. However, the difference between the two protein variants was that only PPARγ1 induced suppression of the genes that were down-regulated in VAT Treg cells (11). More importantly, PPARγ is a key controller for the accumulation and phenotype of Treg cells that are present in VAT. Mice lacking PPARγ specifically in Treg cells had lower frequencies and numbers of VAT Treg cells, but did not exhibit any reductions in Treg cells in lymphoid organs. Additionally, PPARγ-mut mice exhibited reduced expression of VAT Treg cell signature genes (11).

Pioglitazone is a well-known insulin-sensitizing agent that improves metabolic indices in obese mice and humans by targeting and activating PPARγ. Pioglitazone treatment induced an impressive enrichment of the fraction and number of Treg cells in epididymal adipose tissue in both lean and obese mice (11). Conversely, a few days of treatment with an irreversible PPARγ inhibitor, GW9662, decreased the fraction of $Gata3^+$ Treg cells in VAT, although the fraction and number of total Treg cells in VAT showed no significant differences (11). Since Gata3 expression was reduced in the absence of PPARγ, these observations suggested that PPARγ is important for both development and continuous maintenance of VAT Treg cell phenotype (11).

The transcriptional signature of VAT Treg cells from obese mice showed a strong correlation with the transcriptional changes induced by deficiency of PPARγ, suggesting that obesity might exert its impact on VAT Treg cell through either direct or indirect modulation of PPARγ (14). However, the level of transcripts encoding PPARγ in obese mice was not altered compared with lean mice (14), suggesting that the genes abnormally expressed in the obese state are not associated with a reduced expression of PPARγ. Instead, reduction of PPARγ activity was shown to be mediated through phosphorylation by protein kinase Cdk5 (cyclin-dependent kinase 5), a kinase that is activated in adipocytes of diet-induced obese mice. As a result, Cdk5 mediated phosphorylation of the serine residue at position 273 of PPARγ and led to dysregulation of a large number of genes involved in the pathogenesis of insulin resistance (37). Intriguingly, the transcriptional signature of obese VAT Treg cells was also dependent on PPARγ's phosphorylation of the serine residue at position 273, which was provoked by obesity (14). Therefore, it is likely that the post-translational modification of PPARγ might affect VAT Treg cells in obesity.

IL-33 and its Receptor ST2

IL-33 is a member of the IL-1 family, which includes IL-1α, IL-1β, IL-18, IL-36α, IL-36β, IL-36γ, IL-33, receptor antagonists IL-1Ra, IL-36Ra, IL-38, and anti-inflammatory cytokine IL-37 (38). IL-33 protein is produced mainly by non-hematopoietic cells (39), particularly specialized populations of epithelial and endothelial cells (40, 41). IL-33 was first detected in human lymph node high endothelial venules (42) and it is highly expressed in lymph node and spleen fibroblastic reticular cells in mice and humans (40, 41). In contrast to humans, expression of IL-33 in mouse endothelium seems to be limited to adipose tissue, liver and female reproductive tract (43, 44). ST2 and the shared signaling chain, IL-1RAcP, make up the receptor complex for IL-33. ST2 is thought to be the only functional receptor for IL-33, because systemic inflammation caused by the loss of IL-33

nuclear localization signals or constitutive over-expression of IL-33 was abrogated by deficiency of ST2 (45, 46). ST2 is expressed in many different kinds of cells in VAT, including adipocytes, mast cells, ILC2s, Th2 cells and Treg cells (47).

Many Treg cells in VAT display ST2 and ST2$^+$ Treg cell fraction increases with age to achieve nearly 90% of VAT Treg cells in mice at age of 30 weeks, while they are barely detected in the spleen (17). In addition to VAT Treg cells, other tissue-resident Treg cells, like Treg cells in intestine (48) and muscle (9), also show higher percentage of ST2$^+$ cells compared with lymphoid organs. It seems that ST2 expression on tissue-resident Treg cells is maintained by IL-33 itself as IL-33 stimulates the expression of ST2 in Treg cells (19).

Accumulating evidence supports the role for IL-33/ST2 axis as a positive regulator of VAT Treg cells by counteracting obesity-induced adipose inflammation and insulin resistance. Mice lacking ST2 or IL-33 that were kept on a normal chow diet had reduced frequencies and numbers of VAT Treg cells (17, 19, 49), while injection of exogenous IL-33 into lean or obese mice stimulated an impressive expansion of VAT Treg cell population (17, 19, 50). In obese mice, IL-33 treatment reduced inflammation of VAT, as well as improved the metabolic indices (50, 51). However, a recent study reported that although administration of IL-33 induced VAT Treg cell expansion, it also promoted insulin resistance. Moreover, while blockade of IL-33/ST2 signaling by anti-ST2 antibody reduced VAT Treg cells, at the same time the treatment increased insulin-stimulated glucose uptake, suggesting a potential for detrimental effects of IL-33 signaling in metabolism (27). The reason for this discrepancy is not clear, but could be due to different mouse colonies utilized, husbandry practices and facilities utilized in the studies.

Studies suggest both direct and indirect mechanisms for IL-33 modulation of VAT Treg cells. One study suggested that IL-33 had an indirect influence on VAT Treg cells through activation of ILC2, which led to co-stimulatory interactions between Treg cell ICOS and ILC2 expressed ICOSL (49). Another study demonstrated that IL-33 could also act directly on VAT Treg cells. When ST2 expression was specifically ablated in Treg cells, the number of Treg cells in both VAT and SAT decreased, while there was no significant change in spleen. Furthermore, the frequency and number of ILC2 did not change in VAT of these mice, which excluded the effects of ILC2 on VAT Treg cells. In addition, other studies also showed direct effects of IL-33 on tissue-Treg cells *in vitro*, including Treg cells from VAT and other tissues (19, 48, 52, 53). Vasanthakumar et al. have shown that myeloid differentiation factor MyD88, an adaptor protein within IL-33 signaling pathway, was essential for the development and maintenance of VAT Treg cells (19).

The main source of IL-33 in adipose tissue was thought to be primarily derived from multiple stromal cell populations, such as podoplanin$^+$ (Pdpn$^+$) stromal cells (54). IL-33 protein levels in VAT increase with age, accompanied with Treg cell accumulation (19, 54). IL-17A derived from γδ T cells has been identified as a key cytokine that can induce IL-33 production in adipose stromal cells and sustain VAT Treg accumulation, which was evidenced by the observation that mice lacking IL-17A or γδ T cells exhibited significant reduction in IL-33 protein and Treg cell frequencies in adipose tissue but not in spleen (54).

TCR:MHCII Interactions and Co-stimulation

T cell activation requires MHC-mediated antigen presentation and co-stimulation. TCR:MHCII interaction provides a fundamental signal to sustain and activate CD4$^+$ T cells (55). MHCII expression on APCs, especially on adipocytes, is important for the activation of Th1 cells in adipose tissue in obese state (24, 56–58). In lean condition, MHCII-mediated antigen presentation is required for the development and maintenance of VAT Treg cells (17, 19). In MHCII-deficient mice, the number of VAT Treg cells was dramatically decreased and few of the remaining VAT Treg cells expressed Gata3, a typical VAT Treg cell marker (17). In addition, a close association between myeloid cells expressing MHCII and Treg cells in VAT was observed in wild type mice, but not in MHCII deficient mice (17). In another study, VAT Treg cells were found to express higher level of Nur77 and lower level of TCF7 than splenic Treg cells. Since transcription factors Nur77 and TCF7 were up- and down-regulated in response to TCR signaling respectively, these results suggested TCR signals are critical to maintain VAT Treg cells (19). Further studies revealed that TCR signals induced transcriptional regulators BATF and IRF4, and subsequently turned on the expression of PPARγ and ST2, two key regulators of VAT Treg cell phenotype (19). B7 molecules (CD80 and CD86], the most classical T cell co-stimulators, were also involved in reducing HFD-induced inflammation by maintaining the number of Treg cells in adipose tissue, liver and lymphoid tissues (59, 60). CD80/CD86 double knockout (B7 KO) mice showed a reduction of VAT Treg cells (59, 60), likely a result from the systemic defects of Treg cell development in these mice (61).

Myeloid cells, including macrophages and dendritic cells, have been proposed to be the major MHCII$^+$ APCs that provide necessary TCR signals to VAT Treg cells (17). Indeed, adipose tissue macrophages (ATMs) have been shown to contribute to induction and proliferation of VAT Treg cells (62). ATMs from normal mice induced PPARγ-high Treg cells while ATMs from obese mice induced PPARγ-low Treg cells. Moreover, depletion of ATMs resulted in the reduction of adipose-resident Treg cells, while adoptive transfer of ATMs showed opposite result *in vivo* (62), strongly suggesting that the enrichment of Treg cells in adipose tissue is at least partly due to interactions with ATMs. However, deficiency of MHCII specifically in myeloid cells did not change the fraction of VAT Treg cells in chow fed lean mice, although the reduction was observed in obese mice (58). Further studies are warranted to identify or confirm APC populations that are essential to sustain Treg cells in VAT.

Other Regulators

In addition to the regulators mentioned above, the adipose-resident Treg cells could be regulated by other factors, including secretory factors [IL-21 (63), IL-2 (64), and insulin (65)] and transcriptional factors [STAT3 (66), Foxp3 (18), and STAT6 (13)]. Increased accumulation of Treg cells was demonstrated in VAT

of IL-21 knockout mice in comparison with wild-type mice fed normal chow or HFD (63). Accordingly, IL-21 knockout mice exhibited improved insulin sensitivity and decreased adipose inflammation, compared with wild-type mice when fed on HFD. This phenotype was accompanied by a higher induction of IRF4 in VAT, which was negatively regulated by IL-21 (63). IL-2 plays a critical role in Treg cell survival and function (67). A recent study reported that adipose invariant natural killer T cells regulated the proliferation and function of adipose Treg cell through IL-2 production (64). Treg cells express insulin receptor and high levels of insulin could impair the ability of Treg cells to produce IL-10 via AKT/mTOR signaling pathway, suggesting that insulin acts as a negative regulator of Treg cells in hyperinsulinemic obese mice (65).

STAT3 expression in T cells was shown to play a crucial role in adaptive immunity in VAT, which may contribute to adipose tissue inflammation and insulin resistance. Treg cell percentages in VAT were increased in T cell specific STAT3 knockout mice compared with wild-type mice fed a HFD (66). However, since reduction in VAT Treg cells was correlated with HFD-induced obesity, and mice lacking STAT3 in T cells gained less body and VAT weights when fed HFD, it is hard to exclude the possibility that the increase of VAT Treg cells is a secondary effect of weight loss in these mice.

Foxp3 is a master regulator in the function and development of Treg cells (68). Although the role of Foxp3 in lymphoid organ Treg cells is well studied, its role in VAT Treg cell is less clear. A recent study has demonstrated Foxp3 was not only a marker to distinguish Treg cells and Tconv cells; it was also essential for Treg cell enrichment in VAT. When Foxp3 was transduced into Tconv cells; these cells accumulated in VAT, while control transduced cells did not. Moreover, almost all the Foxp3 transduced Tconv cells expressed PPARγ and ST2, markers normally associated with in VAT Treg cells (18).

Few studies have investigated potential regulators of Treg cells in BAT and SAT. So far STAT6 has been found to play a role in regulation of these cells. STAT6 knockout mice displayed lower BAT and SAT Treg cell frequencies compared with wild-type mice. *In-vitro*, Treg cell induction of naïve T cells isolated from BAT and SAT in STAT6 knockout mice was also significantly blunted. Moreover, STAT6 inhibitor suppressed Treg cell induction from naïve $CD4^+$ T cells in inguinal lymph nodes of BALB/c Foxp3-GFP reporter mice (13).

Regulatory Network Controlling Adipose-Resident Treg Cells

Many regulators interact to form a network necessary to maintain proper number and function of adipose resident Treg cells **(Figure 1A)**. PPARγ transcription factor and IL-33/ST2 cytokine/cytokine-receptor pair are key regulators of accumulation and phenotype of VAT Treg cells. ST2 may be regulated by PPARγ, since VAT Treg cells lacking PPARγ show a lower percentage of $ST2^+$ cells (17). However, it is likely that PPARγ expression is not affected by IL-33 stimulation (19). Interestingly, both PPARγ and ST2 are direct target genes of transcriptional factor BATF and IRF4, which are induced by TCR signaling (17, 19). Some factors regulate VAT Treg cells indirectly by modulating either the production or function of IL-33. IL-17 secreted by γδ T cells promotes production of IL-33 from stromal cells to sustain VAT Treg cells (54), while production of IFNγ from Th1 cells could inhibit IL-33 signaling to decrease the proliferation of adipose-resident Treg cells (24). Besides IL-33, other cytokines can also act directly on Treg cells in VAT. IL-2, produced by regulatory iNKT cells, can regulates VAT Treg cell enrichment (64). IL-21, production of which is regulated by STAT3 through an autocrine positive-feedback loop (69), negatively regulates IRF4 to decrease Treg cells (63).

REGULATION OF ADIPOSE-RESIDENT TREG CELLS IN PHYSIOLOGICAL AND PATHOLOGICAL PROCESSES

In accordance with the important function of adipose-resident Treg cells, they have been described as a highly dynamic population in physiological and pathological processes. A growing number of studies have investigated the regulation of adipose-resident Treg cells under healthy and disease conditions in both humans and mice. These studies help us to better understand the critical role of adipose-resident Treg cells in metabolic diseases, and have identified potential pathways for specific manipulation of adipose-resident Treg cells to improve metabolic homeostasis.

Age

In lean male mice, VAT Treg cells increase between 5 and 25 weeks of age, then drop rapidly at 40 weeks of age, while splenic Treg cells remain unchanged (10, 14). Consistently, the transcriptional profiles of VAT Treg cells from mice at different ages revealed age-dependent evolution of VAT Treg cell signatures, and demonstrated that numeric changes in VAT Treg cells coincided with functional skewing in aging mice (14). Aging leads to the development of insulin resistance. The decrease of VAT Treg cells with aging is accompanied by a decline in insulin sensitivity, suggesting a protective role of VAT Treg cells in aging-associated insulin resistance (14). Another study showed that the accumulation of VAT Treg cells mainly occurred at age of 4–9 months and declined with further aging at 15 months of age (49). These studies support the idea that development and accumulation of VAT Treg cells are dependent on age and aging leads to the decline in their number. In contrast, a recent study reported a rise of VAT Treg cells in aging mice at age of 44 weeks vs. the young mice at age of 12 weeks (27), suggesting an opposite viewpoint. This discrepancy may be caused by different ways to define control and aging. If the mice at 12 weeks of age are defined as controls, in which the VAT Treg percentage is quite low (∼10% of $CD4^+$ T cells), we will easily conclude that aging mice (any mice beyond 12 weeks of age) have increased VAT Treg cells. However, if we define the mice at the age of 25 weeks as controls, which are still young and in which the VAT Treg cells reach the peak (∼50% of $CD4^+$ Tells), we will conclude that aging mice (any mice beyond 25 weeks of age) have decreased VAT Treg cells. Thus, as to the age-dependent VAT Treg cell accumulation, it is likely that all these studies are correct and different controls lead to different

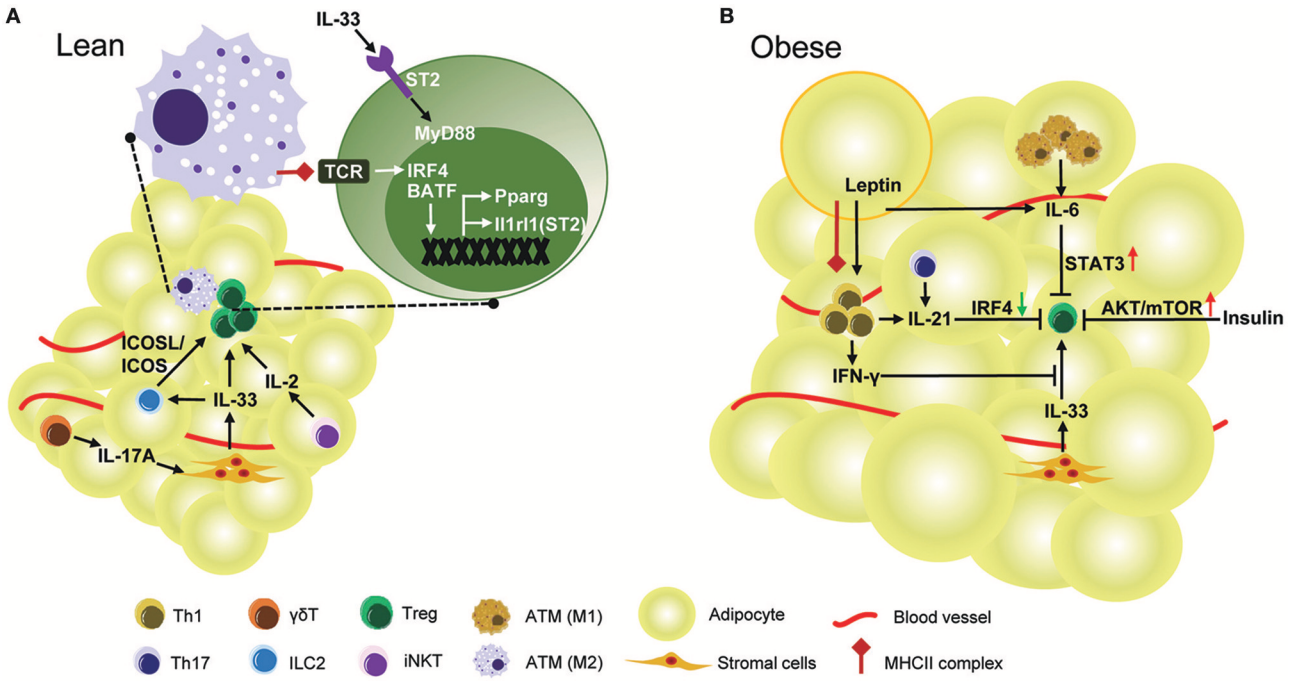

FIGURE 1 | Regulation of Treg cell in mouse VAT. **(A)** In lean mice, healthy adipose tissue is enriched with anti-inflammatory Treg cells. TCR:Antigen recognition and IL-33 signaling are required for the development and accumulation of Treg cells in VAT. The TCR signaling triggered by MHCII complex provided by ATMs up-regulates transcription factors IRF4 and BATF, both of which directly control expression of *Il1rl1* (ST2) and *Pparg* in VAT Treg cells. *Pparg* is the key transcription factor contributing to the unique gene expression profile and phenotype of VAT Treg cells. ST2, encoded by *Il1rl1* gene, is the receptor for IL-33 mediated signaling which promotes proliferation of VAT Treg cells through the adaptor protein MyD88. IL-33 also induces VAT Treg cells indirectly by interactions between Treg ICOS and ILC2 ICOSL. IL-33 is mainly produced by stromal cells and its production is dependent on IL-17A derived from γδ T cells. Regulatory iNKT cells also regulate VAT Treg enrichment by production of IL-2. **(B)** In obese mice, the frequency and number of VAT Treg are drastically reduced. IL-6, which is over-produced by adipocytes and M1-like ATMs in obese mice, suppresses VAT Treg generation by activation of STAT3. IL-21 is overexpressed in obese adipose tissue and reduces VAT Treg cells by down-regulation of IRF4. As obesity develops, adipocytes express more leptin, which in conjuction with MHCII expression by adipocytes, stimulates Th1 cell activation. IFNγ produced by Th1 cells strongly inhibits VAT Treg accumulation by IL-33. Obesity is accompanied by hyperinsulinemia. In obesity, the function of Treg cells is impaired by insulin via AKT/mTOR activation.

conclusions. The investigations on the mechanisms underlying age-dependent VAT Treg cell development and accumulation lead to identification of many key regulators of VAT Treg cells, which were discussed in the above section of "regulators of adipose-resident Treg cells." The effects of aging on Treg cells in SAT and BAT are still unclear and the data on age-dependent adipose-resident Treg cell accumulation in humans is currently unavailable.

Cold Stimulation

Young lean BALB/c mice (3–6 weeks of age) showed selective accumulation of Treg cells in BAT and SAT, but not in VAT (13). Cold exposure (4°C, 24 h) or beta-adrenergic stimulation increased Treg cell frequencies in all three fat depots; however, accumulation was more efficient in BAT and SAT (13). A T cell-specific STAT6/Pten axis has been suggested to link cold exposure or ADRB3 stimulation with Foxp3$^+$ Treg cells induction and adipose tissue function (13). C17orf59, a negative regulator of mTORC1 (70), was induced downstream of STAT6 and mediated Treg cell induction in adipose tissue in response to cold exposure (13). Although the *Adrb3* gene had been reported to be expressed in human lymphocytes (71), it is still unknown if exposure to cold enhances Treg cell induction in human adipose tissue.

Obesity

Obesity-induced adipose inflammation is in part characterized by the imbalance of local pro-inflammatory T cells and Treg cells, thereby causing obesity-associated metabolic abnormalities. In male mice, it is quite clear that VAT Treg cells are down-regulated in obesity. In a seminal study, Feuerer et al. reported that the frequency and number of VAT Treg cells were dramatically reduced in three mouse models of obesity, including ob/ob mice, Ay/a obese mice, and HFD-induced obese mice, while SAT and splenic Treg cell frequencies were not affected (10). The VAT-specific reduction of Treg cells in obese mice was recently confirmed by another study (14). In addition to reduction in frequencies, the phenotype and function of VAT Treg cells were also influenced by obesity. The transcriptional profiles of VAT Treg cells from either HFD-induced or ob/ob obese mice were significantly different from those of lean mice (14). Some of these differentially expressed genes are important for maintaining the phenotype and function of VAT Treg cells. For example, the

expression levels of IL-10 (a key anti-inflammatory cytokine representing Treg cell function) and ST2 (IL-33 receptor, a key receptor defining VAT Treg cells) were substantially reduced in VAT Treg cells, but not in splenic Treg cells isolated from obese mice (19, 65). The transcriptome changes of VAT Treg cells provoked by obesity were thought to be mediated by activation of Cdk5, which deactivated PPARγ through phosphorylation of serine residue 273 (Ser273] (14). While studies in male mice led to the conclusion that obesity decreased the number and changed the transcriptome of VAT Treg cells, the observation did not extend to female mice. In a model of HFD-induced obesity, VAT Treg cells increased proportionally with increase of fat tissue in female, but not in male mice. Accordingly, female mice were better at maintaining metabolic homeostasis, unlike male mice of similar weight, which developed hyperinsulinemia (72). These findings imply that Treg cells can be affected in either a direct or indirect fashion by sexual hormones. Nevertheless, the mechanisms behind the difference in obesity-induced changes of VAT Treg cells between male and female mice are unclear and warrant further investigation. Moreover, the effects of obesity on SAT and BAT Treg cells have not been addressed and remain unknown.

The mechanisms underlying obesity-induced down-regulation of VAT Treg cells in male mice is not fully understood **(Figure 1B)**. IL-21 and STAT3 may be involved in this process. Both IL-21 and STAT3 have been found to be elevated in VAT of mice fed a HFD (63, 66). The mice lacking IL-21 or mice with specific deletion of STAT3 in T cells fed with HFD showed an increase of VAT Treg cells and an improvement of adipose inflammation and insulin sensitivity, suggesting an important role for IL-21 and STAT3 signaling in regulating VAT Treg cells in obesity (63, 66). However, these genetically modified mice were resistant to HFD-induced obesity (63, 66), raising the possibility that the increase of VAT Treg cells is caused by weight loss in these mice, since the abundance of VAT Treg cells is negatively correlated with adiposity in mice. Thus, it is still questionable whether STAT3 and IL-21 have a direct inhibitory effect on VAT Treg cell accumulation in an obese state.

More recently, direct TCR ligation by MHCII expressed by adipocytes was shown to mediate IFNγ production from Th1 cells, which resulted in reduction of VAT Treg cells in obese mice. Obesity increased MHCII expression in adipocytes, while deletion of MHCII specifically in adipocytes (aMHCII$^{-/-}$) led to a decrease of adipose IFNγ expression, an increase of VAT Treg cell abundance and reduced insulin resistance without any changes in weight gain in obese mice. IFNγ treatment effectively blocked IL-33-induced VAT Treg cell proliferation and ST2 expression, whereas HFD-fed IFNγR1$^{-/-}$ mice had similarly high levels of Treg cells in adipose tissue, as did aMHCII$^{-/-}$ mice, and were more insulin sensitive (24). These results suggested a critical role for adipocyte MHCII in the obesity-induced adipose T cell subset switch and insulin resistance.

Although almost all the data from animal models show a decrease in VAT Treg cells in obesity, their regulation by obesity in humans is controversial **(Table 1)**. In most human studies, given that the samples were frozen and cannot be analyzed by flow cytometry, the Foxp3 mRNA levels, presumably an indicator of Treg cells, were determined by quantitative PCR. Consistent to results obtained from mouse models, a human study showed a decrease of Foxp3 gene expression in VAT in obese subjects when compared with lean controls (73). In obese subjects, Foxp3 mRNA levels were higher in SAT than in VAT, and the relative drop in Treg cells in VAT vs. SAT was positively correlated with BMI, suggesting that VAT Treg cells are negatively correlated with obesity in humans (10). In addition, in a cohort of overweight or obese patients, VAT Treg cell number was found to be inversely correlated with plasma fasting glucose and positively correlated with homeostasis model assessment (HOMA)-β (74). These results suggested that consistent with mouse studies, VAT Treg cells in humans are negatively affected by obesity, and have an important role in maintaining glucose. In contrast, several other quantitative PCR-based studies reported an increase of Foxp3 expression and Foxp3/CD3ε ratio in both SAT and VAT in obese subjects (75–77). Frequency of Treg cells measured by flow cytometry analysis was also higher in VAT of obese subjects (78). Moreover, in metabolically healthy obese subjects (MHOS), Foxp3 expression in SAT and VAT were increased as well (79). Analogous to mouse studies, it appears that there is a gender difference in human VAT Treg cell accumulation in obesity. Analysis of CD4$^+$CD25$^+$Foxp3$^+$ or CD8$^-$Foxp3$^+$ cells showed no difference between overweight and lean female subjects (80). It is important to consider, however, that the inconsistency in results from human studies could be due not only to gender, but also differences in race and age among the cohorts used in the studies. It is also notable that in many studies the Treg cell abundance was indicated by Foxp3 mRNA levels, which is not an ideal indicator of Treg cells because Foxp3 is expressed by a significant proportion of non-Treg cells in humans (81). Additionally, almost all the current human studies recruited limited number of subjects (N = 2–44), which may also affect the accuracy of the conclusions drawn. To clarify the regulation of adipose-resident Treg cells in humans, it will be necessary to perform more in-depth analyses on Treg cells from fresh adipose tissue obtained from larger cohorts.

THE THERAPEUTIC POTENTIAL OF ADIPOSE-RESIDENT TREG CELLS IN OBESITY-ASSOCIATED DISEASES

Obesity and its related metabolic diseases have been considered as chronic inflammatory diseases. As one of the most potent anti-inflammatory immune cells, Treg cells have been tested as targets for the treatment of many inflammation-related diseases (82). Accumulating evidence shows that modulation of Treg cells, especially VAT Treg cells, represents a potential new strategy for the treatment of obesity-associated diseases **(Table 2)**.

Adoptive transfer of CD4$^+$Foxp3$^+$ Treg cells into db/db mice increased Treg cell numbers in VAT and significantly improved insulin sensitivity (22). Other strategies have focused on Treg cell induction *in vivo*. Brief treatment with the mitogenic CD3-specific antibody in HFD-fed C57BL/6 and leptin deficient ob/ob obese mice restored Treg cells numbers in VAT, thereby greatly improving glucose tolerance and insulin sensitivity (83).

TABLE 1 | Change of adipose-resident Treg cells in adipose inflammation of human.

Subject	Sex	Mean Age (year)	Tissue	Method	Main findings	References
Control subjects (BMI <30 kg/m^2, $n = 7$) and obese subjects without overt type 2 diabetes (BMI ≥30 kg/m^2, $n = 13$)	No data available	No data available	VAT	Real-time quantitative PCR (Foxp3)	Foxp3 gene expression was lower in VAT in obese subjects compared to control subjects.	(73)
Obese subjects (BMI: 30 to 39.9 kg/m^2, $n = 2$) and morbidly obese subjects (BMI >40 kg/m^2, $n = 9$)	Male and female	No data available	VAT and SAT	Real-time quantitative PCR (Foxp3)	The frequency of Treg cells in VAT vs. SAT was negatively correlated with BMI.	(10)
Overweight subjects (BMI ≥25 kg/m^2, $n = 44$)	Male and female	41.5	VAT and SAT	Flow cytometry	VAT Treg cells were negatively correlated with fasting glucose and MCP-1 and positively correlated with HOMA-β.	(74)
Waist circumferences in lean (<94 cm, $n = 10$), overweight (94–102 cm, $n = 10$) and obese (>102 cm, $n = 10$)	Male	Lean: 43.5, overweight: 48 and obese: 45.2	SAT	Real-time quantitative PCR (Foxp3)	Foxp3 gene expression was higher in SAT in obese subjects compared to control subjects.	(75)
Control subjects (BMI: 17.6 to 26.7 kg/m^2, $n = 15$) and obese subjects (BMI: 38.7 to 66.0 kg/m^2, $n = 36$)	Female	Lean: 41.5 and obese: 45.2	VAT and SAT	Real-time quantitative PCR (Foxp3)	Foxp3 gene expression was higher in both VAT and SAT, and the proportion of Treg cells was increased in SAT in obese subjects compared to control subjects	(76)
Control subjects (BMI <30 kg/m^2, $n = 20$) and highly obese subjects (BMI >40 kg/m^2, $n = 20$)	Male and female	No data available	VAT and SAT	Real-time quantitative PCR (Foxp3)	Foxp3 gene expression and the proportion of Treg cells were increased in VAT and SAT in obese subjects compared to control subjects.	(77)
Control subjects (BMI: 18 to 24.9 kg/m^2, $n = 16$) and obese subjects (BMI ≥30 kg/m^2, $n = 15$)	Male and female	Lean: 46.07 and obese: 32.83	VAT	Flow cytometry	The frequency of Treg cells was increased in VAT in obese subjects compared to control subjects and the frequency was positively correlated with BMI.	(78)
Control subjects (BMI <25 kg/m^2, $n = 15$) and highly obese subjects (BMI >40 kg/m^2, $n = 16$)	Male and female	27–55	VAT and SAT	Real-time quantitative PCR (Foxp3)	Foxp3 gene expression was increased in both VAT and SAT, and its expression in SAT was positively correlated with weight and BMI, while its expression in VAT was positively correlated with BMI and body fat percentages.	(79)
Control subjects (BMI <25 kg/m^2, $n = 11$) and overweight subjects (BMI >25 kg/m^2, $n = 15$)	Female	38.7	Adipose tissue	Flow cytometry	No difference in the percentage of Treg cells between overweight and control subjects.	(80)

Consistently, injection of the non-mitogenic F(ab′)2 fragment of CD3-specific antibody into obese mice restored Treg cells in VAT, which was accompanied by a long-term improvement in glucose tolerance (83). Similarly, oral administration of anti-CD3 antibody in conjunction with oral GC in ob/ob obese mice increased Foxp3$^+$ T cells and decreased CD11b$^+$F4/80$^+$ macrophages in adipose tissue, resulting in decreased adipose inflammation and reversal of insulin resistance (23). In addition, expansion of Treg cells in VAT by administration of IL-2 and IL-2-specific monoclonal antibody complex (10) or recombinant IL-33 (50, 51) improved adipose inflammation and insulin resistance in obese mice. All these studies indicated that modulation of VAT Treg cells has a strong potential for treatment of obesity-associated insulin resistance, and provided the rationale to identify new therapeutic targets and develop novel reagents capable of inducing Treg cells in adipose tissues.

In-vivo administration of β3 adrenergic receptor agonist CL-316243, which was once used for treatment of obesity and type 2 diabetes (92), enhanced abundance of Foxp3$^+$ CD4$^+$ T cells in inguinal lymph nodes as well as in adipose tissues, including BAT, SAT, and VAT (13). Other studies found that male mice treated with TUG89, a selective free fatty acid receptor 4 agonist, or resveratrol after being subjected to sleep fragmentation (SF) exposure showed a reversion of SF-associated low M2/M1 ratio, an increase in Treg cell fraction in VAT and improvement of VAT inflammation and insulin resistance (84, 85). Oral administration with γ-aminobutyric acid in HFD-fed mice significantly increased the frequency of Treg cells and reduced infiltration of macrophages in adipose tissues, which correlated with a decrease in fat mass and adipocyte size, improved glucose tolerance and insulin sensitivity (86). Surprisingly, C57BL/6 and ob/ob mice fed with 5% Eicosapentaenoic acid mixed into chow

TABLE 2 | Therapeutic strategies involving the modulation of adipose-resident Treg cells in metabolic disorders.

Strategy	Subject	Mechanism	Changes in Metabolic parameters	References
Adoptive Treg cells transfer	db/db mice	Upregulate Foxp3 expression in mVAT; decrease the percentage of pro-inflammatory mVAT-infiltrating $CD8^+CD69^+$ effector T cells	Decrease blood glucose levels and mVAT cell diameter; improve insulin sensitivity	(22)
Injection of mitogenic αCD3 anti-T cell antibody	HFD-fed C57BL/6 mice and ob/ob mice	Restore Treg cell numbers in VAT	Improve glucose tolerance and insulin sensitivity; lose weight transiently	(83)
Injection of the non-mitogenic F(ab′)2 fragment of αCD3	HFD-fed C57BL/6 mice	Restore Treg cell numbers in VAT; increase the MMR^{hi} pool and reduce the MMR^- pool; generate an increase in IL-10	Improve glucose tolerance and fasting insulin level	(83)
Oral anti-CD3 in conjunction with oral GC	ob/ob mice	Increase $Foxp3^+$ T cells in adipose tissue; decrease $CD11b^+$ $F4/80^+$ monocytes in adipose tissue	Reduce the level of glucose; reduce pancreatic hyperplasia and hepatic fat accumulation	(23)
Injection of IL-2 and IL-2-specific monoclonal antibody complex	HFD-fed C57BL/6 mice	Increase the fraction of Treg cells in the abdominal fat and spleen	Improve glucose tolerance and insulin sensitivity	(10)
Injection of rIL-33	HFD-fed C57BL/6 mice	Reverse the reduction of Treg cells in obese VAT; reduce VAT inflammation	Reduce fasting glycemia and insulin resistance.	(50)
Treatment with recombinant IL-33	ob/ob mice	Induce accumulation of Th2 cytokines and Th2 cells in WAT; improve differentiation of M2 macrophages in both adipose and liver	Decrease VAT weight and body fat; reduce adipocyte size and blood glucose levels; improve insulin sensitivity	(51)
ADRβ3 stimulation *in vivo* with the β3 receptor agonist CL-316243 (CL)	BALB/c Foxp3-GFP reporter mice	Enhance Foxp3 abundance in $CD4^+$ T cells from lymph nodes as well as fat tissue	No data available	(13)
Treatment with TUG891	Male mice subjected to SF exposures	Reduce M1/M2 ratios; increase the number of Treg cells in VAT; attenuate VAT inflammation	Reduce food consumption, weight gain and VAT mass; prevent insulin residence	(84)
Treatment with resveratrol (Resv)	Male mice subjected to SF or sleep control conditions	Attenuate the increase of M1 and decrease of M2 induced by SF; abrogate SF-induced reduction in Treg cells; attenuate VAT inflammation	Abrogate the increased fasting insulin and leptin levels associated with SF; attenuate insulin resistance	(85)
Oral Treatment with γ-Aminobutyric Acid	HFD-fed C57BL/6 mice	Increase the frequency of $CD4^+$ $Foxp3^+$ Treg cells; reduce the infiltration of macrophage in the adipose tissues	Reduce fasting blood glucose; improve glucose tolerance and insulin sensitivity; inhibit the gain in body weight	(86)
Oral treatment with EPA	C57BL/6/ mice and ob/ob mice	Increase the number of adipose tissue Treg cells	Lower the weight of body and adipose tissues in C57BL/6 mice	(87)
Oral treatment with pioglitazone	HFD-fed C57BL/6 mice	Enrich the fraction and number of Treg cells in epididymal adipose tissue; augment levels of CD36 on the surface of macrophages	Normalize systemic metabolic parameters, including insulin sensitivity and glucose tolerance; increase the level of serum adiponectin	(11)
Oral administration of Akkermansia muciniphila	HFD-fed C57BL/6 mice	Induce Foxp3 Treg cells in the VAT; attenuate adipose tissue inflammation	Improve glucose tolerance; reduce the concentrations of insulin and leptin	(88)
Oral treatment of VAT mixture antigens	HFD-fed C57BL/6 mice	Restore decrease of VAT Treg cells; decrease $CD8^+$ T cells infiltration in VAT; limit the switch of M2 to M1 macrophages	Inhibit the gain of body weight and fat mass; improve insulin sensitivity	(89)
Stimulating $CD4^+CD25^-$ cells with the CD3/CD28 antibodies and IL-2/TGF-β	$CD4^+CD25^-$ cells separated from the blood of children with MS or control	Convert conventional $CD4^+CD25^-$ cells into Treg cells *in vitro*	No data available	(90)
Treating isolated Treg cells with EGCG	Treg cells isolated from normal-weight and obese subjects	Enhance the proliferation and IL-10 production of Treg cells *in vitro*; decrease NF-kappaB activity; increase histone deacetylase (HDAC) activity and HDAC-2 expression in Treg cells	No data available	(91)

diet had an increase in the number of Treg cells in epididymal adipose tissues compared with control mice fed with normal chow. However, only C57BL/6 mice showed lowered body and adipose tissue weight than controls (87). Recently, it was reported that oral administration of thiazolidinediones (TZDs), PPARγ agonists with insulin-sensitizing function, in HFD-fed mice enhanced the fraction and number of Treg cells specifically in epididymal adipose tissue (11, 27). However, whether the insulin-sensitizing effects of TZDs are dependent on the TZD-induced expansion of VAT Treg cells is still a matter of debate (11, 27).

In addition to conventional medicines, novel strategies have been tested to enhance VAT Treg cells and counteract

obesity-related metabolic disorders. A recent study reported that oral administration of Akkermansia muciniphila significantly induced Foxp3 Treg cells in the VAT, subsequently attenuated adipose tissue inflammation and improved glucose tolerance (88). Another study showed that oral treatment of VAT mixture antigens in HFD-fed C57BL/6J mice could not only restore the decrease of VAT Treg cells and reduce the infiltration of $CD8^+$ T cells in VAT, but also limit M2-type macrophages changing into M1-type, thereby, reducing the gain of body weight and fat mass, as well as improving insulin sensitivity (89).

Most of the studies discussed so far were carried out in animal models, while only a limited number of studies were performed on human cells or in human subjects. The $CD4^+CD25^-$ cells isolated from the peripheral blood of children with metabolic syndrome (MS) can be converted into Treg cells *in vitro* by the treatment with the CD3/CD28 antibodies and IL-2/TGF-β, suggesting a viable source of functional Treg cells exists in children with MS (90). Another *in vitro* study found that treatment with epigallocatechin gallate (EGCG) enhanced the proliferation and IL-10 production of Treg cells isolated from normal-weight and obese subjects (91). As EGCG is a potent anti-inflammatory agent from green tea, its effects on human Treg cells support an idea that dietary modulation of Treg cells could be a therapeutic strategy for patients with obesity.

Despite great potential benefits of expanding adipose tissue Treg cells, its clinical use is associated with possible risks. The major concern is that excessive Treg cell numbers may suppress the response to infection or promote tumor occurrence (93). Since metabolic diseases are chronic conditions, the treatment options are held to the highest standard for safety. The potential side effects may dampen the enthusiasm for Treg cell therapy in metabolic diseases. The majority of currently available approaches used to expand Treg cells *in vivo* promote systemic increase of Treg cells. Although PPARγ agonist can induce Treg cells specifically in VAT, its unexpected side effects in heart and kidney limit its application. The ideal strategies should include identification of reagents that specifically promote adipose tissue resident Treg cells, or invention of delivery systems specifically targeting adipose tissue.

CONCLUDING REMARKS AND FUTURE OUTLOOKS

In recent years, the focus has shifted from lymphoid tissue to tissue resident Treg cells (9, 94). Among them, adipose-resident Treg cell population is one of the best-characterized examples, which displays a unique phenotype. PPARγ has been described to be a key regulator of VAT Treg cell in maintaining its accumulation, phenotype and function. In addition, the proliferation and maintenance of VAT Treg cells are also regulated by other immune cells and cytokines, such as adipose tissue macrophages, iNKT cells, γδT cells, and IL-33. In mice, VAT Treg cells are down-regulated by obesity and may serve as a decisive cell population in the pathogenesis of obesity-related metabolic disorders. Most mouse studies focused on adipose-resident Treg cells support their beneficial function in suppressing inflammation and promoting thermogenesis, suggesting a potential strategy for the treatment of obesity-related metabolic disorders.

However, many questions regarding adipose-resident Treg cell development, tissue accumulation and lineage maintenance still remain to be addressed. Here we highlight several questions that particularly intrigue us and are interesting realms to explore. What antigen(s) are recognized by VAT Treg cells and stimulate their expansion? How do adipose-resident Treg cells acquire the PPARγ activity? Except PPARγ, are there any other regulators that provide adipose-resident Treg cells with unique characteristics? What are the phenotypes and functions of adipose-resident Treg cells in the humans and how are they regulated and modified in human diseases? Finally, how can we specifically manipulate adipose-resident Treg cells? Better understanding of VAT resident Treg cells could be a new strategy to treat or prevent obesity and related metabolic diseases. Studies aimed to address these questions may ultimately represent a potential novel strategy for treating chronic inflammation and metabolic disorders.

AUTHOR CONTRIBUTIONS

TD conceived the manuscript. QZ, XS, and TD wrote the manuscript. TD, MB, ZX, and LX revised the manuscript.

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REVIEW
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Regulation, Communication, and Functional Roles of Adipose Tissue-Resident CD4$^+$ T Cells in the Control of Metabolic Homeostasis

Haiyan Zhou[1] *and Feng Liu*[1,2]*

[1] Department of Metabolism and Endocrinology, Metabolic Syndrome Research Center of Central South University, The Second Xiangya Hospital, Central South University, Changsha, China, [2] Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, TX, United States

Evidence accumulated over the past few years has documented a critical role for adipose tissue (AT)-resident immune cells in the regulation of local and systemic metabolic homeostasis. In the lean state, visceral adipose tissue (VAT) is predominated by anti-inflammatory T-helper 2 (Th2) and regulatory T (Treg) cell subsets. As obesity progresses, the population of Th2 and Treg cells decreases while that of the T-helper 1 (Th1) and T-helper 17 (Th17) cells increases, leading to augmented inflammation and insulin resistance. Notably, recent studies also suggest a potential role of CD4$^+$ T cells in the control of thermogenesis and energy homeostasis. In this review, we have summarized recent advances in understanding the characteristics and functional roles of AT CD4$^+$ T cell subsets during obesity and energy expenditure. We have also discussed new findings on the crosstalk between CD4$^+$ T cells and local antigen-presenting cells (APCs) including adipocytes, macrophages, and dendritic cells (DCs) to regulate AT function and metabolic homeostasis. Finally, we have highlighted the therapeutic potential of targeting CD4$^+$ T cells as an effective strategy for the treatment of obesity and its associated metabolic diseases.

Keywords: obesity, CD4$^+$T cells, antigen-presenting cells, inflammation, insulin resistance, energy homeostasis

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Texas A&M University, United States
Liqing Yu,
University of Maryland, United States

***Correspondence:**
Feng Liu
liuf@uthscsa.edu

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INTRODUCTION

Obesity, which is associated with various metabolic and cardiovascular diseases such as insulin resistance, type 2 diabetes, hypertension, and stroke, is among the most severe health threats to modern society (1, 2). With excessive nutrient intake and/or reduced energy expenditure, obesity triggers a state of chronic low-grade inflammation in the adipose tissue (AT). Specifically, visceral adipose tissue (VAT) is more prone to obesity-induced inflammation (3). Under the condition of overnutrition, the composition, number, and function of AT-resident immune cells are significantly altered, especially in white adipose tissue (WAT). Recent studies have demonstrated a dynamic crosstalk between adipocytes and immune cells, including both innate and adaptive immune cells, within AT. In response to nutritional or other environmental stimuli, altered AT-resident immune cells may initiate a low-grade inflammatory process, leading to insulin resistance and impaired metabolic homeostasis. Understanding the mechanisms underlying immune cell-initiated inflammatory responses in AT of obese individuals is thus of great clinical importance.

Naïve CD4+ T cells are developed in the thymus and then reside in secondary lymphoid organs such as spleen and lymphocytes and non-lymphoid organs such as AT (4). After activation by antigen-presenting cells (APCs), naïve CD4+ T cells may differentiate into one of the several lineages of T helper (Th) cells, including T-helper 1 (Th1), T-helper 2 (Th2), T-helper 17 (Th17), and regulatory T (Treg) cells, as defined by their specific patterns of cytokine production and function. Compared with secondary lymphoid tissues, AT contains few naïve CD4+ T cells but a large proportion of effect memory T cells that can regulate adaptive immunity based on interactions with APCs (5, 6). Indeed, AT-resident CD4+ T cells are one of the immune cells that rapidly respond to HFD challenge (7). However, the roles of AT-resident CD4+ T cells in metabolic homeostasis are not well established. In the lean state, WAT is dominated by anti-inflammatory Th2 and Treg cells, which help to maintain an anti-inflammatory milieu and metabolic homeostasis. The total number of CD4+ T cells in WAT is significantly increased after HFD feeding. However, as obesity progresses, the populations of Th2 and Treg cells are decreased (8), concurrently with increased Th1 and Th17 cells (9, 10). While accumulative studies have demonstrated a critical role of CD4+ T cells in obesity-induced inflammation, their roles in adaptive thermogenesis in subcutaneous WAT (SAT) and brown adipose tissue (BAT) remain in its infancy.

It is well documented that CD4+ T cell activation is initiated by antigen presentation. However, how AT-resident CD4+ T cells are activated during obesity remains controversial. There is some evidence suggesting that adipocytes, macrophages, and dendritic cells all could act as APCs to promote CD4+ T cell activation in AT (11–13).

In this review, we discuss recent findings on how AT-resident CD4+ T cells are involved in the regulation of local and systemic metabolic homeostasis. We also attempt to highlight the therapeutic potentials of targeting CD4+ T cells to treat obesity and its associated metabolic syndrome.

ORCHESTRATION OF CD4+ T CELL SUBSETS IN IMMUNE RESPONSES

As an important component of adaptive immunity, CD4+ T cells play critical roles in defending against a large variety of pathogens. Besides, they are also involved in the pathogenesis of autoimmune diseases, asthma, and allergic responses. Naïve CD4+ T cells are activated by two signals including Class II major histocompatibility complex (MHCII)-mediated antigen presentation and co-stimulatory molecule- mediated co-stimulation, both provided by APCs. After activation, CD4+ T cells are differentiated into distinct subsets, depending on the cytokine signals in the microenvironment.

The four major lineages of CD4+ T cells, including the classical Th1 and Th2 cells, as well as Th17 and Treg cells, each have a characteristic cytokine profile (14). IL-12 and IFN-γ induce high expression levels of the Th1 master regulator T-box expressed in T cells (T-bet) and signal transducer and activator of transcription 4 (STAT4), promoting the naïve CD4+ T cells to differentiate into Th1 cells. With robust production of IFN-γ, Th1 cells mediate immune responses against intracellular pathogens (15). On the other hand, IL-4 induces high-level expression of STAT6 and GATA binding protein 3 (GATA3) in naïve CD4+ T cells, facilitating the differentiation of naïve CD4+ T cells to Th2 cells. With high expression levels of IL-4, IL-5, and IL-13, Th2 cells mediate host defense against extracellular parasites including helminths (15). Inappropriate Th2 responses are the major cause of allergic diseases such as asthma (16). IL-17-producing Th17 cells, induced by TGF-β, IL-6, IL-23, and IL-1β, contribute to the host defense against fungi and extracellular bacteria, with the high expression of their master regulator retinoic acid receptor-related orphan receptor-γt (RORγt) (15). The pathogenicity of Th1 and Th17 cells has been recognized in various autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis (17, 18). Treg cells represent a subset of CD4+ T cells characterized by high suppressive capacity. As a key transcription factor of Treg cells, Foxp3 is indispensable for Treg cell development. Treg populations have also been identified and characterized in other non-lymphoid tissues such as skin, intestine, lung, liver, fat, muscle and placenta, clearly indicating an important role of Treg cells in the maintenance of tissue homeostasis (2, 19).

Cross-regulation among CD4+ T cell subsets by specific cytokine networks and transcription factors is critical for determining CD4+ T cell fates (14). Indeed, T-bet$^{-/-}$mice exhibit a severe disease after virus infection and display asthma-like phenotype independent of allergen exposure (20). In addition, T cell-specific deletion of Gata3 results in impaired Th2 differentiation, permitting Th1 differentiation in the absence of IFN-γ and IL-12 (21). Similar to the crosstalk between Th1 and Th2 cells, cross-regulation has also been reported between Th17 and Th1 or Th2 cells. Both Th1-specific cytokine IFN-γ and Th2-specific cytokine IL-4 inhibit Th17 differentiation and induction of IL-17 (22). The immune homeostasis of Th1, Th2, and Th17 cells has also been found to be regulated by Treg cells via production of TGF-β and IL-10 (14).

ROLES OF VAT-RESIDENT CD4+ T CELLS IN OBESITY

Treg Cells

Treg cells are thought to be one of the most crucial defenses against inappropriate immune responses including autoimmunity, allergy, inflammation, and infection (23). Treg cells are highly enriched in the VAT of lean mice, but their numbers in this fat depot are markedly and specifically reduced in animal models of obesity and insulin resistance (2, 8). Treg cells contribute to the maintenance of insulin sensitivity in WAT by limiting inflammation and producing insulin-sensitizing factors such as IL-10 (24). IL-10 suppresses the expression of monocyte chemotactic protein-1 (MCP-1) in adipocytes to limit M1 macrophage infiltration of WAT. Besides, IL-10 could inhibit the ability of TNF-α to downregulate glucose transporter 4 (GLUT-4) expression and impair insulin action in adipocytes (25). A loss-of-function experiment by diphtheria toxin receptor

(DTR)-mediated depletion of Treg cells and a gain of function experiment by injection of recombinant IL-2 and particular an IL-2-specific monoclonal antibody (mAb) have revealed that manipulating Treg cells can affect the inflammatory state of AT (2, 26).

Treg cell homeostasis in VAT is regulated by iNKT cells via the production of IL-2 (27). Besides, IL-33, which is mainly secreted by a number of different stromal cell types including Cadherin11$^+$ (Cdh11$^+$) mesenchymal cells, podoplanin$^+$ (Pdpn$^+$) fibroblasts, and CD31$^+$ endothelial cells, is required for Treg cell accumulation in VAT through binding to its receptor Interleukin 1 receptor-like 1, also known as IL1RL1 or ST2 (28, 29). In addition, IL-33 and IFN-γ counter-regulate group 2 innate lymphoid cells (ILC2) activation to control Treg cell numbers (30). The control of Treg cells by ILC2 is independent of the cytokines of ILC2 but mediated by a direct interaction of co-stimulatory molecules inducible co-stimulator (ICOS) and ICOS ligand (ICOSL) (30).

What is the origin of AT-resident Treg cells? It is reported that the AT-resident Treg and conventional T (Tconv) cell populations have different repertoires, suggesting that the accumulation of Foxp3$^+$ Treg cells in VAT is not due to the local conversion of Tconv cells (2). The VAT-resident Treg cells are also found not to be originated from circulating Treg cells. On the other hand, there is strong evidence suggesting that VAT-resident Treg compartment comes from thymus and their accumulation depends on interactions with local APCs (28).

VAT-resident Treg cells have a distinct transcriptome and antigen-receptor repertoire from those of their counterparts in the spleen and lymph nodes (31). Notably, peroxisome proliferator-activated receptor (PPAR-γ), the master regulator of adipocyte differentiation, is identified as a crucial molecular orchestrator of VAT Treg cell accumulation, phenotype, and function (31). Specifically, knockout of PPAR-γ in Treg cells significantly lowered the fractions and numbers of Treg cells in VAT but not in lymphoid organs. The thiazolidinedione (TZD) drug pioglitazone (Pio), a well-known insulin-sensitizing agent, is a synthetic agonist for PPAR-γ. Pio treatment specifically promotes VAT-resident Treg cell numbers and phenotype in HFD-fed wild-type (WT) mice but not in PPAR-γ mutant mice (31).

Th2 Cells

Similar to VAT-resident Treg cells, the IL-4- and IL-13-expressing Th2 cells accumulate in VAT of older animals. Compared with VAT-resident Treg cells, the numbers of VAT-resident Th2 cells are relatively rare and their function in obesity progression is much less studied. VAT Th2 cells also express ST2 and treatment of ob/ob mice with IL-33 leads to the production of strong Th2 cytokines in WAT, resulting in improved insulin sensitivity (32, 33).

Adoptive transfer of CD4$^+$ T cells into HFD-fed Rag1-null mice has normalized obesity-associated insulin resistance (34). Interestingly, the beneficial effects of CD4$^+$ T cells in the adoptive transfer model are found to be contributed by Th2 cells but not Treg cells, since mice transferred with Foxp3$^{-/-}$ or IL-10$^{-/-}$ CD4$^+$ T cells show no obvious changes in the phenotypes compared with mice transferred with WT CD4$^+$ T cells. By contrast, reconstitution with STAT6$^{-/-}$ CD4$^+$ T cells leads to the loss of the insulin-sensitizing effects of the WT CD4$^+$ T cells, suggesting that Th2 cells are important controllers of obesity and insulin resistance (34).

Th1 Cells

Under over-nutrition conditions, VAT expansion creates an environmental milieu that potentiates the influx of proinflammatory cells and the production of type 1 cytokines such as IL-6, TNF-α, IL-1β, and IFN-γ (35, 36). The immune homeostasis in VAT is consequently disrupted with a decrease of Treg and Th2 cell populations, concurrently with a significant increase of proinflammatory T cells, especially IFN-γ-producing Th1 cells, CD8$^+$ T cells, and Th17 cells (9, 10, 12, 37, 38). Consistent with these findings, T-bet deficient mice display enhanced insulin sensitivity though increased VAT mass (39), suggesting that deficiency of Th1 cells may lead to metabolic restoration. Increasing Th1 cells accelerate insulin resistance by producing TNF-α and IFN-γ in WAT (11). IFN-γ is a robust proinflammatory cytokine that activates M1 macrophages (40), promotes Th1 cell polarization (41), and induces inflammatory mediators such as MHCII (9, 42). The mRNA level of IFN-γ rapidly increases even after just 1-week HFD feeding (42). High level of IFN-γ in human VAT is also associated with increased waist circumference (7). IFN-γ-deficient mice show a significant decrease in inflammatory gene expression and accumulation of leukocytes, as well as improved glucose tolerance (9). Taken together, these findings reveal that decreased IFN-γ levels and/or Th1 cell expansion in AT are beneficial for suppressing inflammation and improving insulin sensitivity.

Th17 Cells

Th17 cells are usually regarded as proinflammatory cells. However, the role of AT-resident Th17 cells in obesity remains largely elusive. One study shows that, although the total number of Th17 cells in VAT remains unchanged, the percentage of Th17 cells is decreased during the development of obesity (34). To the contrary, other studies show that the numbers or percentages of Th17 cells are increased in AT of obese humans compared to their lean controls (12, 43). Th17 cells have been suggest to block insulin receptor signaling and contribute to metabolic dysfunction via promoting the secretion of IL-17 and IL-22 (43–45). HFD feeding is also able to stimulate splenic Th17 cell development, thus accelerating the onset of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) (10, 46). Mechanistically, obesity boosts Th17 cell polarization by upregulating Acetyl-CoA carboxylase 1 (ACC1) expression, which promotes the binding of RORγt to the IL-17 gene locus (10). RORγt$^{-/-}$ mice fed a normal-chow diet display glucose intolerance hyperinsulinemia, and slightly insulin resistance. However, RORγt$^{-/-}$ mice fed a HFD rapidly lost weight and die within 1 month, probably due to a deleterious effect of the lipotoxicity. Of note, after HFD feeding, heterozygous RORγt$^{+/-}$ mice have impaired glucose tolerance

and increased insulin resistance compared with WT control mice (47).

IL-17, a major effector cytokine produced by Th17 cells, functions as a negative regulator of adipogenesis (48, 49). IL-17 deficiency enhances diet-induced obesity in mice and accelerates fat mass accumulation even in mice fed a low-fat diet (49). It has been shown that γδ T cells, but not Th17 cells, are the predominant cells that produce IL-17 in WAT under both ND and HFD conditions (49, 50). Thus, the role of IL-17 is not equal to the role of Th17 cells under either ND or HFD conditions. Mice lacking γδ T cells or IL-17A had impaired ability to regulate core body temperature at thermoneutrality and after cold challenge due to the decreased $ST2^+$ Treg cells and IL-33 abundance in AT (50). Nevertheless, the function of Th17 cells in obesity-related WAT inflammation is complex and requires further investigation.

$CD4^+$ T CELLS CROSSTALK WITH APCS

$CD4^+$ T cells in VAT are increasingly recognized as a key regulator of AT inflammation and systemic insulin action. APCs are indispensable for the activation and differentiation of $CD4^+$ T cells (11, 51). Importantly, VAT resident $CD4^+$ T cells, regardless of their specific lineages, demonstrate distinct and selective T cell receptor (TCR) repertoires compared with their circulating counterparts, suggesting an AT-specific antigen expansion (2, 31, 34). This finding further strengthens the view that APCs also exist in AT and are required for AT-resident $CD4^+$ T cell polarization. Although the exact nature of the antigens is still largely unknown, recent studies have revealed different types of APCs including macrophages, adipocytes and dendritic cells in the course of obesity-related AT inflammation (11, 42, 52).

Adipose Tissue Macrophages (ATMs)

Macrophages have been implicated as one of the important types of APCs found in AT (11). Based on their characteristics and functions, adipose tissue macrophages (ATMs) can be categorized into "classically activated" M1 macrophages and "alternatively activated" M2 macrophages (36). Obesity is accompanied by a switch in macrophage activation from the protective M2 macrophages to the proinflammatory M1 macrophages (53). ATMs are thought to be the predominant MHCII-expressing cells in VAT under both ND and HFD feeding conditions (51). MHCII-deficient mice are protected from HFD-induced insulin resistance with the reduction of ATMs and $CD4^+$ T cells accumulation in VAT (11, 42). It is believed that AT-resident M2 macrophages are the predominant APCs in lean mice and humans (11, 36). MHCII in M2 macrophages is required to translate obesogenic cues into $CD4^+$ T cell immune responses at the initial stage of obesity (11). During the development of obesity, M2 macrophages may progressively obtain a proinflammatory phenotype and induces Th1 cell polarization, accelerating the development of insulin resistance (54). Immunofluorescence and intravital imaging analysis show that ATMs physically interact with $CD4^+$ T cells in an antigen-dependent manner (11). Macrophage-specific deletion of MHCII has no effect on AT-resident T cells in the lean state, but significantly prevents the generation of effector memory AT-resident $CD4^+$ T cells and insulin resistance in AT (11). Diet-induced obesity also promotes the expression of T-cell co-stimulatory molecules, such as CD80 and CD40, on the surface of ATMs in VAT (55). CD40 deficiency affects ATM infiltration into VAT and decreases T cell accumulation during diet-induced obesity.

In contrast to the classical view of ATMs being grouped into M1 and M2 macrophages, a recent study shows that CD9 and Ly6c define unique populations of ATMs in obese AT, with CD9 ATMs predominating in crown-like structures (CLS) and Ly6c ATMs uniformly distributed in AT (56). CD9 ATMs contain high levels of intracellular lipid and express proinflammatory transcriptomes while Ly6c ATMs express factors that support angiogenesis and tissue organization (56). In addition, adoptive transfer of CD9 ATMs, but not Ly6c ATMs, from obese donor mice to lean recipients confers an inflammatory response to the AT of lean mice (56). Nevertheless, whether these two subsets of ATMs function distinctively in antigen presentation is not explored. Since MHCII expression ATMs are concentrated in CLS in obese AT (51), There is a possibility that CD9 ATMs, rather than Ly6c ATMs, may be the main ATMs that activate $CD4^+$ T cells in AT. Further studies are needed to address this possibility.

Adipocytes

While the role of ATMs in AT inflammation is well documented, several studies report that macrophages do not infiltrate into AT until 10 weeks after HFD feeding (7, 42). Indeed, $CD8^+$ effector T cells are believed to contribute to the later macrophage recruitment (37). On the other hand, an early infiltration of lymphocytes is observed soon after HFD feeding (7, 37, 42). The early presence of T cells in VAT at the time of manifest insulin resistance raises a possibility that there may be other APCs that initiate T cell activation in AT. Consistent with this view, adipocytes are recently implicated as APCs that influence T cell activation in obesity (42). Expression of MHCII in adipocytes begins to increase within 2 weeks of HFD challenge, paralleled with early changes of AT-resident $CD4^+$ T cells which show enhanced expression of the proinflammatory Th1 marker genes (42). HFD-fed $MHCII^{-/-}$ mice show less adipose inflammation and insulin resistance (42). Mechanically, it is suggested that, as obesity advances, leptin secreted by adipocytes stimulates IFN-γ production from $CD4^+$ T cells, which further promotes adipocyte MHCII expression and thus Th1 differentiation, leading to a vicious cycle of AT inflammation (42). The specific contribution of this vicious cycle to metabolic dysfunction is further verified by adipocyte-specific disruption of MHCII. AT-specific knockout of MHCII suppresses AT IFN-γ production and increases Treg accumulation, leading to reduced AT inflammation and insulin resistance in obese mice (13). Inhibition of MHCII expression in adipocytes by adrenomedullin 2 treatment restores the HFD-induced early insulin resistance due to decreased $CD4^+$ T cell activation (57). It is suggested that IL-10 produced by adipocytes may dampen the APC function of ATMs, thus showing the superiority of adipocytes over ATMs as APCs at the early stage of obesity (42). Indeed, recruitment

of M1 macrophages into WAT is induced by inflammatory mediators such as MCP1, C-X-C motif chemokine 12 (CXCL12) produced by dead and neighboring adipocytes (58, 59). While APCs may shape $CD4^+$ T cells, $CD4^+$ T cells can also influence the recruitment and activation of APCs. CD40L is induced in AT $CD4^+$ T cells after HFD feeding, which can further stimulate activation of ATMs as well as adipokine production of adipocytes through ligation with CD40 (55, 60).

Adipocytes secret various adipokines such as leptin, adiponectin, and resistin, which are implicated in the regulation of $CD4^+$ T cell immune responses. Leptin receptor is expressed in human AT T cells and its expression increases with obesity (61). Impairment of leptin receptor signaling improves Treg cell immune responses (62, 63). However, how does leptin signaling regulate Treg responses remains elusive. Although both IL-33 and ILC2 are found to promote AT Treg accumulation (30), studies show that IL-33 expression positively correlates with leptin expression in human AT (64). Obesity-associated elevation of leptin also contributes to the increased susceptibility of asthma via modulation of Th2 and ILC2 response (65). These findings suggest that leptin may regulate Treg immune response independent of IL-33 and ILC2. On the other hand, leptin receptor signals are required for Th17 differentiation via activation of signal transducer and activator of transcription 3 (STAT3) and through cooperate with IL-6 (45, 66, 67). Leptin can also stimulate Th1 cell differentiation through promoting IFN-γ secretion (42, 68). Adiponectin is another adipokine that has been shown to directly enhance Th1 differentiation by activating the p38-STAT4-T-bet axis (69). Adiponectin activates DCs leading to enhanced Th1 and Th17 responses (70). Lastly, resistin has been found to stimulate $CD4^+$ T cell chemotaxis in a concentration-dependent manner (71).

During obesity, bioactive lipids released by adipocytes also involve in the regulation of $CD4^+$ T cells. Ceramide synthesis is elevated under obesity conditions, correlating positively with the degree of insulin resistance (72). Ceramides are localized predominantly within the cell membrane and are suggested to enhance Th1 cell differentiation together with IL-12 (73). Many ceramide derivatives have been found to inhibit IL-4 production in T cells (74). n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can alter the biochemical and biophysical properties of $CD4^+$ T cell plasma membranes, thus modulating cytoskeletal dependent $CD4^+$ T cell activation and differentiation (75). It is also suggested that n-3 PUFA suppress Th1/Th17 immune responses in diverse tissues in obese mice following the induction of colitis (76).

Whether adipocytes in WAT of lean mice also play a role as APCs is not clear. VAT Adipocytes of lean mice show low but detectable MHCII expression. When cocultured with $CD4^+$ T cells *in vitro*, adipocytes of lean mice could stimulate $CD4^+$ T cells IFN-γ and IL-2 production though to a much less extent than that of obese mice (42). The antigen-presentation capacity of adipocytes from lean AT-specific MHCII knockout mice is remarkably reduced compared with lean WT mice (13). It is possible that adipocytes of lean mice with low-level MHCII expression could also act as APCs. However, whether and how adipocytes of lean mice function as APCs to regulate $CD4^+$ T cell activation *in vivo* remain to be further determined.

Adipose Tissue Dendritic Cells (ATDCs)

Dendritic cells (DCs) are professional APCs and play an important role in promoting $CD4^+$ T cell activation and polarization (77). However, it has been difficult to clarify the contribution of ATDCs to AT inflammation since clear discrimination between ATDCs and ATMs in AT is limited. It is suggested that, in lean mice, the majority of $CD11c^+$ cells are ATDCs but not ATMs (78). HFD feeding for 16 weeks led to a substantial increase in $CD11c^+$ infiltrating M1 macrophages and the maintenance of a prominent population of $CD11c^+$ ATDCs (78). Since ATMs and ATDCs are both $CD11c^+$ cells in WAT of obese mice, macrophage-specific marker CD64 is thus adopted to distinguish the two populations, with $CD11c^+CD64^+$ identified as infiltrating M1 macrophages and $CD11c^+CD64^-$ identified as ATDCs (11). Both populations have similar capacities to stimulate $CD4^+$ T cell proliferation (78).

Another study defines $CD11b^-CD11c^+$ cells as ATDCs, which express higher levels of MHCII than $CD11b^+CD11c^+$ ATMs (28). Confocal analysis reveals that both Treg and Tconv cells are in close contact with ATMs and ATDCs (28). The distance between T cells and APCs is dramatically increased in mice treated with an anti-MHCII mAb, suggesting that ATMs and ATDCs may contact with T cells through MHCII. (28). Ablation of $CD11c^+$ cells by DTR normalizes insulin sensitivity in obese and insulin resistant mice (79). Since CD11c is commonly recognized as a marker of DCs, this finding suggests that the deletion of DCs, at least in part, may contribute to the increased insulin sensitivity (80).

The majority of ATDCs in the lean state are thought to be $CD11c^{high}F4/80^-CD103^+$ cells. Since $CD103^+$ DCs are able to induce the development of Treg cells (81), it is suggested that this $CD11c^{high}F4/80^-CD103^+$ ATDCs play a role in the induction of AT Treg cells to restrain AT inflammation (12). Some atypical $CD11c^{high}F4/80^{low}CX3CR1^+$ ATDCs are also detectable at a very low frequency (<1%) in the AT of lean mice. Both the frequencies and absolute numbers of these two ATDCs populations are increased after HFD feeding, accompanied by enhanced antigen-presenting abilities to induce Th17 differentiation (12). It's worth mentioning that the increased atypical $CD11c^{high}F4/80^{low}CX3CR1^+$ ATDCs, regarded as inflammatory DCs in AT, are the major contributors to the induction of Th17 cells in AT of obese mice possibly via expressing high levels of IL-6, TGF-b, and IL-23 (12, 52). This observation is in accordance with previous studies that demonstrate the importance of obesity in the expansion of Th17 cells (10, 46).

Although much progress has been made on our understanding of the role of AT-resident $CD4^+$ T cells in regulating metabolism, it is still unclear which cells are the major APCs at different stages of obesity and whether these APCs cooperate to activate $CD4^+$ T cells. To define distinct populations within

each APCs with unique transcriptomes and functions is of great importance, which will help to develop APCs-based therapies for the treatment of obesity and related inflammatory comorbidities.

THE ROLES OF CD4+ T CELLS IN ENERGY HOMEOSTASIS IN SAT AND BAT

Despite extensive studies on the functional roles of adipose-immune crosstalk in VAT, the role and regulation of $CD4^+$ T cells in adaptive thermogenesis are much less clear. Several recent studies have uncovered a potential function of Treg cells in SAT and BAT in regulating energy homeostasis (4, 82). BAT-resident Treg cells share many similar characteristics with VAT-resident Treg cells, although BAT harbors more Treg cells than VAT (82). Systemic depletion of Treg cells impairs oxygen consumption under cold stimulation conditions (82). In fact, treatments known to enhance sympathetic tone and promote BAT thermogenesis such as cold exposure, short-term high-calorie input, and β-adrenergic stimulation, greatly increase Treg cells in WT but not in β-less mice in which all of the three β-adrenergic receptors are deleted (4). These results indicate an essential role for thermogenic response in BAT Treg cell accumulation. Indeed, UCP-$1^{-/-}$ mice exhibit reduced Treg cells in BAT and SAT compared with WT control mice (4). Furthermore, loss-of-function and gain-of-function experiments all suggest that Treg cells are critical for BAT thermogenic capacity and lipolytic function (4). The T cell-specific Stat6/Pten axis is believed to link beta3-adrenergic stimulation to Treg cell induction in BAT and SAT, which is consistent with a previous report that inhibition of PI3K/AKT could promote Treg differentiation (4, 83). Interestingly, IL-17-producing γδ T cells are recently reported to regulate thermogenesis via BAT Treg cells, further supporting an important role of Treg cells in energy expenditure (50).

In contrast to the role of Treg cells, the role of Th2 cells in energy expenditure is largely unknown. However, given that both the transcription factors and cytokines are functionally similar between Th2 cells and ILC2s (32, 84), it is possible that the Th2 cells may also play a part in energy expenditure. Further investigations will be needed to address this question.

Rag$1^{-/-}$ mice that lack both T and B lymphocytes display excess weight gain under HFD-feeding conditions, which is at least in part due to decreased energy expenditure resulted from decreased UCP-1 expression in BAT (85). In contrast, another study showed that even in the lean state, Rag$1^{-/-}$ mice display more energy expenditure and upregulated of UCP1 expression in SAT than WT mice at room temperature (86). Decreased $CD8^+$ T cells, but not $CD4^+$ T cells, are believed to contribute to promote beige fat development, mainly due to the decreased IFN-γ secretion (86). However, given that Th1 cells are also major producers of IFN-γ and that HFD feeding increases both the percentage and the total number of Th1 cells in SAT, it is possible that Th1 cells may also be involved in the regulation of energy expenditure.

THERAPEUTIC IMPLICATIONS OF TARGETING CD4+ T CELLS

Targeting Chemokines and Their Receptors

Infiltration of proinflammatory $CD4^+$ T cells into VAT is now recognized as one of the primary events in obesity-induced chronic inflammation. Chemokines and their receptors play crucial roles in the trafficking of leukocytes to lesions and areas of inflammation (87, 88). Antagonizing chemokines and/or their receptors by small molecules or antibodies have been shown to be another promising approach to suppress inflammation and potentially, improve metabolic dysfunction.

Indeed, $CD4^+$ T cells, as well as $CD8^+$ T cells and B cells, from ob/ob mice had a greater propensity to migrate specifically to inflamed tissues (89). The regulated on activation normal T cell expressed and secreted (RANTES), also known as CCL5, is a chemokine that plays an active role in recruiting leukocytes into inflammatory sites. RANTES and its chemokine receptor CCR5 have been implicated in T cell trafficking to VAT in the setting of murine and human obesity (88). The expression of RANTES and CCR5 in WAT, especially the SVF fraction, is increased in a gender-dependent fashion in obese mice (88). Interestingly, monoclonal antibodies against RANTES have been shown to significantly reduce T-cell chemotaxis (88, 90).

The CCR5/RANTES axis also plays an important role in the progression of hepatic inflammation and fibrosis. Maraviroc, a CCR5 antagonist that has already been approved by FDA for the treatment of human immunodeficiency Virus (HIV) (91), ameliorates hepatic steatosis in an experimental model of NAFLD (87). Another CCR5 ligand, CCL3, is also secreted at significantly high levels in the omentum of patients with an obesity and inflammation-driven cancer oesophagogastric adenocarcinoma (OAC). Antagonizing CCL3 receptor, including CCR5 and CCR1, significantly reduces T cell migration to the omentum of OAC patients (92). As obesity develops, human adipocytes release the chemokine CCL20 and promote T cell migration into VAT via its receptor CCR6 (61).

Pharmacological inhibition of chemokines may exert beneficial pleiotropic effects in several metabolically active organs since these organs are likely to be affected by similar cellular, molecular, or endocrine pathways (93). Elucidation of the mechanisms that recruit inflammatory $CD4^+$ T cell to AT should improve our knowledge for developing novel therapeutics for inflammation-associated metabolic dysfunctions.

Promoting Treg Cell Accumulation in AT

Induction of Treg cells is one of the major goals in immunotherapy of autoimmune diseases and transplantation. The emerging notion that Treg cells in AT are important for immune homeostasis and thermogenesis has evoked an exciting possibility to expand Treg cells as a therapeutic strategy for the treatment of obesity-induced metabolic dysfunctions (94) (**Figure 1**). In some studies, mitogenic anti-CD3 antibody is utilized to promote T cell self-tolerance through a global but transient T cell depletion, which leads to a selective increase of Treg cell pools at sites of tissue inflammation (95, 96).

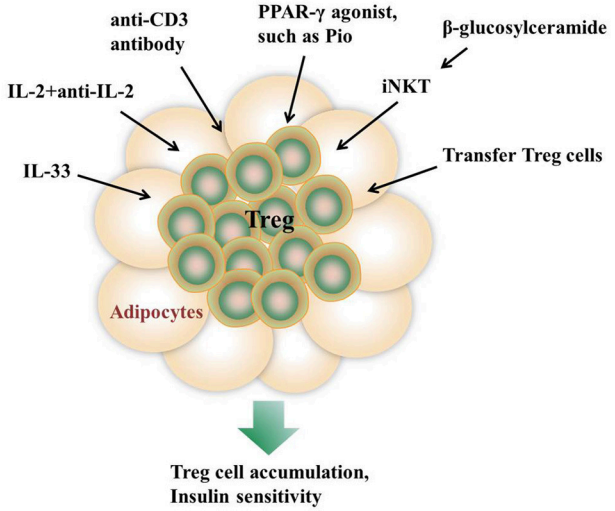

FIGURE 1 | Approaches to promote AT Treg cell accumulation *in vivo*. Injection of IL-33, the anti-CD3 antibody, or a complex consisting of recombinant IL-2 and a particular anti-IL-2 monoclonal antibody all show a long-lasting effect on Treg cell expansion in both lymphoid tissues and VAT. β-glucosylceramide induces iNKT cells activation which promotes Treg cells expansion via IL-2 production. In addition, adoptive transfer of Treg cells into recipient mice is recognized as a straight way to increase Treg populations. PPAR-γ agonist Pio treatment also specifically promotes VAT Treg cell accumulation.

Injection of an anti-CD3 antibody to HFD-fed mice for 5 days greatly improves glucose tolerance and insulin sensitivity (34). In addition, the normalizing effects on insulin resistance and glucose tolerance last for over 4 months even under the condition of sustained HFD feeding, suggesting a long-lasting therapeutic effect (34). Oral administration of an anti-CD3 antibody plus β-glucosylceramide displays a decrease in pancreatic islet cell hyperplasia, fat accumulation in the liver, and inflammation in adipose tissue via induction of Treg cells (24). In addition, a complex consisting of recombinant IL-2 and a particular anti-IL-2 mAb is used to induce *in situ* expansion of Treg cells (2, 97). IL-33 injection shows a long-lasting effect on Treg cell expansion in both lymphoid tissues and VAT (28), while PPAR-γ agonist Pio treatment specifically promoted VAT Treg cell accumulation (31). In some studies, adoptive transfer of Treg cells into recipient mice is recognized as a straight way to increase Treg populations (4).

Targeting Gut Microbiota

Profound gut microbiota alterations are found to be closely associated with obesity and metabolic syndrome in recent years (98). HFD feeding induces prominent alterations in the gut microbiota composition by increasing the Firmicutes to Bacteroidetes ratio, which positively correlates with body weights in humans (99–101). The development and maturation of $CD4^+$ T cells are influenced by gut microbiota. Eating purified probiotic microbe alone, namely Lactobacillus reuteri, prevented weight gain irrespective of the baseline diet due to the promotion of IL-10-producing Treg cells (102). HFD-fed mice supplemented with a mixture of foodborne lactic acid bacteria show reduced VAT mass with increased Treg cells (103). There is some evidence showing that HFD-derived ileum microbiota is responsible for a decrease of Th17 cells in the lamina propria, while microbiota from synbiotic-treated obese mice increases the number of intestinal Th17 cells and improves glucose tolerance (47). In addition, delivery of Th17 cells to the intestines of obese mice leads to expansion of commensal microbes that maintain metabolic homeostasis (104). However, the precise mechanisms by which microbiome regulates $CD4^+$ T cells and thus metabolic homeostasis remain largely unknown and require further investigations.

TABLE 1 | Summary of the timeline of appearance or changes of the major cell types or factors that contribute to the proinflammatory status of adipose tissue after HFD feeding.

Major cell types or factors	Timeline of appearance or changes	References
Leptin	Within 1 week	(42)
Adipocyte MHCII	Within 2 weeks	(42)
T-bet	2 weeks	(42)
GATA3	3 weeks	(42)
Foxp3	12 weeks	(42)
IFN-γ	2 weeks	(42)
M1	10–12 weeks	(42), (7)
CD3	5 weeks	(7)
$CD11c^{high}F4/80^{low}$	15 weeks	(12)

CONCLUSIONS AND PERSPECTIVES

New evidence accumulated over the past several years strongly implicates an important role of AT-resident immune cells such as Th2 and Treg cells in the housekeeping functions of animals or humans via regulation of inflammation. During the progression of obesity, specific antigens in VAT are produced, captured, and presented to $CD4^+$ T cells by APCs, leading to decreased Th2 and Treg cell populations and a shift to increased proinflammatory Th1 and Th17 cell populations. The timeline of appearance or changes of the major cell types or factors that contribute to the proinflammatory status of adipose tissue after HFD feeding is summarized in **Table 1**. Although the roles of $CD4^+$ T cells in obesity have been largely investigated, more efforts will be needed to elucidate the function of $CD4^+$ T cells in the regulation of energy expenditure in AT.

It remains to be controversial as to which cell types are the major APCs in the AT. Both ATMs and adipocytes show enhanced MHCII gene expression under obesity conditions. Deficiency of MHCII in either macrophages or adipocytes shows improved metabolic phenotypes in mice (11, 13). However, several studies report that macrophages do not infiltrate into AT until 10 weeks after HFD feeding while MHCII family genes are upregulated at 2 weeks after HFD, indicating that adipocytes but not ATMs are the APCs that initiate T cells

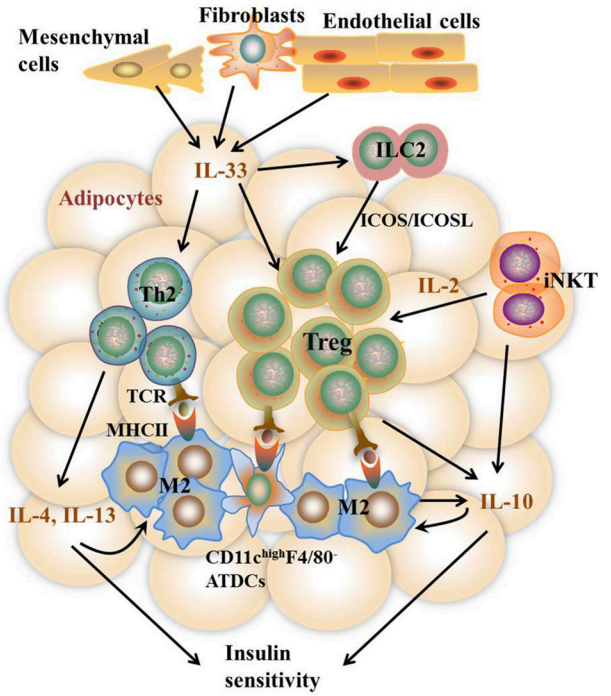

FIGURE 2 | Th2 and Treg cell-mediated immune responses in VAT under the lean state. In the lean state, activation and proliferation of AT-resident Th2 and Treg cells are mediated by APCs including M2 macrophages, CD11c ^{high}F4/80^{-} ATDCs, and maybe adipocytes with low MHCII expression. Activated Th2 cells produce type 2 cytokines including IL-4, IL-5, and IL-13, which together with iNKT and Treg cell-produced IL-10 further stimulate M2 activation. IL-10 also acts directly on adipocytes to promote insulin sensitivity. The homeostasis of Th2 and Treg cells is promoted by constitutively produced IL-33 from Cdh11^{+} mesenchymal cells, Pdpn^{+} fibroblasts, and CD31^{+} endothelial cells via high expression of ST2 on the surface of both cells. In addition, IL-33 and IFN-γ counter-regulate ILC2 activation to control Treg cell numbers. Besides, iNKT cells are necessary to sustain Treg cells via the production of IL-2. Together, these pathways contribute to metabolically healthy VAT.

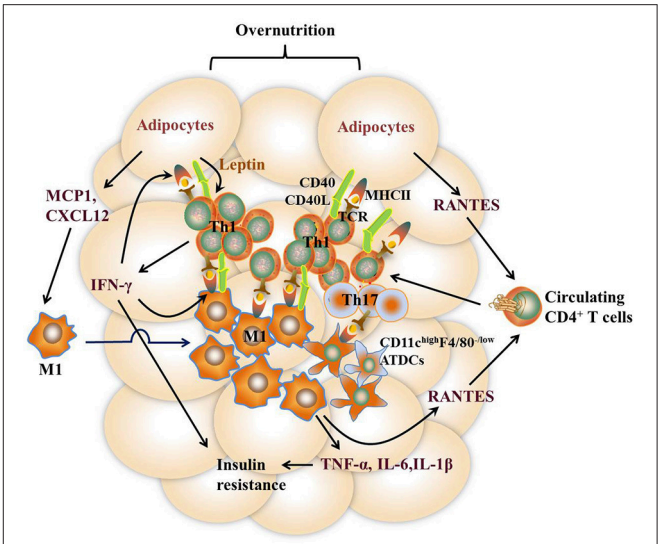

FIGURE 3 | Th1 and Th17 cell-mediated immune responses in VAT under the obese state. Overnutrition causes adipocyte hypertrophy, leading to the release of chemokines such as RANTES, which recruit proinflammatory CD4^{+} T cell accumulation in VAT via its receptor CCR5. Leptin secreted by adipocytes stimulates IFN-γ production from CD4^{+} T cells, which further promotes adipocyte MHCII expression and antigen-presentation to induce Th1 cell differentiation, leading to a vicious cycle of AT inflammation. Dead and neighboring adipocytes recruit M1 macrophages to WAT by producing inflammatory mediators such as MCP1 and CXCL12. Likewise, the expression of MHCII in M1 macrophages is promoted by IFN-γ, thus facilitating M1 macrophage-mediated antigen-presentation to induce Th1 cell differentiation. In addition, diet-induced obesity also promotes the expression of inflammatory receptor CD40 expression on ATMs and adipocytes as well as CD40L on CD4^{+} T cells, which reinforce the crosstalk between CD4 $^{+}$ T cells and these APCs. CD11c ^{high}F4/80 ^{low} ATDCs are also regarded as APCs to induce Th17 reactivation via production of TGF-β, IL-6, and IL-23. Type 1 cytokines such as TNF-α and IFN-γ, act directly on adipocytes to impair insulin action. Together with IL-6 and IL-1β, these cytokines elicit sustained chronic inflammation that eventually leads to insulin resistance.

activation (7, 42). On the other side, several studies suggest that ATMs are the predominant MHCII-expressing cells in VAT under both ND and HFD feeding conditions, arguing that non-macrophage cells such as adipocytes play a minor role in MHCII expression in adipose tissue (11, 28, 36, 53). Thus, it is possible that different cell types may act as APCs at different stages of obesity. At early stages, adipocytes of lean mice with a low expression of MHCII, AT-resident M2 macrophages, and CD11c^{high}F4/80^{-} ATDCs may act as APCs, leading to the homeostatic proliferation of Th2 and Treg cells. As obesity develops, adipocytes of obese mice with markedly increased MHCII expression, infiltrating M1 macrophages, as well as CD11c^{high}F4/80^{-} and CD11c^{high}F4/80^{low} ATDCs become dominant in the AT that act as APCs instead [**Figures 2**, **3**). This notion, to a certain extent, has conciliated different views on the regulation and function of APCs in AT.

It's also debatable about the phenotypes and function of Th17 cells in obesity development. Most studies suggest that HFD feeding promotes the percentages of Th17 cells in AT and periphery, which contributes to the acceleration of obesity and some autoimmune diseases in which obesity is recognized as a risk factor (10, 45, 46, 52). However, HFD-derived gut microbiota decreases Th17 cells in the lamina propria (47). Heterozygous RORγt$^{+/--}$ mice promote diet-induced obesity and insulin resistance compared with WT mice (47). Delivery of Th17 cells to the intestines of obese mice results in expansion of commensal microbes that maintain metabolic homeostasis (104). One explanation for the discrepancies may be due to the differences in the functions of local APCs. HFD-induced gut microbiota impairs the gene expression profile and function of lamina propria APCs required for Th17 Cell Differentiation (47), whereas the APCs in AT of obese mice show higher levels of cytokines secretion or surface markers expression that facilitate Th17 cell differentiation (12, 52). Still, there is a possibility that Th17 cells in distinct organs may function differently.

The recent groundbreaking research on the roles of AT-resident CD4^{+} T cells in the regulation of insulin sensitivity and energy homeostasis has shed new light on our understanding

of the communication between immune cells and adipocytes, paving a road to the development of novel therapeutic strategies for the treatment of obesity and its associated diseases. However, many important questions remain to be addressed. Identifying the molecular nature of antigens associated with AT inflammation during obesity is of great importance, which can help to restrain proinflammatory $CD4^+$ T cell immune responses. A future challenge will also involve ascertaining the possibilities and molecular mechanisms of the functional interplay between other immune cells and $CD4^+$ T cells, especially in SAT and BAT. Unlike their circulating counterparts, Treg cells express an AT specific marker PPAR-γ (31) and show a high degree of adaptation to the surrounding milieu. Thus, it is assumable that the metabolic microenvironment in AT may also endow other $CD4^+$ T cells with specific characters. An answer to this question would provide new insights into developing organ-specific therapies for obesity and its related metabolic disorders. Nevertheless, translating the preclinical findings into clinical applications remains a great challenge, waiting for a further understanding of the mechanisms regulating the interaction between AT-resident immune cells and adipocytes under both physiological and pathological conditions.

AUTHOR CONTRIBUTIONS

HZ organized and wrote the draft. FL revised the whole manuscript and figures and offered constructive comments.

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Identifying a Novel Role for Fractalkine (CX3CL1) in Memory CD8+ T Cell Accumulation in the Omentum of Obesity-Associated Cancer Patients

*Melissa J. Conroy[1], Stephen G. Maher[1], Ashanty M. Melo[1], Suzanne L. Doyle[2], Emma Foley[1], John V. Reynolds[1,3], Aideen Long[4] and Joanne Lysaght[1]**

[1] Department of Surgery, St. James's Hospital, Trinity College Dublin, Trinity Translational Medicine Institute, Dublin, Ireland, [2] School of Biological Sciences, Dublin Institute of Technology, Dublin, Ireland, [3] Gastro-Intestinal Medicine and Surgery, St. James's Hospital, Dublin, Ireland, [4] Department of Clinical Medicine, St. James's Hospital, Trinity College Dublin, Trinity Translational Medicine Institute, Dublin, Ireland

The omentum is enriched with pro-inflammatory effector memory CD8+ T cells in patients with the obesity-associated malignancy, esophagogastric adenocarcinoma (EAC) and we have identified the chemokine macrophage inflammatory protein-1alpha as a key player in their active migration to this inflamed tissue. More recently, others have established that subsets of memory CD8+ T cells can be classified based on their surface expression of CX3CR1; the specific receptor for the inflammatory chemokine fractalkine. CD8+ T cells expressing intermediate levels (CX3CR1^{INT}) are defined as peripheral memory, those expressing the highest levels (CX3CR1^{HI}) are effector memory/terminally differentiated and those lacking CX3CR1 (CX3CR1^{NEG}) are classified as central memory. To date, the fractalkine:CX3CR1 axis has not been examined in the context of CD8+ T cell enrichment in the omentum and here we examine this chemokines involvement in the accumulation of memory CD8+ T cells in the omentum of EAC patients. Our data show that fractalkine is significantly enriched in the omentum of EAC patients and drives migration of T cells derived from EAC patient blood. Furthermore, CX3CR1 is endocytosed specifically by CD8+ T cells upon encountering fractalkine, which is consistent with the significantly diminished frequencies of CX3CR1^{INT} and CX3CR1^{HI} CD8+ T cells in the fractalkine-rich environment of omentum in EAC, relative to matched blood. Fractalkine-mediated endocytosis of CX3CR1 by CD8+ T cells is sustained and is followed by enhanced surface expression of L-selectin (CD62L). These novel data align with our findings that circulating CX3CR1^{NEG} CD8+ T cells express higher levels of L-selectin than CX3CR1^{INT} CD8+ T cells. This is consistent with previous reports and implicates fractalkine in the conversion of CX3CR1^{INT} CD8+ T cells to a CX3CR1^{NEG} phenotype characterized by alterations in the migratory capacity of these T cells. For the first time, these findings identify fractalkine as a driver of T cell migration to the omentum in EAC and indicate that CD8+ T cells undergo sequenced fractalkine-mediated alterations in CX3CR1 and L-selectin expression. These data implicate fractalkine as more than a chemotactic cytokine in obesity-associated meta-inflammation and reveal a role for this chemokine in the maintenance of the CX3CR1^{NEG} CD8+ T cell populations.

Keywords: fractalkine, CX3CR1, T cells, obesity, upper gastrointestinal cancer, inflammation, adhesion, omentum

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****Correspondence:***
Joanne Lysaght
jlysaght@tcd.ie

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INTRODUCTION

The flexibility and multifaceted functionality of chemokines together with their involvement in multiple inflammatory diseases has ignited an interest in their potential as targets for immunotherapy (1, 2). Indeed, there has been a paradigm shift in the classification of chemokines as solely chemotactic cytokines and emerging evidence has uncovered their additional functions in the generation of effector and memory T cells and the co-stimulation of cytokine secretion by T cells (3–5). Most recently, the specific receptor for the inflammatory chemokine fractalkine, CX3CR1 has been used to classify memory $CD8^+$ T cell subsets (5).

Fractalkine (CX3CL1) exists in both a soluble and transmembrane form, and plays a role in both immune cell migration and adhesion and has been implicated in multiple inflammatory diseases such as asthma, dermatitis, diabetes, and neuropathic pain (6–12). The involvement of this chemokine in several inflammatory disorders is facilitated through its induction by inflammatory cytokines such as TNF-α, IFN-γ, and IL-1 (13). In the context of obesity, fractalkine has already been implicated in obesity-associated diseases such as diabetes and cardiovascular disease and in the chronic inflammation that precedes metabolic dysfunction; meta-inflammation (7, 8, 10, 14). Fractalkine is known to be expressed by endothelial cells and facilitates vascular recruitment and adhesion of macrophages, NK cells, and T cells, however, its expression by both the stromal vascular fraction (SVF) and the adipocyte fraction has been demonstrated in the adipose tissue (8). Most studies have focused on the role of fractalkine in macrophage-mediated adipose tissue inflammation (8, 15, 16), with previous work demonstrating a role for fractalkine in macrophage recruitment and macrophage-adipocyte adhesion in adipose tissue but, to our knowledge there are no reports of fractalkine-driven migration of T cells in obesity (8).

The omentum forms the largest component of the visceral adipose tissue (VAT) compartment and is enriched with leukocyte aggregates which liken it to the follicles of secondary lymphoid tissues and promote immune responses and inflammation (17). We have previously shown that the omentum is a hot bed of T cell-mediated inflammation in patients with esophagogastric adenocarcinoma (EAC), a malignancy that arises in a background of inflammation and importantly has one of the strongest associations with obesity of all malignancies (18). Furthermore, we have identified the macrophage inflammatory protein-1alpha (MIP-1α)/CCR1 axis as a key pathway governing T cell migration to the omentum of EAC patients and reported its therapeutic potential in the space of obesity-associated inflammation (19). However, the fractalkine pathway may present a more attractive and more specific therapeutic target in this setting as there is only one known receptor for this chemokine (CX3CR1), in contrast to the redundancy observed in other chemokine pathways (1, 20, 21). We have already demonstrated that the omentum of EAC patients is primed for fractalkine-mediated inflammation through an abundance of TNF-α-producing $CD8^+$ and $CD4^+$ T cells and secreted IL-1β and here, we address the role of fractalkine in inflammatory and cytotoxic T cell trafficking and retention in this inflamed tissue (22, 23). These questions are extremely relevant at a time when the global obesity problem has reached epidemic proportions and shows no signs of abating (www.who.int).

For the first time, this study identifies a role for fractalkine in the recruitment of T cells to the inflamed omentum of EAC patients implicating this chemokine as a player and potential therapeutic target in pathological T cell-mediated inflammation in obesity and obesity-associated malignancies. Importantly, our data also reveal fractalkine-mediated regulation of CX3CR1 and L-selectin expression by $CD8^+$ T cells, placing this inflammatory chemokine as an orchestrator of cytotoxic T cell trafficking and memory in EAC. The data generated here has ramifications for fractalkine as a therapeutic target for inflammation particularly in the context of cancer indicating that antagonism of this pathway might have consequences for L-selectin-dependent cytotoxic T cell homing to lymph nodes and anti-tumor immunity.

MATERIALS AND METHODS

Subjects

Forty-seven consecutive consenting patients with EAC, attending the National Esophageal and Gastric Center at St. James's Hospital, Dublin from 2011 to 2018 were enrolled in this study. The patient cohort was similar in age and ethnicity, and 81% had received neoadjuvant chemo-radiotherapy. The patient group included 37 males and 10 females, representative of the male predominance in EAC, with an average age of 65.9 years. The mean BMI at time of surgery was 26.4 and CT-defined visceral fat area (VFA) was 126.54 cm^2 (**Table 1**). Control blood and omentum were taken from a group of non-cancer control patients attending St. James's Hospital, Dublin for laparoscopic cholecystectomy. All

TABLE 1 | Demographic data.

Age (range years)	65.9 (35–93)
Sex ratio (M:F)	37:10
Esophageal adenocarcinoma	38
Gastric adenocarcinoma	9
Tumor stage[a]	
T0	4
T1	11
T2	8
T3	20
T4	1
Nodal status[a]	
Positive	21
Negative	23
Mean BMI (kg/m^2) (range)[b]	26.4 (17.8–36.4)
Underweight (BMI < 19.9)	4
Normal weight (BMI 20–24.9)	12
Overweight (BMI 25–29.9)	13
Obese (BMI > 30)	14
Mean waist circumference (cm) (range)	93.8 (68–115)
Centrally obese by waist circumference[c]	57.75%
Mean visceral fat area (VFA) (cm^2) (range)	126.54 (4.65–353.05)
Viscerally obese by VFA[d]	30%
Received Neoadjuvant CRT	81%

[a]*Tumor stage and nodal status was not available for three patients.*
[b]*BMI was not available for four patients.*
[c]*Obese waist circumference ≥94 cm for men and ≥80 cm for women (25).*
[d]*Obese VFA >160 cm^2 for men and >80 cm^2 for women (24).*

cancer patients were evaluated by a dietician. Body mass index, waist circumference, and anthropometric variables were measured as described previously (23). VFA was assessed by computer tomography as previously described, with more than 160 and 80 cm^2 defining visceral obesity in males and females, respectively (24). Serum C-reactive protein (CRP) levels were determined as part of routine testing at time of surgery. Metabolic syndrome was measured as per the IDF definition (25).

Ethics Approval Statement

The work was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Patients provided informed consent for sample and data acquisition and the study received full ethical approval from the St. James's Hospital Ethics Review Board. Patient samples were pseudonymized to protect the privacy rights of the patients.

Sample Preparation

Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation using Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden). Omental samples (10 g from each patient) were digested enzymatically to obtain SVF as previously described (22, 23). Adipose tissue conditioned media (ACM) was prepared as previously described (22).

Quantification of Soluble Fractalkine and CX3CR1 Levels in Serum and Omentum

The V-PLEX™ Fractalkine plate (Meso Scale Discovery) was used to detect the levels of fractalkine in the serum and ACM of 19 EAC patients according to the manufacturer's instructions and read using an MSD Sector S600. The Human CX3CR1 ELISA Kit (ELISA Genie) was used to measure secreted CX3CR1 in the serum and ACM of 10 EAC patients and read using a VersaMax™ ELISA microplate reader.

Examining Secreted Adhesion Molecule Levels in the Omentum of EAC Patients

The Evidence Investigator Adhesion Molecules Array (RANDOX) was used to detect the concentrations of VCAM-1, ICAM-1, L-selectin, P-selectin, and E-selectin in the ACM of five EAC patients according to the manufacturer's instructions and read using a RANDOX Evidence Investigator.

T Cell Chemotaxis Assays

To activate the T cells for chemotaxis, PBMC were prepared from the blood of five EAC patients and three non-cancer controls, and incubated with CD3/CD28 T-cell expander Dynabeads (Invitrogen) at a bead:cell ratio of 1:1. Following activation, the beads were removed and the T cells were counted and resuspended at densities of 2×10^6 cells/ml serum-free RPMI media. Chemotaxis was assessed using a 5-μm pore transwell filter system (Corning Inc., USA). Cells were added to the top chamber in a volume of 100 μl RPMI, while the bottom chamber contained 600 μl of the following; M199 media alone (negative control) or M199 media supplemented with fractalkine at a concentration of 30 ng/ml (based on the mean concentration in EAC ACM) or ACM derived from four obese and three non-obese EAC patients or ACM derived from two obese and three non-obese non-cancer controls. The assay was incubated for 2 h at 37°C before removal of the insert. The media in the bottom chambers were taken and centrifuged at 1,300 RPM for 3 min and then stained for CD3 expression. CountBright Absolute Counting Beads (Invitrogen) were used to enumerate the absolute number of $CD3^+$ T cells that had migrated.

Quantification of CX3CR1 Expressing T Cells in Blood and Omentum and Phenotyping of $CX3CR1^{NEG}$ and $CX3CR1^{INT}$ $CD8^+$ T Cell Populations

Freshly isolated PBMC and SVF were stained with monoclonal antibodies specific for human surface markers; PE-efluor-labeled CD3 and APC-eFluor780-labeled CD27 (eBioscience, Hatfield, UK), PE-Cy5.5-labeled CD4, APC-labeled ICAM-1 (CD54), V500-labeled CD45RA, and BV421-labeled CD8 (BD Biosciences, Oxford, UK), PE-Cy5-labeled L-selectin (Abcam, Cambridge, UK), and PE-labeled CX3CR1 (Miltenyi Biotec, Germany). Cells were acquired using CyAn ADP flow cytometer (Beckman Coulter) and analyzed with FlowJo software (TreeStar Inc.).

CX3CR1 Surface Expression by T Cells Following Recombinant Fractalkine Treatment

To examine the effects of fractalkine on CX3CR1 expression by $CD8^+$ T cells, PBMC from 17 EAC patients were seeded in RPMI media at 1×10^6 cells/ml and treated with M199 media or 30 ng/ml of recombinant fractalkine for 15 min, 30 min, 1 h, 2 h, and 24 h and subsequently analyzed for CX3CR1 surface expression using flow cytometry, as described above. To test whether CX3CR1 was endocytosed, PBMC from 10 EAC patients were treated with M199 media (untreated) or 30 ng/ml of recombinant fractalkine for 2 h at 4°C (cold treatment) or in the presence of 80 μM of the GTPase inhibitor dynasore (Sigma Aldrich). To examine whether CX3CR1 is recycled to the surface of $CD8^+$ T cells following fractalkine treatment, PBMC from three donors were seeded in RPMI media at 1×10^6 cells/ml and treated with M199 media alone or 30 ng/ml of recombinant fractalkine for 2 h. Following 2 h, cells were washed and placed in fractalkine-free media for 24 and 48 h or placed in fractalkine-free media supplemented with 50 ng/ml of TNF-α or 100 ng/ml of IFN-γ, IL-6, IL-7, IL-17, IL-18, IL-15, or IL-36 or 50 U/ml of IL-2 for 24 h and subsequently analyzed for CX3CR1 surface expression using flow cytometry.

Examining CX3CR1 Protein Expression Following Treatment With Recombinant Fractalkine

$CD8^+$ T cells from three healthy control subjects were isolated from PBMC using the EasySep™ Human $CD8^+$ T Cell Isolation Kit (Stemcell Technologies) and subsequently seeded at a density of 1×10^6 cells/ml RPMI media and treated with 30 ng/ml of fractalkine for 24 and 48 h. Flow cytometry was used to confirm reduction of CX3CR1 surface expression on a subset of treated

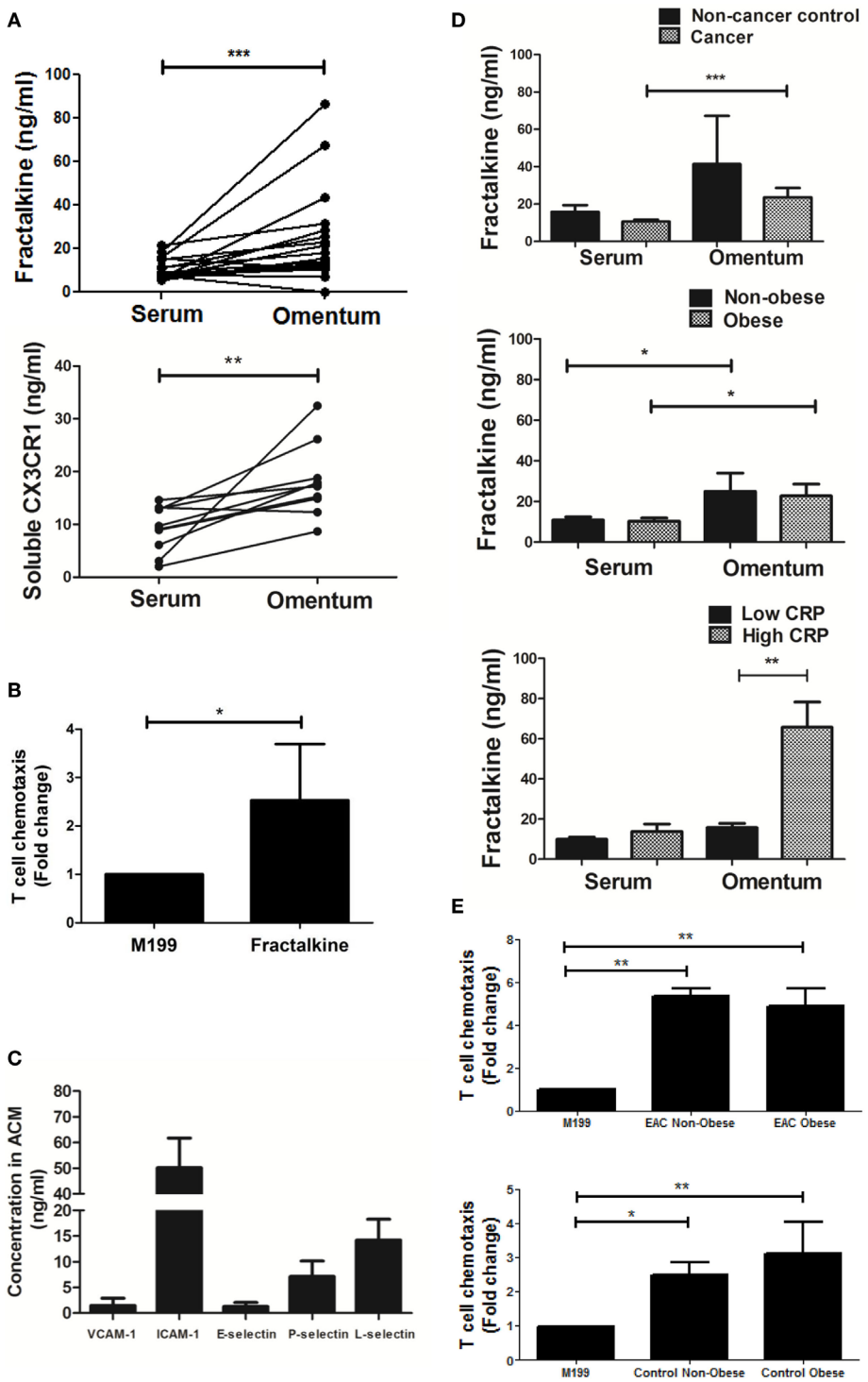

FIGURE 1 | Both soluble fractalkine and CX3CR1 are secreted in abundance in the omentum of esophagogastric adenocarcinoma (EAC) patients and fractalkine can drive migration of EAC patient-derived T cells. **(A)** Scatter plots showing the fractalkine (top) and soluble CX3CR1 levels (bottom) in paired samples of serum and adipose tissue conditioned media (ACM) from 10 to 19 EAC patients. **(B)** Bar charts showing significant fold change migration of peripheral blood-derived T cells from five EAC patients to M199 media supplemented with 30 ng/ml of recombinant fractalkine, relative to M199 alone negative control. **(C)** Bar charts showing the levels of soluble VCAM-1, ICAM-1, E-selectin, P-selectin, and L-selectin in omental adipose tissue conditioned media (ACM) from five EAC patients. **(D)** Bar charts showing the fractalkine levels in serum and ACM from 3 non-cancer control subjects and 20 EAC patients, from 9 non-obese and 11 obese EAC patients, and 17 EAC patients with serum C-reactive protein (CRP) <5 mg/l (low CRP) and 3 EAC patients with serum CRP >5 mg/l (high CRP). **(E)** Bar charts showing significant fold change migration of peripheral blood-derived T cells to ACM from three non-obese and four obese EAC patients and three non-obese and two obese non-cancer controls. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by paired, unpaired *t* tests and one-way ANOVA with Tukey *post hoc* analysis.

and untreated cells after 24 h and the remainder of the cells were lysed. Protein was extracted using a 6 M urea lysis buffer (15 min on ice). Protein was quantified against a standard curve using the Pierce BCA Protein Assay Kit (Thermofisher). Protein (60 μg per sample) was loaded onto a 10% SDS page gel and electrophoresed for 80 min. Separated protein was subsequently transferred to a PVDF membrane. Membrane was blocked in 0.5% Bovine Serum Albumin (in TBST) for 1.5 h at room temperature. The membrane was incubated for 1.5 h at room temperature with mouse anti-human CX3CR1 antibody (H-70) (Santa Cruz) at 1:1,000 dilution in 0.5% BSA TBST. The membrane was incubated for 1 h at room temperature with secondary antibody (goat anti-mouse IgG-HRP at 1:5,000 dilution in 0.5% BSA TBST). Bands were visualized using the West Pico chemiluminescent substrate on a gel doc. Beta-actin primary mouse anti-human at 1:10,000 dilution (Santa Cruz) was used as a loading control. Protein extract quality was confirmed visually *via* coomassie blue staining (10% gel, 20 μg protein per sample). $CD8^+$ T cells from three control subjects were isolated from PBMC using the EasySep™ Human $CD8^+$ T Cell Isolation Kit (Stemcell Technologies) and subsequently seeded in RPMI media at 1×10^6 cells/ml and treated with 30 ng/ml of fractalkine for 24 and 48 h. Cell supernatant was collected after 24 and 48 h and the Human CX3CR1 ELISA Kit (ELISA Genie) was used to compare secreted CX3CR1 in the untreated and fractalkine-treated cells.

Assessing Integrin and Adhesion Molecule Expression Together With Memory Phenotype of $CD8^+$ T Cells Following Fractalkine Treatment

To examine the effects of fractalkine on CX3CR1 expression by $CD8^+$ T cells, PBMC from six EAC patients were treated with M199 media alone or M199 media supplemented with 30 ng/ml of recombinant fractalkine for 24 h and subsequently analyzed for VLA-4, LFA-1, alpha4 integrin, beta7 integrin, ICAM-1, L-selectin, CD45RA, and CD27 surface expression using flow cytometry, as described above.

Statistical Analyses

Statistical analysis was carried out using Prism GraphPad Version 5.0. Differences between groups were assessed using two-tailed paired, Wilcoxon sign-rank test, unpaired non-parametric Mann–Whitney *U* tests, and one-way ANOVA with Tukey *post hoc* analysis where appropriate. Significant associations between fractalkine, CX3CR1, and clinical parameters were investigated using Spearman's rank-order correlation test. p Values of <0.05 were considered to be significant.

RESULTS

Significantly High Levels of Soluble Fractalkine in the Omentum of EAC Patients Can Drive Migration of EAC Patient-Derived T Cells

Secreted fractalkine was quantified by MSD V-Plex ELISA in the matched serum and omental adipose tissue conditioned media (ACM) of 19 EAC patients revealing that levels of this chemokine were significantly higher in ACM (mean: 23.66 ng/ml) compared to serum (mean: 10.56 ng/ml) ($p < 0.001$, **Figure 1A**). Soluble CX3CR1 ELISA revealed that the soluble form of the fractalkine receptor was also significantly abundant in the ACM of 10 EAC patients, compared to matched serum suggesting that the fractalkine receptor can be shed in the omental microenvironment (mean: 18.1 versus 9.3 ng/ml, $p < 0.01$, **Figure 1A**). To confirm whether T cells respond and migrate toward fractalkine-elicited chemotactic signals in EAC, patient-derived T cell chemotaxis to 30 ng/ml of fractalkine was measured using a transwell system. Significantly higher fold-levels of T cell migration toward recombinant fractalkine, compared to serum-free M199 media were observed supporting our hypothesis of fractalkine-driven T cell migration to omentum in EAC (M199 versus fractalkine fold change: 1 versus 2.456, $p < 0.05$, **Figure 1B**). An adhesion molecule array was used to screen omentum-derived ACM from five EAC patients for soluble adhesion molecules and ICAM-1 and L-selectin were shown to be most abundant, indicative of the adhesive properties of the omental microenvironment (**Figure 1C**). Secreted fractalkine levels were significantly higher in omentum compared to matched serum regardless of patient obesity status and interestingly, there were significantly higher levels of soluble fractalkine in the omentum of EAC patients with elevated serum CRP, i.e., >5 mg/l compared to those with lower serum CRP, i.e., <5 mg/l (mean: 65.8 versus 13.87 ng/ml, $p < 0.05$) (**Figure 1D**; **Table 2**). Furthermore, our data show significant levels of T cell migration to ACM in both non-cancer and EAC patients, irrespective of obesity status (**Figure 1E**).

Low Frequencies of $CX3CR1^+$ $CD8^+$ T Cells in the Omentum of EAC Patients Are due to Significantly Diminished Frequencies of Both the $CX3CR1^{INT}$ and $CX3CR1^{HI}$ Subsets of $CD8^+$ T Cells

The frequencies of $CD4^+$ and $CD8^+$ T cells expressing the chemokine receptor CX3CR1 were examined in the blood and omentum of a total of 24 EAC patients by flow cytometry. Our results identified significantly higher frequencies of $CX3CR1^+$ $CD8^+$ T cells in the circulation compared to their $CD8^+$ counterparts in omentum (blood versus omentum mean: 48.87 versus 23.64%, $p = 0.0002$, **Figure 2A**) and compared to proportions of

TABLE 2 | Correlations of CX3CL1 levels and frequencies of $CX3CR1^{NEG}$ expressing T cells with waist circumference, visceral fat area (VFA), and body mass index.

R^2 values for correlations of CX3CR1 and the following measurements	Waist circumference R^2 value	VFA R^2 value	Body mass index R^2 value
Serum CX3CL1	0.028	0.058	0.119
Omental CX3CL1	0.003	0.126	0.134
Peripheral $CX3CR1^+$ $CD4^+$ T cells	−0.15	0.008	−0.054
Omental $CX3CR1^+$ $CD4^+$ T cells	0.193	0.159	0.036074
Peripheral $CX3CR1^+$ $CD8^+$ T cells	−0.298	−0.216	−0.164
Omental $CX3CR1^+$ $CD8^+$ T cells	0.464	0.044	0.073

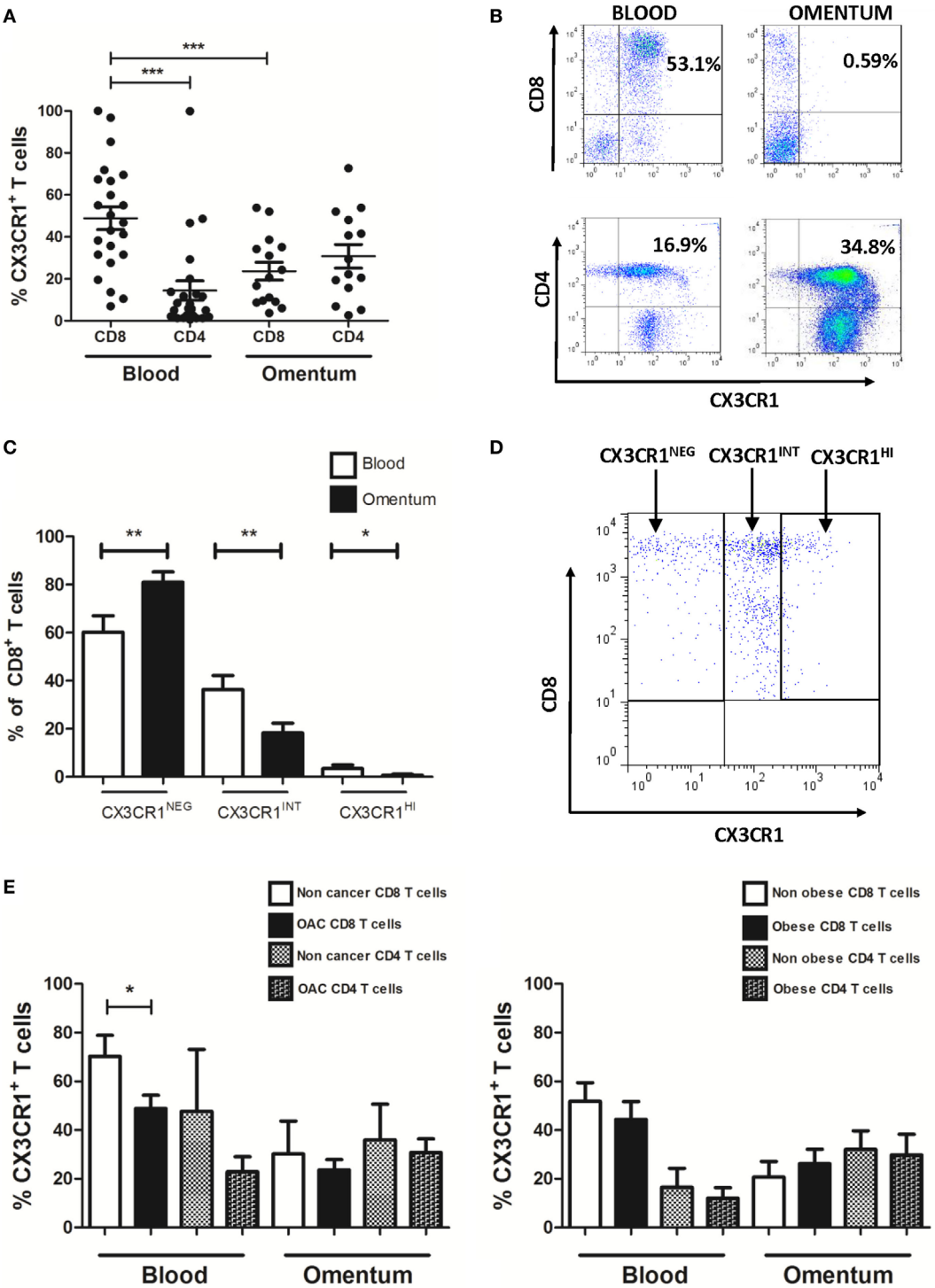

FIGURE 2 | Frequencies of CX3CR1$^+$ CD8$^+$ T cells are significantly lower in omentum of esophagogastric adenocarcinoma (EAC) patients compared to frequencies of both peripheral blood CX3CR1$^+$ CD8$^+$ T cells and CX3CR1$^+$ CD4$^+$ T cells. Peripheral blood mononuclear cells and stromal vascular fraction of omentum were isolated from EAC patients. **(A)** Scatterplots show the frequencies of CX3CR1$^+$ CD8$^+$ and CD4$^+$ T cells in the blood and omentum of a total of 24 EAC patients. **(B)** Representative dot plots of CX3CR1$^+$ CD8$^+$ and CX3CR1$^+$ CD4$^+$ T cells (gated on and shown as a percentage of total CD3$^+$ population) in blood and omentum. **(C)** Bar chart showing the frequencies of CD8$^+$ cells expressing no CX3CR1 (CX3CR1^{NEG}), intermediate (CX3CR1^{INT}), and high levels of CX3CR1 (CX3CR1^{HI}) in the blood (white) and omentum (black) of 24 EAC patients. **(D)** Representative dot plot of circulating CD8$^+$ T cells expressing no CX3CR1 (CX3CR1^{NEG}), intermediate levels of CX3CR1 (CX3CR1^{INT}), and high levels of CX3CR1 (CX3CR1^{HI}). **(E)** Bar charts showing the CX3CR1$^+$ CD8$^+$ and CD4$^+$ T cells in the blood and omentum of 8 non-cancer control subjects and 24 EAC patients (left) and 14 non-obese and 10 obese EAC patients (right). *$p < 0.05$, *$p < 0.01$, ***$p < 0.001$ by paired and unpaired *t* tests.

circulating CX3CR1^{+} CD4^{+} T cells (mean: 14.48%, $p < 0.0001$, **Figure 2A**). These differences were paralleled by substantially but not significantly higher frequencies of CX3CR1^{+} CD4^{+} T cells in EAC omentum (mean: 30.76%, **Figures 2A,B**) compared to blood. Further analysis revealed that the diminished numbers of CX3CR1^{+} CD8^{+} T cells within the omentum were due to significantly lower frequencies of CD8^{+} T cells expressing both intermediate and high levels of CX3CR1 and significantly higher frequencies of CX3CR1^{NEG} populations in omentum, compared to blood (blood versus omentum mean of CX3CR1^{NEG}: 60.24 versus 81.03%, $p < 0.01$, CX3CR1^{INT}: 36.34 versus 18.25%, $p < 0.01$, CX3CR1^{HI}: 3.41 versus 0.72%, $p < 0.05$, **Figure 2C**). The highest proportions of circulating CX3CR1^{+} cells were identified within the CX3CR1^{INT} CD8^{+} T cell population (**Figures 2C,D**). While such observations in CX3CR1 expression by T cells were consistent among obese and non-obese EAC patients, there were significantly higher frequencies of CX3CR1^{+} CD8^{+} T cells in the circulation of non-cancer controls, compared to EAC patients (non-cancer versus EAC: 70.33 versus 48.87%, $p < 0.05$, **Figure 2E**; **Table 2**).

CX3CR1 Expression by Peripheral Blood but Not Omental CD8^{+} T Cells Is Significantly Diminished Following Treatment With Recombinant Fractalkine

To ascertain why enrichments of CX3CR1^{+} CD4^{+} T cells were detected in the omentum, while highest frequencies of CX3CR1^{+} CD8^{+} T cells were detected in the circulation, we assessed whether CX3CR1^{+} CD8^{+} T cells convert to CX3CR1^{NEG} CD8^{+} T cells upon encountering their ligand, which is secreted in abundance in the omental microenvironment. Blood-derived T cells from 17 EAC patients were treated with M199 media or recombinant fractalkine for 2 h to simulate the effects of the high fractalkine levels in the omental microenvironment. Flow cytometric analysis revealed that surface expression of CX3CR1 was significantly decreased on peripheral blood CD8^{+} but not CD4^{+} T cells or omental CD8^{+} T cells following 2 h treatment with recombinant fractalkine (untreated versus treated CD8^{+} T cells: 52.2 versus 4.238, $p = 0.0001$, **Figures 3A,B**). Further analysis revealed that CX3CR1 expression by peripheral blood CD8^{+} T cells is reduced between 15 and 30 min post-treatment with most significant reductions observed at 2 and 24 h post-treatment (**Figure 3C**, $p < 0.001$). Fractalkine treatments performed at a temperature of 4°C or in combination with treatment of 80 μM dynasore, a GTPase inhibitor, demonstrated significantly reduced efficacy to decrease CX3CR1 expression on CD8^{+} T cells thus confirming that the observed reductions were due to endocytosis (**Figure 4A**).

CX3CR1 Internalization by CD8^{+} T Cells Is Sustained Following Treatment With Recombinant Fractalkine and the Receptor Is Not Degraded or Recycled to the Cell Surface

Surface expression of CX3CR1 by CD8^{+} T cells was not restored when 2 h treatment with fractalkine was followed by culture

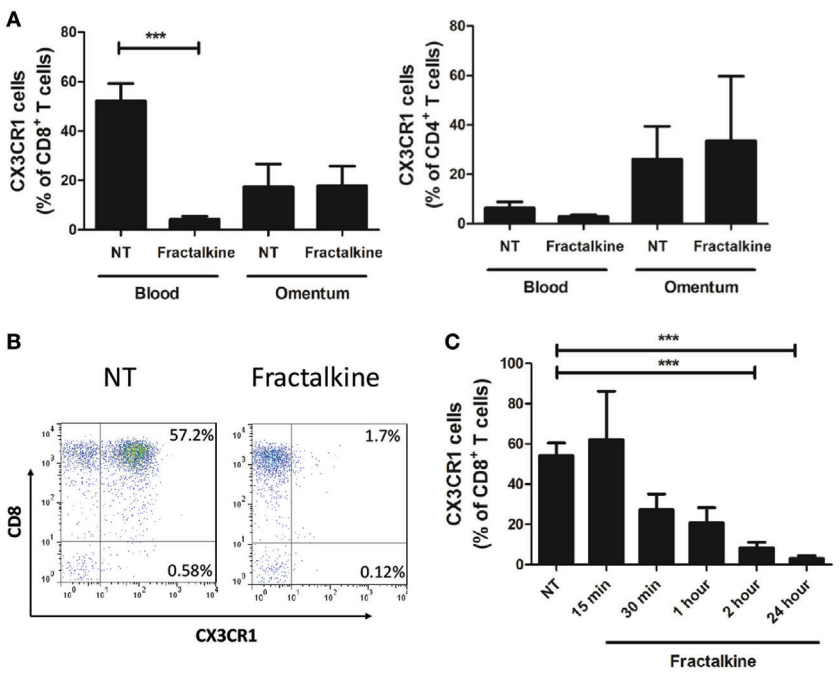

FIGURE 3 | Peripheral blood CD8^{+} T cells exhibit a significant and sustained reduction in CX3CR1 surface expression following treatment with recombinant fractalkine. **(A)** Frequencies of CX3CR1^{+} cells, as a percentage of CD8^{+} T cells (left) and CD4^{+} T cells (right) following; treatment with M199 media alone (NT) or 30 ng/ml of recombinant fractalkine for 2 h ($n = 17$); **(B)** representative dot plots of CX3CR1 expression following treatment with M199 media alone (NT) or 30 ng/ml of recombinant fractalkine for 2 h. **(C)** Frequencies of CX3CR1^{+} cells, as a percentage of CD8^{+} T cells following treatment with M199 media alone (NT) or 30 ng/ml of recombinant fractalkine across a time course of 15 min, 30 min, 1 h, 2 h, or 24 h ($n = 4$). ***$p < 0.001$ by paired t tests.

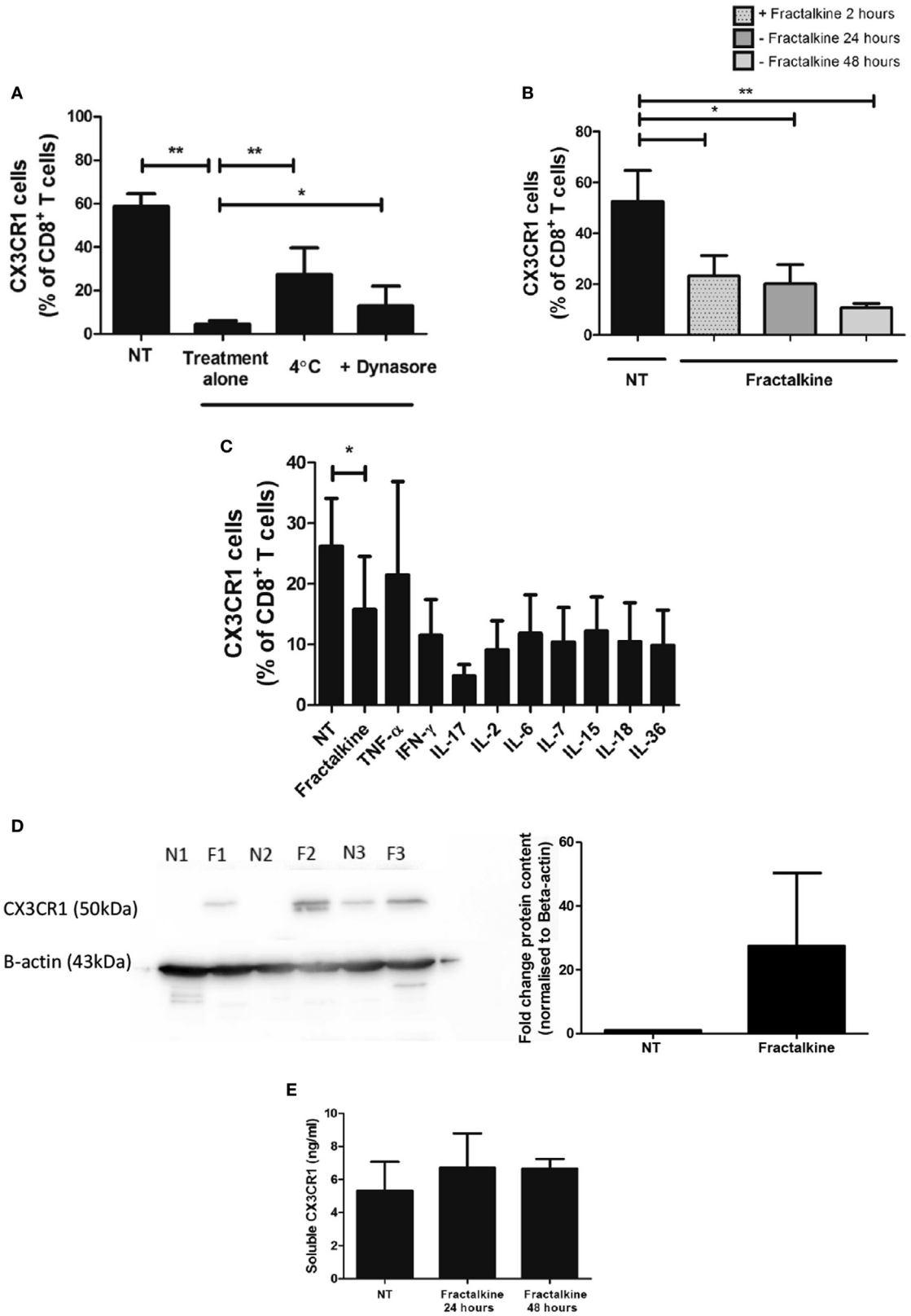

FIGURE 4 | Continued

FIGURE 4 | CX3CR1 is endocytosed following fractalkine treatment and is not subsequently recycled to the surface of CD8+ T cells or secreted but, intracellular accumulations of the protein are detectable. **(A)** Frequencies of CX3CR1+ cells, as a percentage of CD8+ T cells following treatment with M199 media alone (NT), 30 ng/ml recombinant fractalkine alone (treatment alone), 30 ng/ml recombinant fractalkine at 4°C and 30 ng/ml recombinant fractalkine plus 80 μM Dynasore ($n = 10$). **(B)** Bar chart showing frequencies of CX3CR1+ cells, as a percentage of CD8+ T cells following treatment with M199 media alone (NT) or 30 ng/ml of recombinant fractalkine for 24 h (fractalkine, light gray dot pattern) followed by removal from a fractalkine-free environment for 24 h (fractalkine, dark gray no pattern) or 48 h (fractalkine, light gray no pattern) ($n = 6$). **(C)** Bar chart showing frequencies of CX3CR1+ cells, as a percentage of CD8+ T cells following treatment with M199 media alone (NT) or 30 ng/ml of recombinant fractalkine for 24 h (fractalkine, light gray dot pattern) followed by removal from a fractalkine-free environment for 24 h and treatment with 50 ng/ml of TNF-α, 100 ng/ml of IFN-γ, IL-17, IL-6, IL-7, IL-15, IL-18, IL-36, and 50 U/ml of IL-2 ($n = 3$). **(D)** Western Blot (left) showing CX3CR1 and beta-actin protein in peripheral blood-derived CD8+ T cells from three donors following 24 h of no treatment (N1, N2, and N3) or fractalkine treatment (F1, F2, and F3) and densitometry data (right) from this western blot displayed as fold change bar chart. **(E)** Soluble CX3CR1 levels in the supernatant of peripheral blood mononuclear cells treated with M199 alone or fractalkine for 24 or 48 h ($n = 3$). *$p < 0.05$, **$p < 0.01$ by paired t-test.

in fractalkine-free media for 24 and 48 h, suggesting that the receptor is not recycled to the surface following endocytosis (**Figure 4B**). Stimulation with 50 ng/ml of TNF-α or 100 ng/ml of IFN-γ, IL-6, IL-7, IL-17, IL-18, IL-15, or IL-36 or 50 U/ml of IL-2 for 24 h following removal of fractalkine, did not induce recycling of CX3CR1 to the surface of CD8+ T cells (**Figure 4C**). Western blot revealed that there are consistent accumulations of CX3CR1 protein levels in CD8+ T cells following 24 h fractalkine treatment compared to untreated CD8+ T cells demonstrating that the receptor is not degraded following fractalkine-mediated endocytosis (**Figure 4D**). Furthermore, ELISA revealed that the levels of CX3CR1 shed by T cells are not increased following 24 or 48 h of fractalkine treatment (**Figure 4E**).

CX3CR1^{NEG} and CX3CR1^{INT} Populations of CD8+ T Cells in the Peripheral Blood Have Significantly Different Memory Phenotype and Adhesion Molecule Expression

Since the CX3CR1+ compartments of CD8+ T cells were predominantly CX3CR1^{INT} and this subpopulation has been shown to have the potential to convert to CX3CR1^{NEG} CD8+ T cells, we focused our phenotypical comparisons between this subset and the CX3CR1^{NEG} CD8+ T cell subset [**Figure 2C**; (5)]. Significant differences in memory phenotype were identified between the CX3CR1^{NEG} and CX3CR1^{INT} CD8+ T cells based on their CD45RA and CD27 surface expression using the Dieli scheme; terminally differentiated (CD45RA+CD27−), naive (CD45RA+CD27+), effector memory (CD45RA−CD27−), and central memory (CD45RA−CD27+) (26). Our data revealed that circulating CX3CR1^{INT} CD8+ T cells were significantly different to CX3CR1^{NEG} CD8+ T cells in memory phenotype; CX3CR1^{INT} CD8+ T cells were predominantly central memory and effector memory phenotype while CX3CR1^{NEG} CD8+ T cells contained more naive and terminally differentiated cells (**Figure 5A**, central memory: $p = 0.0001$, effector memory: $p < 0.05$, naive: $p = 0.001$, terminally differentiated: $p = 0.002$). These phenotypical differences were attributable to significantly lower CD45RA surface expression and significantly higher CD27 surface expression within the CX3CR1^{INT} CD8+ T cell population (CX3CR1^{INT} versus CX3CR1^{NEG} CD45RA: 27.2 versus 96.4%, $p < 0.001$, CD27, 94.9 versus 68.8%, $p < 0.05$, **Figure 5B**). To elucidate whether our data compared to previous reports of lower L-selectin surface expression within CX3CR1^{INT} T cell population, we examined the expression of L-selectin and ICAM-1 on circulating CX3CR1^{NEG} and CX3CR1^{INT} CD8+ T cells (5). Our data revealed significantly lower frequencies of ICAM-1+ and L-selectin+ cells within the CX3CR1^{INT} CD8+ T cell population compared to their CX3CR1^{NEG} counterparts, indicating differential migratory and adhesive properties of these subsets (CX3CR1^{NEG} versus CX3CR1^{INT} ICAM-1: 68.4 versus 47.1%, $p = 0.02$, L-selectin: 60.4 versus 35.4%, $p = 0.02$, **Figure 5C**).

Increased L-Selectin Surface Expression Follows Fractalkine-Mediated CX3CR1 Endocytosis on CD8+ T Cells

To simulate the high fractalkine levels in the omental microenvironment and examine whether this affects adhesion molecule expression and memory phenotype; fractalkine treatment of peripheral blood T cells from three donors was performed for 24 h. Fractalkine treatment induced a significant increase in surface expression of L-selectin but not ICAM-1 on CD8+ T cells following 24 h, indicating that it alters their adhesiveness and lymphoid tissue homing capacity (L-selectin: 24.6 versus 35.5%, $p < 0.05$, **Figure 6A**). Fractalkine treatment had no significant effects on ICAM-1, LFA-1, VLA-4, or integrin alpha4 and beta7 surface expression or memory phenotype of CD8+ T cells defined by CD45RA and CD27 expression (**Figures 6B–D**). In data not shown, 24 h fractalkine treatment had no significant effects on pro-inflammatory cytokine production by the CD8+ T cells.

DISCUSSION

High Levels of Soluble Fractalkine in Omentum Can Drive T Cell Migration and May Represent a Hallmark of Meta-Inflammation in Obesity-Associated Cancer Patients

For the first time, this study has revealed significantly high levels of soluble fractalkine in the omental adipose tissue conditioned media (ACM) derived from patients with the obesity-associated cancer EAC. This is in line with previous observations of fractalkine enrichment in VAT (8). Known inducers of fractalkine include the inflammatory cytokines IFN-γ, TNF-α, and IL-1β (13). Such an abundance of fractalkine was unsurprising as we have reported enrichments of IFN-γ and TNF-α producing T cells together with secreted IL-1β in

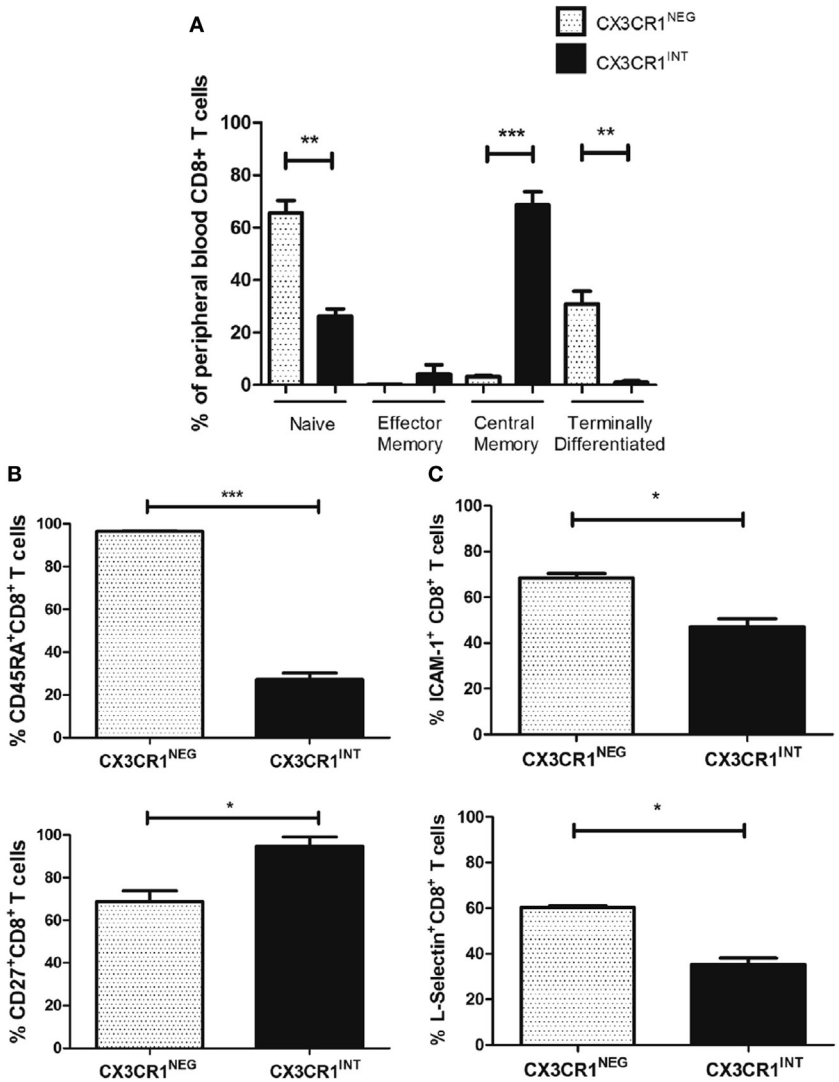

FIGURE 5 | CX3CR1^{NEG} and CX3CR1^{INT} populations represent significantly different CD8$^+$ T cells in the peripheral blood with significantly different memory phenotypes, L-selectin, and ICAM-1 expression. **(A)** Bar charts showing the frequencies of naive, central memory, effector memory, and terminally differentiated CD8$^+$ T cells, characterized by CD45RA and CD27 expression within the CX3CR1^{NEG} and CX3CR1^{INT} populations in peripheral blood of six non-cancer controls. **(B)** Bar chart showing frequencies of peripheral blood-derived CD8$^+$ T cells expressing CD45RA (top) and CD27 (bottom) within CX3CR1^{NEG} and CX3CR1^{INT} populations ($n = 3$). **(C)** Bar chart showing frequencies of peripheral blood-derived CD8$^+$ T cells ICAM-1 and L-selectin within CX3CR1^{NEG} and CX3CR1^{INT} populations ($n = 3$). $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by unpaired *t* tests.

the omentum of EAC patients (13, 22, 23). Interestingly, significantly higher fractalkine levels were observed in the patients with highest serum CRP levels, which is indicative of its role in meta-inflammation and in line with previous reports of the circulating levels of CRP and fractalkine in metabolic dysfunction (7, 27). However, this is the first reported association of high fractalkine in the omentum and high serum CRP in obesity-associated cancer. Soluble fractalkine levels were significantly high in both obese and non-obese omentum in both cancer and non-cancer subjects. Furthermore, soluble cues from both obese and non-obese and both cancer and non-cancer omental microenvironments drove T cell migration suggesting that this tissue is a rich source of this chemokine and recruits significant numbers of T cells, regardless of obesity status. Interestingly, while fractalkine was secreted in abundance in the omentum and could drive *in vitro* EAC-derived T cell migration, this was not paralleled by high frequencies of T cells expressing its specific receptor in this tissue. In fact, the highest proportions of CX3CR1^{INT} cells were identified in circulating CD8$^+$ T cell populations and not the omentum. Such frequencies were still significantly lower than CD8$^+$ T cell frequencies in non-cancer controls suggesting that the fractalkine:CX3CR1 axis might be perturbed by malignancy in this study cohort. Together with our previously reported data, our investigations suggest that fractalkine together with MIP-1α in the omentum recruit inflammatory T cells to this tissue in EAC, most likely

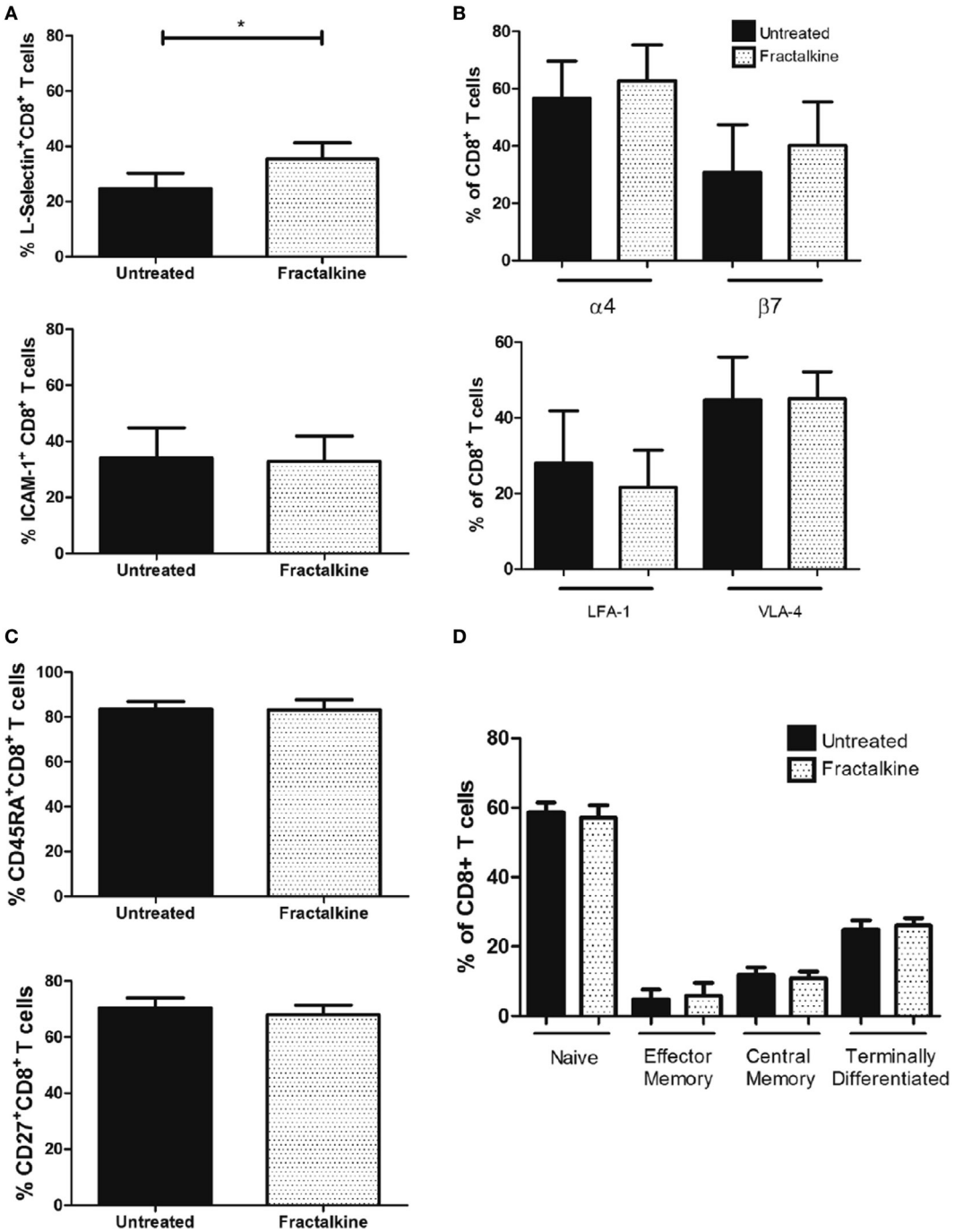

FIGURE 6 | The fractalkine-mediated CX3CR1^{INT} to CX3CR1^{NEG} conversion of CD8$^+$ T cells is followed by enhanced L-selectin expression. **(A)** Bar charts showing frequencies of peripheral blood-derived CD8$^+$ T cells expressing L-selectin (top) and ICAM-1 (bottom) following treatment with M199 media alone (untreated, black) or 30 ng/ml of recombinant fractalkine for 24 h (fractalkine, white pattern) ($n = 6$). **(B)** Bar chart (top) showing frequencies of peripheral blood-derived CD8$^+$ T cells expressing alpha4 and beta7 integrins following treatment with M199 media alone (untreated, black) or 30 ng/ml of recombinant fractalkine for 24 h (fractalkine, white pattern) ($n = 6$). **(B)** Bar chart (bottom) showing frequencies of peripheral blood-derived CD8$^+$ T cells expressing LFA-1 and VLA-4 following treatment with M199 media alone (untreated, black) or 30 ng/ml of recombinant fractalkine for 24 h (fractalkine, white pattern) ($n = 6$). **(C)** Bar chart showing the frequencies of total CD45RA$^+$ and CD27$^+$ populations of CD8$^+$ T cells following treatment with M199 media alone (untreated, black) or 30 ng/ml of recombinant fractalkine for 24 h (fractalkine, white pattern) ($n = 6$). **(D)** Bar chart showing the frequencies of naive, effector memory, central memory, and terminally differentiated CD8$^+$ T cells, characterized by CD45RA and CD27 expression following treatment with M199 media alone (untreated, black) or 30 ng/ml of recombinant fractalkine for 24 h (fractalkine, white pattern) ($n = 6$). *$p < 0.05$ by paired t tests.

contributing to the pathological adipose tissue inflammation at the expense of effective anti-tumor immunity (19). As other studies have shown that fractalkine serves distinctive roles in different inflammatory diseases such as diabetes, dermatitis, asthma, and neuropathic pain, we propose that it may also serve functions additional to T cell chemotaxis in the omentum of EAC patients (8, 11, 12, 28).

Diminished Frequencies of CX3CR1^{+} CD8^{+} T Cells and Increased Frequencies of CX3CR1^{NEG} CD8^{+} T Cells in Omentum Are Indicative of Fractalkine-Mediated CX3CR1^{INT} to CX3CR1^{NEG} Conversion

Our data reveal significantly higher proportions of CX3CR1^{HI} and CX3CR1^{INT} CD8^{+} T cells in the peripheral blood of EAC patients and significantly diminished frequencies in the omentum. CX3CR1^{HI} and CX3CR1^{INT} CD8^{+} T cells represent two of three distinct memory CD8^{+} T cell subsets which have recently been defined by their CX3CR1 expression (5). This classification includes a central memory CX3CR1^{NEG} population that expresses higher levels of L-selectin and is more prevalent in the lymphoid tissue, a CX3CR1^{INT} population that expresses lower levels of L-selectin and circulates between the peripheral tissue and the blood, and a CX3CR1^{HI} population that lacks L-selectin and the migratory capacity of the other populations and is predominantly effector memory/terminally differentiated in phenotype (5). CX3CR1^{INT} CD8^{+} T cells have been described as peripheral memory T cells (5). They undergo more homeostatic divisions than any other memory subset to maintain self-renewal and can also convert to CX3CR1^{NEG} to feed the central memory pool of CD8^{+} T cells (5). However, the trigger or mediators for such transition have not been previously identified. To date, few studies have looked at the tissue localization of CX3CR1^{+} CD8^{+} T cell populations with recent reports of their migration to lymph nodes, spleen, bone marrow, lung, and liver in murine models of viral infection (5, 29). Here, treatment of peripheral blood CD8^{+} T cells with the CX3CR1 ligand fractalkine resulted in significant reduction in their CX3CR1 surface expression and thus expanded the CX3CR1^{NEG} pool of CD8^{+} T cells. Due to the abundance of fractalkine in the omentum and due to previous reports that CX3CR1^{INT} CD8^{+} T cells can replenish the CX3CR1^{NEG} CD8^{+} T cell pool, we propose that fractalkine recruits circulating T cells to omentum and subsequently mediates the conversion from CX3CR1^{INT} CD8^{+} T cells to CX3CR1^{NEG} CD8^{+} T cells, thus implicating the omentum as a key site in the accumulation of the memory CD8^{+} T cell pool in EAC and potentially other conditions. While CX3CR1 is the specific receptor for fractalkine, it must be noted that Eotaxin-3 (CCL26) has been identified as a functional chemoattractant for CX3CR1^{+} CD8^{+} T cells (30). However, our data have revealed that EAC omentum is not an abundant source of Eotaxin-3 (data not shown), compared to chemokines such as MIP-1α and fractalkine thus reducing the likelihood of this chemokine governing CX3CR1^{+} CD8^{+} T cell trafficking to omentum in EAC.

Fractalkine Mediates CX3CR1^{INT} to CX3CR1^{NEG} Conversion in Peripheral Blood-Derived CD8^{+} T Cells *via* Receptor Endocytosis

CX3CR1 has recently been identified as a marker of CD8^{+} T cell memory during viral infection in murine models but the role if any of fractalkine in the differentiation of such T cells has not been described (5). Here, a significant decrease in CX3CR1 surface expression following fractalkine treatment was observed in the CD8^{+} T cell compartment in the peripheral blood but not in the T cell populations containing lower levels of CX3CR1 expression; circulating CD4^{+} T cells or omental CD4^{+} or CD8^{+} T cells. Such cell type- and compartment-specific cell surface regulation of a chemokine receptor is not unique to CX3CR1 and similar findings have been reported with CXCR4 (31). Indeed, the internalization of CXCR4 facilitated its association with the T cell receptor and enhanced co-stimulation of cytokine production by T cells thus emphasizing the multifaceted functions of chemokines in immunity (3). Further work performed in this study has revealed that the reduction in CX3CR1 expression is due to endocytosis in line with previous reports on macrophages in sepsis patients (32). To our knowledge, this is the first report of CX3CR1 endocytosis on CD8^{+} T cells following ligand binding and implicates fractalkine as master regulator of CX3CR1^{INT} to CX3CR1^{NEG} conversion. Such endocytosis is not followed by CX3CR1 recycling to surface within 48 h of ligand binding even when fractalkine is removed. Furthermore, fractalkine-mediated endocytosis of its receptor does not lead to increased levels of CX3CR1 shedding. In fact, our western blot data show that the protein accumulates within the cell and stimulation with an array of inflammatory cytokines simulating the soluble cues of an inflammatory microenvironment could not trigger its recycling to the cell surface. These data suggest that fractalkine elicits significant and long-lasting changes in CD8^{+} T cells, which may culminate in their retention in the omentum.

Enhanced L-Selectin Expression by Peripheral Blood-Derived CD8^{+} T Cells Following Fractalkine-Mediated Conversion From a CX3CR1^{INT} to a CX3CR1^{NEG} Phenotype

An abundance of soluble ICAM-1 and L-selectin levels were detected in the omentum-derived ACM of EAC patients, suggesting their shedding by cells in this inflamed tissue and may be indicative of their role in immune cell arrest and retention in the VAT. Significant higher frequencies of ICAM-1^{+} and L-selectin^{+} cells within the CX3CR1^{NEG} CD8^{+} T cell population are also reported here. Since CX3CR1^{NEG} CD8^{+} T cells are the predominant subset within the omentum, our data suggest that such cells have altered adhesive and migratory properties and this may serve a function in retention within the omentum. Interestingly, our data also revealed significantly different memory phenotypes between the peripheral blood CX3CR1^{NEG} and CX3CR1^{INT} CD8^{+} T cell subsets and such characteristics

have been previously described together with differences in their migratory properties and those of $CX3CR1^{HI}$ $CD8^+$ T cells (5). Others have shown that $CX3CR1^{INT}$ $CD8^+$ T cells in mice can increase their L-selectin expression and convert to $CX3CR1^{NEG}$ $CD8^+$ T cells, unlike $CX3CR1^{HI}$ $CD8^+$ T cells (5). For the first time, we identify fractalkine as a mediator of increased L-selectin expression by peripheral blood $CD8^+$ T cells following their fractalkine-mediated conversion from a $CX3CR1^{INT}$ to $CX3CR1^{NEG}$ phenotype and we propose that these sequenced alterations occur in the fractalkine-rich environment of the omentum changing the migratory properties of $CD8^+$ T cells in EAC. While soluble fractalkine induces changes in L-selectin surface expression, that of ICAM-1, VLA-4, LFA-1, alpha-4, and beta-integrin together with memory phenotype are unchanged. Contrary to ICAM-1 expression being highest within the $CX3CR1^{NEG}$ $CD8^+$ T cell population in blood, these data reveal that fractalkine-mediated conversion of $CX3CR1^{INT}$ to $CX3CR1^{NEG}$ $CD8^+$ T cells is not accompanied by an increase in ICAM-1 expression and this event may be mediated by another soluble factor. These data identify a novel role for fractalkine in the alteration of migratory and lymphoid tissue homing capacity of $CD8^+$ T cells and align with previous reports of fractalkine and CX3CR1 regulating immune cell responses *via* distinct disease-dependent mechanisms (28, 32). For instance, this chemokine axis promotes T cell survival in asthma, while it promotes T cell retention in inflamed skin in dermatitis and plays a role in immune paralysis in sepsis (11, 28, 32). However, for the first time, this study identifies fractalkine as a player in T cell recruitment to omentum and a master regulator of CX3CR1 expression, $CX3CR1^{INT}$ to $CX3CR1^{NEG}$ conversion and L-selectin expression. Together with our previously published data, we propose that such alterations change the fate of $CD8^+$ T cell trafficking and $CD8^+$ T cell-mediated immunity in EAC and may lead to their retention in the fractalkine-rich omentum where they contribute to pathological inflammation to the detriment of effective anti-tumor immunity.

CONCLUSION

Fractalkine-driven migration of T cells to omentum in EAC is likely to contribute to the $CD8^+$ T cell-mediated adipose tissue inflammation previously demonstrated in these patients (22, 23). In pilot data not shown, we have also found lower levels of soluble fractalkine in tumor compared to omentum in our patient cohort. Since the fractalkine:CX3CR1 pathway has been shown to have a selective bias for cytotoxic lymphocytes such as $CD8^+$ T cells and NK cells, the preferential migration of $CD8^+$ T cells to omentum over tumor might be detrimental for anti-tumor immunity in EAC and other obesity-associated cancers (33). Antagonizing the CX3CR1:fractalkine pathway might attenuate $CD8^+$ T cell-mediated inflammation in the omentum but secondary to this it might prevent the $CX3CR1^{INT}$ to $CX3CR1^{NEG}$ conversion and disrupt frequencies and migration of peripheral and central memory $CD8^+$ T cells, which would also be detrimental to anti-tumor immunity. Therefore, our findings place fractalkine as more than a chemotactic cytokine driving T cell-mediated inflammation in EAC. Further *in vivo* work will be necessary to elucidate the consequences of blocking this pathway in obesity-associated cancer. These novel data advance our knowledge on the multifaceted functionality of fractalkine and may inform T cell therapies and chemokine-targeted therapies in the future.

AUTHOR CONTRIBUTIONS

MC obtained Irish Research Council funding; performed the acquisition, analysis, and interpretation of data; drafted and finalized the manuscript; completed the statistical analysis; and drove study concept and design. SM performed the acquisition, analysis, and interpretation of data. SD acquired data. AM, EF, and JR provided material support. AL provided technical support and performed a critical revision of the manuscript for intellectual content. JL obtained Health Research Board funding and provided technical and material support, drove study concept and design, and performed a critical revision of the manuscript for intellectual content.

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