Inflammatory and anti-inflammatory states of adipose tissue in transgenic mice bearing a single TCR

Ayaka Matsumoto[1], Kaori Taniguchi[1,5], Naoki Takeda[2], Ken-ichi Yamamura[2], Satoko Arai[1] and **Toru Miyazaki[1,3,4]**

[1]Laboratory of Molecular Biomedicine for Pathogenesis, Center for Disease Biology and Integrative Medicine, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[2]Center for Animal Resources and Development, Kumamoto University, Kumamoto 860-0811, Japan
[3]AMED-CREST, Japan Agency for Medical Research and Development, Tokyo 113-0033, Japan
[4]Max Planck–The University of Tokyo Center for Integrative Inflammology, Tokyo 113-0033, Japan
[5]Present address: Department of Demyelinating Disease and Aging, National Institute of Neuroscience, Tokyo 187-8502, Japan

Correspondence to: T. Miyazaki; E-mail: tm@m.u-tokyo.ac.jp

Abstract

Obesity is accompanied by chronic, low-grade inflammation in adipose tissue, which is associated with insulin resistance and consequent multiple metabolic diseases. In addition to M1 macrophage infiltration, multiple involvements of adipose tissue T lymphocytes in the progression of inflammation have been highlighted recently. Here, we isolated a specific Vα5/Vβ8.2 TCR-bearing T cell that accumulated in obese adipose tissue of mice, and generated transgenic mice expressing this TCR. Under lean conditions with a normal chow diet, CD4+FoxP3+ T$_{reg}$ cells and M2 macrophages increased in adipose tissue with ageing in wild-type mice, but not in transgenic mice. However, both mice exhibited no obvious adipose tissue inflammation such as the formation of crown-like structures (CLSs) of infiltrating macrophages. When fed a high-fat diet, the proportion of adipose tissue T$_{reg}$ cells was markedly small at a similar level in transgenic and wild-type mice. Both types of mice exhibited comparable inflammatory states in adipose tissue, including vast formation of macrophage CLSs, accompanied by insulin resistance. Together, our findings suggest that the absence of an increase in T$_{reg}$ cells and M2 macrophages is not sufficient to initiate inflammatory macrophage infiltration in lean adipose tissue and also provide a new view about the involvement of T cells in promoting obesity-associated inflammation.

Keywords: adipose tissue inflammation, obesity, T cell, TCR transgenic mouse

Introduction

The population suffering from obesity keeps increasing in modern society because of rapid and extreme changes in lifestyle, particularly eating habits. Obesity causes insulin resistance and consequent type 2 diabetes as well as multiple metabolic and cardiovascular diseases. It is well known that obesity-associated insulin resistance is associated with chronic inflammation in visceral adipose tissue (1–5), which is mainly dependent on the innate immune system through overproduction of inflammatory mediators, including TNFα, IL-6, IL-1β and MCP-1, by both infiltrating inflammatory macrophages (classically activated inflammatory macrophages; known as M1 macrophages) and adipocytes themselves (6–8). Conversely, lean adipose tissue contains a resident population of alternative activated macrophages (also known

as M2 macrophages), which can suppress adipose tissue inflammation via the production of inflammatory regulators such as IL-10 (9–12). Thus, at the cellular level, it is believed that macrophages are a key mediator of obesity-associated adipose tissue inflammation and the consequent metabolic disorders.

Other than macrophages, the accumulation of other types of immune cells has been documented in obese adipose tissue. Of these, the important roles of T cells in the regulation of adipose tissue inflammation were highlighted recently. Mathis's group reported that lean visceral fat is enriched with a unique population of Foxp3+CD4+ T$_{reg}$ cells harboring a distinct TCR repertoire and transcriptome, which suppress adipose tissue inflammation and, thus,

insulin resistance ([13]). They also showed that such T_{reg} cells are strikingly and specifically reduced with the progression of obesity, leading to an acceleration of adipose tissue inflammation. More recently, they reported that these adipose tissue T_{reg} cells are derived from the thymus at the neonatal stage and accumulate in response to specific antigen recognition by their TCRs and soluble mediators, notably IL-33 ([14], [15]). Conversely, Winer *et al.* demonstrated that CD4 T cells with specific Vα repertoires accumulate in obese visceral adipose tissue and may expand in response to antigen recognition ([16]). Furthermore, they proposed that obesity-associated chronic inflammation and insulin resistance are under the control of specific T_h1 and T_h2 T cells, by showing that the transfer of CD4 T_h2 cells or depleting predominantly T_h1 cells by anti-CD3 antibody treatment reversed diet-induced insulin resistance in mice. In addition, Nishimura *et al.* found that a preceding infiltration of CD8 T cells and their activation in obese visceral adipose tissue are indispensable for macrophage recruitment and adipose tissue inflammation ([17]). All of these observations implicate essential roles for different types of T cells (i.e. T_h1/T_h2 CD4, CD8 and T_{reg}) in controlling macrophage-dependent pathological inflammation in visceral adipose tissue and consequent local and systemic metabolic disorders.

One worthy strategy to characterize further specific adipose tissue T cells and assess their roles is the identification of the TCRs of T cells that accumulate in obese adipose tissue. In the current study, we isolated a specific TCR that was biased in visceral adipose tissue in wild-type mice fed a high-fat diet (HFD) for 9 weeks. Furthermore, we generated transgenic mice in which all T cells expressed the isolated TCR on a TCRα-null background. In the TCR transgenic mice, we analyzed adipose tissue focusing on the development of an anti-inflammatory environment along with ageing in lean conditions, as well as the state of inflammation originating from M1 macrophage infiltration in response to a HFD. Our findings may suggest another view of T cell involvement in obesity-associated chronic inflammation.

Methods

Mice

C57BL/6 (B6) and HFD (HFD32, fat kcal: 60%) were purchased from Crea Inc. (Japan). TCRα$^{-/-}$ mice were purchased from JAX® MICE (The Jackson Laboratory, USA). Generation of TCR transgenic mice using B6 mice was carried out by prenuclear micro-injection of DNA fragments into fertilized eggs from B6 at the Center for Animal Resources and Development, Kumamoto University, and the mice were transferred to the University of Tokyo and used for experiments. All mice were maintained under an specific pathogen-free condition. All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Tokyo (Permit Number: P10-143). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Antibodies and reagents

Antibodies and reagents used for histological, biochemical experiments and flow cytometry are as follows: F4/80 (clone BM8, eBioscience, CALTAG/Invitrogen), αCD4 (clone RM4-5, eBioscience), αCD8 (clone 53-6.7, eBioscience), αCD25 (clone PC61.5, eBioscience), αFoxP3 (clone NRRF-30, eBioscience), αCD3 (clone 145-2C11, eBioscience), αTCRβ (clone H57-597, eBioscience), αCD69 (clone H1.2F3, Pharmingen), αVβ8.2 (clone MR5-2, eBioscience) and αCD16/32 (clone 93, eBioscience). Secondary antibodies and related reagents are: Alexa fluor 488 conjugated anti-rat IgG (Molecular Probes), DAPI (Roche) or Hoechst33342 (Invitrogen) for nuclear staining, Cytofix/Cytoperm solution (BD Bioscience) to permeabilize cells and G-Block (Genostaff Inc., Tokyo, Japan) as a blocking reagent. Specimens were analyzed using a confocal microscope, FV10i-DOC (Olympus, Tokyo). Stained cells were analyzed using an SH800 flow cytometer (Sony Inc.), LSRII (BD) or FlowJo.

Isolation of stromal-vascular cell fraction cells

Stromal vascular fraction (SVF) isolation was performed as previously described ([18]) with some modifications. Briefly, mice were killed after an anesthesia and systemic heparinization by infusion of PBS containing heparin (1 U ml^{-1}). Epididymal fat pads were collected, minced into small pieces, washed in PBS containing heparin (1 U ml^{-1}) for 1 min to remove blood cells and then centrifuged at $1000 \times g$ for 10 min. Floating fat pieces were collected and incubated for 40 min in type 2 collagenase solution (2 mg ml^{-1}; type 2 collagenase was purchased from Calbiochem Inc.). Thereafter, the digested tissue was filtered using a 100 µm cell strainer and centrifuged at 1400 rpm for 8 min, and the resultant pellet containing enriched SVF was washed twice in PBS and FACS wash buffer [PBS supplemented with 2.5% fetal bovine serum (FBS)].

Single-cell sorting and RT–PCR

The SVF cells from epididymal fat were collected as described above and stained for CD4 and CD8. Single CD4$^+$ or CD8$^+$ T cells were sorted using an Aria™ II cell sorter (BD) into a MicroAmp® Fast Optical 96-well Reaction Plate (Life Technologies Inc.), in which each well contains 20 µl of 1× RNA extraction buffer. After 15-min incubation at room temperature, RT–PCR was performed using ReverTra Ace® (TOYOBO, Japan) following the manufacturer's protocol. Nested PCR was then performed using KOD Plus-Ver. 2 *Taq* polymerase (TOYOBO) with degenerate primers for TCRα ([Table 1]) and TCR β ([Table 2]).

Lentivirus construction and transfection

To produce recombinant lentiviruses, 293FT cells were co-transfected with pLenti vector and ViraPower Packaging Mix (Invitrogen) using X-treameGENE9 (Roche). Cells were cultured for 4–5 days in DMEM culture medium containing 10% FBS and 500 µg ml^{-1} G418 (Invitrogen). The culture supernatant containing lentiviral particles was collected and concentrated using Lenti-X Concentrator (Clontech). Afterwards, 1×10^5 of 58α$^-$β$^-$ cells ([19]) were suspended in 10 ml of DMEM medium without antibiotics, and 1 ml of supernatant containing

lentiviral particles and 10 µg ml⁻¹ Polybrene (MILLIPORE) were added. After an overnight culture, the culture medium was removed, followed by additional culture for 10–12 days in new DMEM medium containing 10 µg ml⁻¹ Blasticidin (Life Technologies) to enrich transfected cells. Cells were harvested and analyzed for the TCRα/β expression by flow cytometer.

In vitro T-cell activation assay

To stimulate T cells by concanavalin A (Con A), lymph node cells were plated at 1×10^6 cells per well in a 96-well flat-bottomed culture plate. Then, 1 µg ml⁻¹ of Con A (SIGMA-ALDRICH) was added, and cells were cultured for 6 or 16 h in DMEM culture medium supplemented with 10% FBS. Thereafter, cells were harvested and analyzed for CD25 expression levels using a flow cytometer. To stimulate TCR, 96-well flat-bottomed culture plates were coated with the αCD3 (clone 145-2C11) antibody (0.1 µg per well) at 4°C overnight. Plates were washed twice with PBS and then, lymph node cells were plated at 1×10^6 cells per well. Cells were cultured for 6 or 16 h in DMEM culture medium supplemented with 10% FBS. Thereafter, cells were harvested and analyzed for CD25 expression levels using a flow cytometer.

Histology

Epididymal fat tissues were fixed in 4% paraformaldehyde in PBS for 24 h, and frozen sections were made at 7–10 µm thickness. For immunohistochemistry, frozen sections were treated with G-block (Genostaff Inc.) to block non-specific background, and then incubated with a primary antibody at 4°C overnight, followed by incubation with a fluorescently conjugated secondary antibody at room temperature for 1 h. Nuclei were additionally stained with Hoechst33342, and then, the slides were mounted with Prolong Gold anti-fade reagent (Molecular Probes). The specimens were analyzed using confocal microscopy (FV10i, Olympus).

Insulin tolerance test

Mice were injected intra-peritoneally with 0.75 U kg⁻¹ body-weight of insulin under the same fasting condition. Thereafter, at indicated time points, blood glucose levels were measured.

qPCR assay

The quantitative evaluation of mRNA was performed by the $\Delta\Delta C_T$ method using a QuantStudio3 Real-Time PCR system (Thermo Fisher Scientific). Sequences of the oligonucleotides used are shown in Table 3:

Statistical analysis

Paired results were assessed using parametric tests such as Student's *t*-test. The significance code is added in each figure legend.

Results

Specific Vα5/Vβ8.2 T cells accumulate in adipose tissue of obese mice

We examined the increase of T cells in adipose tissue along with the progression of obesity. To this end, the visceral epididymal fat tissue of wild-type C57BL/6 (B6) mice fed a HFD for 0, 4, 9 or 12 weeks was digested by collagenase

Table 1. Degenerate primers for TCRα

Name	Sequence (5'-3')
f-TCRα NW36	``` A G AA ACCC CCC T GTCCTGACCTCGCATGCTTC T TC TGGTA CC TATT TTG C ```
f-TCRα NW37	``` C C G C AC GT T T T GTCCTGACCTCGCATGCTTCA T TTC T TGGTA TT AC C C C T A ```
f-TCRα NW38	``` GTG GGGCC G A T GTCCTGACCTCGCATGC A GT C GTGGTT ACC CAATT C T C ```
r-TCRα NJ108	GGCCCCATTGCTCTTGGAATC
r-TCRα NJ109	CGGCACATTGATTTGGGAGTC

Table 2. Degenerate primers for TCRβ

Name	Sequence (5'-3')
f-TCRβ NK121	``` T CC C CCG GG T T GTCCTGACCTCGCATGCCA A G T A TGGTA TA T ATA TT C C ```
f-TCRβ NK122	``` A T C C CTG G TT T GTCCTGACCTCGCATGCCA C AG T T TGGTA T A AAA T AC C ```
f-TCRβ NK123	``` A C CACCA CGCC T GTCCTGACCTCGCATGC C T TGGTA A TTAGG AAAT C ```
r-TCRβ MQ284	AGCACACGAGGGTAGCCTT
r-TCRβ MS175	GACAGAACTTTGAATTCCTCTGCTTTTGATGG

Table 3. PCR primers for qPCR

Name	Sequence (5′–3′)
f-GAPDH	AGAACATCATCCCTGCATCC
r-GAPDH	CACATTGGGGGGTAGGAACAC
f-F4/80	CCTGGACGAATCCTGTGAAG
r-F4/80	GGTGGGACCACAGAGAGTTG
f-MCP1	CATCCACGTGTTGGCTCA
r-MCP1	GATCATCTTGCTGGTGAATGAGT
f-IL-1β	CTGGTGTGTGACGTTCCCATTA
r-IL-1β	CCGACAGCACGAGGCTTT
f-iNOS	AATCTTGGAGCGAGTTGTGG
r-iNOS	CAGGAAGTAGGTGAGGGCTTG
f-TNFA	ACGGCATGGATCTCAAAGAC
r-TNFA	AGATAGCAAATCGGCTGACG
f-CD163	CCTGGATCATCTGTGACAACA
r-CD163	TCCACACGTCCAGAACAGTC
f-MR	CCACAGCATTGAGGAGTTTG
r-MR	ACAGCTCATCATTTGGCTCA

treatment (20), and the non-adipocyte SVF was isolated. SVF cells were assessed for the proportion of CD4 and CD8 T cells using a flow cytometer. As shown in Fig. 1(A), a significant increase of both CD4 and CD8 T cells was observed until 9 weeks. The proportion of CD4 T cells was larger than that of CD8 T cells (~2:1), and this was not changed by HFD challenge (Fig. 1A). Thus, we confirmed the recruitment of T cells to adipose tissue with the progression of obesity.

We then investigated the TCR repertoire of T cells that accumulated in visceral adipose tissue at various time points in mice fed a HFD. As the available antibodies for flow cytometry do not sufficiently cover the whole repertoire of the TCR V region, in particular for Vα, we amplified the *TCR V-(D)-J* region by degenerative RT–PCR using total RNA from adipose tissue SVF cells. The PCR fragments were sub-cloned, and 50 independent clones per mouse were sequenced for Vα and Vβ usage at 0, 4, 9 and 12 weeks of HFD challenge. The percentage of different Vα (TRAV) and Vβ (TRBV) in the 50 clones analyzed is presented in Fig. 1(B). No specific skew in Vα was observed at 0 (lean) and 4-week HFD. In contrast, a marked accumulation of T cells with TRAV3 (Vα5) was observed at 9-week HFD (Fig. 1(B), upper panel). In one mouse, 49 out of 50 clones showed Vα5. Interestingly, in the spleen of mice at 9-week HFD, there was no apparent accumulation of Vα5 (Supplementary Figure 1, available at *International Immunology* Online), suggesting that the accumulation of Vα5-bearing T cells was due to the preferential recruitment of these cells to adipose tissue, and not due to their systemic amplification. Winer *et al.* similarly reported Vα5 as one of several TCR Vα repertoires identified in obese adipose tissue (16). Interestingly, at 12-week HFD, the dominancy of TRAV3 (Vα5) clones was no longer observed (Fig. 1B, upper panel). The progression of tissue injury following the prominent recruitment of specific TRAV3 (Vα5) T cells might cause the exposure of multiple tissue antigens, leading to the subsequent infiltration of various T cells. In contrast to Vα5, however, the Vβ repertoire was varied throughout the HFD challenge; no specific skew was observed at 9 weeks (Fig. 1B, lower panel).

Next, we isolated CD4 and CD8 T cells from 9-week HFD adipose tissue and sequenced the whole *V-(D)-J* sequence in single cells. Twelve of the 15 CD4 cells analyzed possessed an identical *Vα5-J* sequence. To our surprise, this *Vα5-J* mRNA was also expressed in all 12 CD8 cells analyzed. In addition, although no specific skew was observed in the Vβ repertoire at 9-week HFD by bulk analysis of RT–PCR products, the *Vβ-D-J* sequence was identical and contained TRBV13-2 (Vβ8.2) in these CD4 and CD8 T cells carrying the specific *Vα5-J* sequence. The entire amino acid sequences of Vα5-J and Vβ8.2-D-J (plus Cα or Cβ, respectively) are presented in Fig. 1(C). Thus, CD4 and CD8 T cells bearing an identical TCR accumulated in visceral adipose tissue in mice fed a HFD for 9 weeks.

To test whether the isolated TCRα (Vα5) and TCRβ (Vβ8.2) chains develop a TCR complex, we generated a lentivirus vector carrying the TCRα and TCRβ coding sequences linked by the 2A peptide sequence. The vector also expressed GFP as a marker protein. A mouse T-cell clone lacking TCRα and β, 58α⁻β⁻ cells (19) was transfected with the lentivirus vector,

and TCR expression on the cell surface was assessed by flow cytometry. The GFP⁺ population, corresponding to successfully transfected cells, was positive for both the TCRβ chain (H57 antibody) and CD3ε (145-2C11 antibody), indicating that the isolated TCRα and β chains formed a TCR complex (Fig. 1D).

Generation of transgenic mice expressing the Vα5/Vβ8.2 TCR

We generated transgenic mice expressing the Vα5/Vβ8.2 TCR in T cells under the control of the human CD2 promoter (Fig. 2A). The transgene contained the cDNA of the TCRα or β chain and the 5.5 kb locus control region sequence to exclude the possible influence of the insertional locus of the transgene in the genome (21, 22). After co-injection of DNA fragments for the TCRα and β transgenes into fertilized eggs from B6 mice, 13 independent transgenic mouse lines that carried both α and β transgenes were obtained, and in three of them (lines #3, #11 and #12), the majority of CD4 and CD8 peripheral T cells expressed only the Vβ8.2 transgenic TCRβ chain, indicating entire allelic exclusion of the endogenous β chain (Supplementary Figure 2, available at *International Immunology* Online). These three founder mice were bred to TCRα-deficient (*TCRα⁻/⁻*) mice to eliminate endogenous TCRα protein.

In the periphery, the TCR level in the T cells of the resulting TCR transgenic mice on a *TCRα⁻/⁻* background [Adipose tissue *T*-cell Transgenic mice (ATT) mice] was comparable to that in wild-type mice in all three independent lines, when lymph node and splenic T cells were analyzed using an anti-H57 antibody (Fig. 2B, histograms). Thus, we employed one mouse line (line #3) for the following analyses. Intriguingly, the proportion of CD8 T cells was larger than that of CD4 T cells in ATT mice, unlike wild-type mice (Fig. 2B, CD4/CD8 profiles).

We addressed whether the transgenic T cells responded to activating stimulation. Lymph node cells from wild-type and ATT mice were stimulated using an anti-CD3ε antibody or Con A, and the cell activation state was assessed by CD25 expression. As shown in Fig. 2(C), CD25 levels on CD4 and CD8 T cells increased in response to CD3 stimulation in both types of mice, suggesting that the transgenic T cells possessed normal responsiveness to TCR stimulation. Parallel results were obtained when the cells were stimulated with Con A (Fig. 2C). Thus, the transgenic T cells harbor normal responsiveness to TCR-dependent and -independent stimulation.

We then assessed the thymic development of the transgenic T cells. The total number of thymocytes was similar in wild-type and ATT mice ($1.66 \times 10^8 \pm 4.33 \times 10^7$ in wild-type mice versus $1.52 \times 10^8 \pm 1.30 \times 10^7$ in ATT mice; mean ± SD, $n = 4$ each). Both CD4 and CD8 T cells were positively selected and developed to single positive (SP) thymocytes in ATT mice (Fig. 2D). As Vα5/Vβ8.2 thymocytes were not negatively selected, the specific antigen that is recognized by Vα5/Vβ8.2 T cells may not be expressed highly in the thymus. The number of CD4 SP cells was smaller in ATT thymus than in wild-type thymus, indicating the less efficient positive selection of CD4 cells in ATT mice. Although the number of CD8 SP cells was much larger than that of CD4 SP cells in ATT mice (Fig. 2D, left panels), it was comparable in CD4 and CD8 cells when
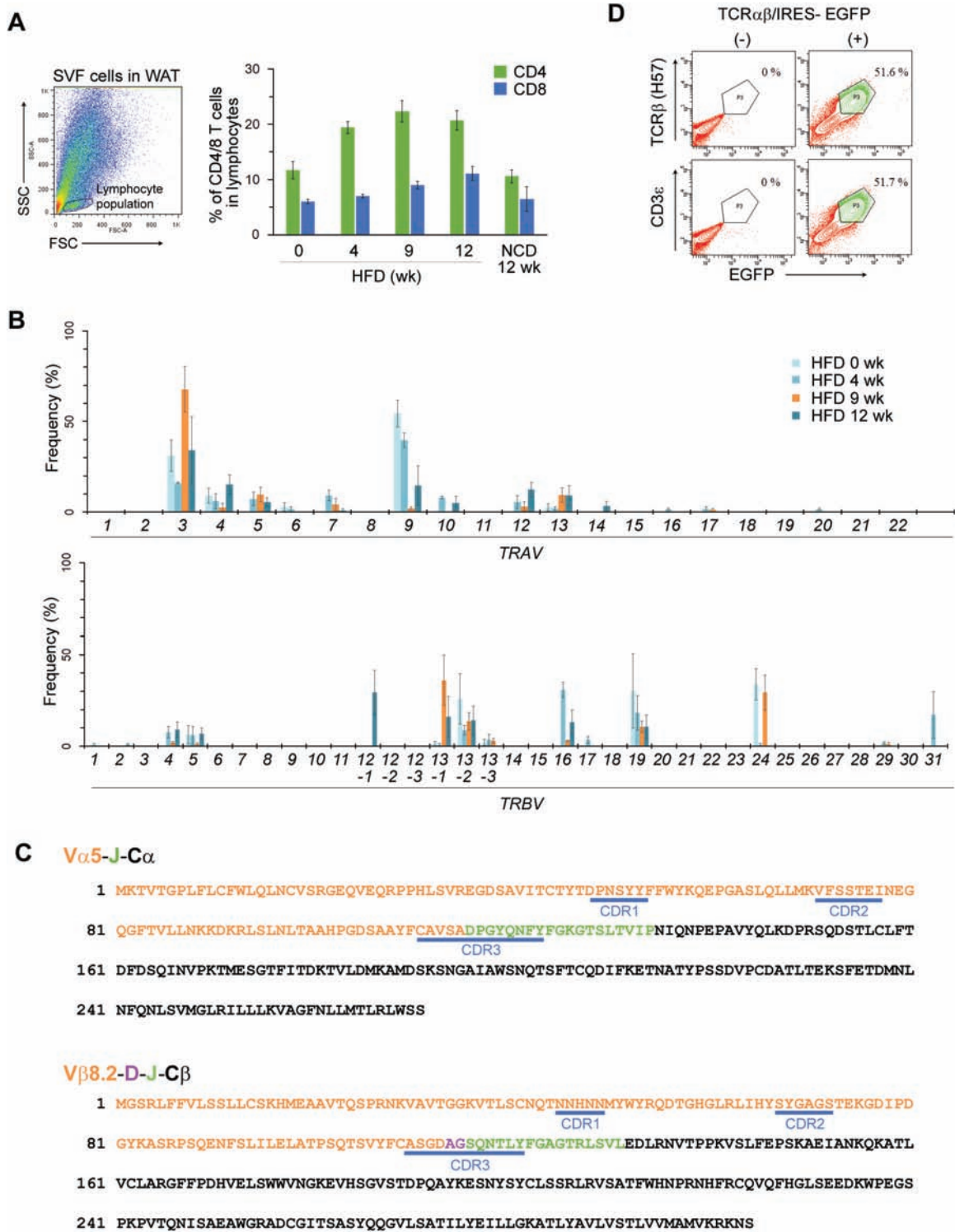
Fig. 1. Accumulation of a specific Vα5/Vβ8.2 T cells in obese adipose tissue. (A) The proportion of CD4 (green bars) and CD8 (blue bars) T cells in the lymphocyte population (indicated by a gate in the FSC/SSC profile) of SVF cells isolated from adipose tissue of wild-type C57BL/6 mice fed a HFD for indicated weeks. The SVF cells isolated from mice fed an NCD for 12 weeks were also analyzed as controls. *n* = 3–4 each. Error bar: SEM. (B) The frequency of adipose tissue T cells bearing indicated TCRα (upper) or β (lower) in mice fed a HFD for the indicated period (0~12 weeks). *n* = 3 each. (C) Amino acid sequence of the isolated TCRα and β. Orange, V region; purple, D region; green, J region; and black, C region. The complementarity determining regions (CDRs) are indicated by blue bars. (D) A Lentivirus encoding the TCRα/β and IRES-EGFP was transfected to 58α⁻β⁻ cells and the expression of TCR complex was assessed in EGFP-positive transfected cells by flow cytometry.
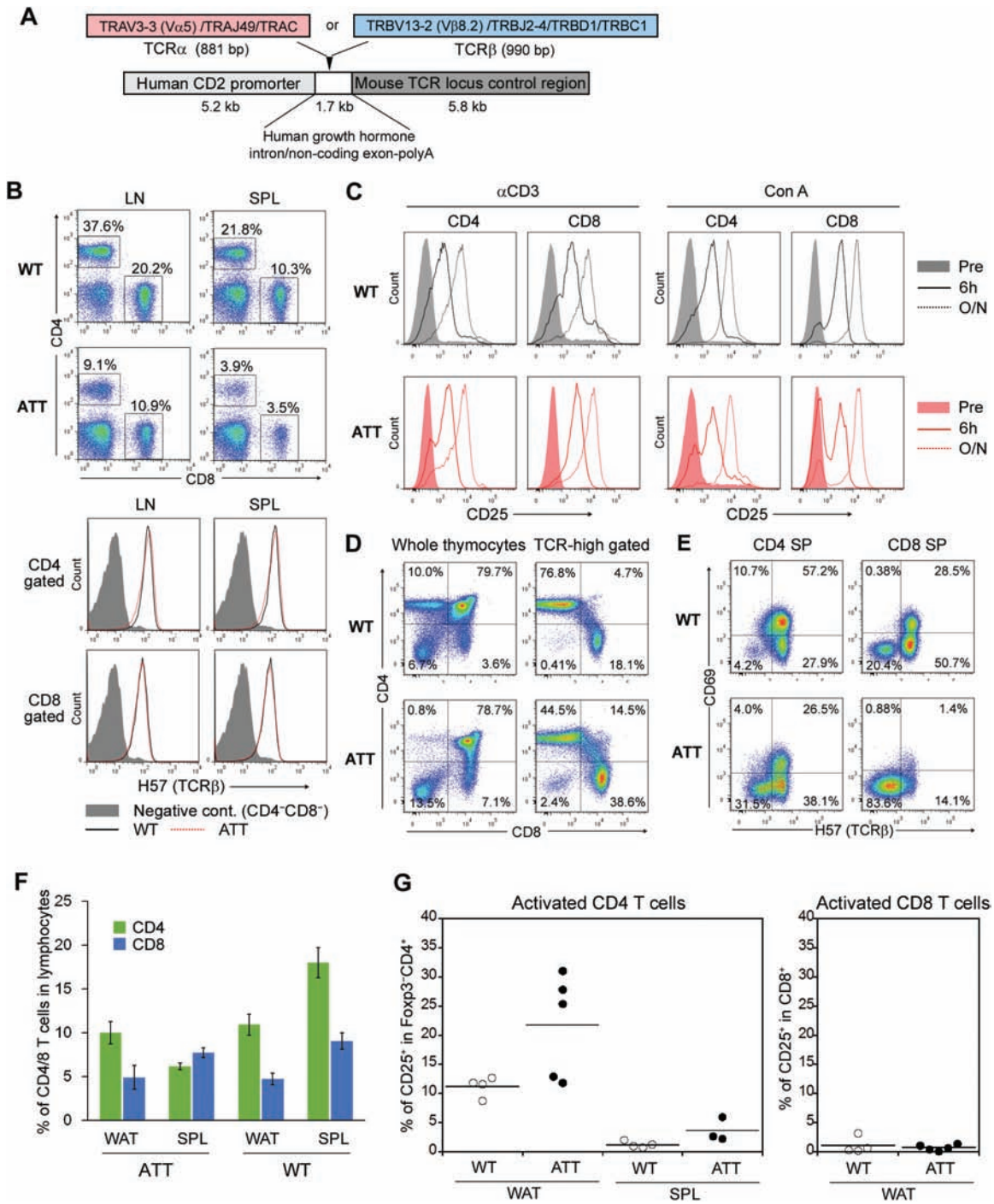
Fig. 2. Generation of Vα5/Vβ8.2 TCR transgenic mice. (A) A scheme of the TCR transgene. The TCRα or β cDNA fragment was inserted into a TCR expression vector cassette that contains a 5.2 kb human genomic DNA fragment covering the human *CD2* gene promoter region, human growth hormone gene non-coding exons and intron and a 5.8 kb mouse genomic DNA fragment of the LCR region. (B) Representative CD4/CD8 profiles of lymph node (LN) and spleen (SPL) cells. The histograms show the TCR levels of CD4 and CD8 T cells in the lymph nodes and spleen. Black lines, wild-type (WT) mice; red lines, ATT mice; shadowed, non-T cells. (C) Lymph node cells were stimulated by an anti-CD3ε antibody (145-2C11) or Con A (1 μg ml^{-1}) for 6 or overnight (O/N; 16 h), and the surface CD25 levels in CD4 and CD8 T cells were analyzed by flow cytometry. Three independent experiments were performed and the representative histograms are presented. (D) Left: the CD4/CD8 profiles of whole thymocytes. The proportion of each cell population is indicated. Right: the CD4/CD8 profiles of thymocytes gated on the TCR^{high} population. Representative profiles are presented after analyzing four mice for each genotype. (E) The CD69/TCRβ profiles of CD4 SP (left) and CD8 SP (right) thymocytes. The proportion of each cell population is indicated. Representative profiles are presented after analyzing four mice for each genotype. (F) The proportion of CD4 (green bars) or CD8 (blue bars) within T cells (H57-positive cells) in the epididymal white adipose tissue (WAT) or splenic cells in wild-type and ATT mice fed a HFD for 12 weeks. Error bar: SEM. (G) The proportion of CD25$^+$ cells in CD4$^+$Foxp3$^-$ or CD8$^+$ T cells in the epididymal white adipose tissue or spleen in wild-type and ATT mice fed a HFD for 12 weeks. Each dot corresponds to the result from an individual mouse. Averages are indicated by bars.

gated on cells that bear high levels of TCR, namely mature SP cells (Fig. 2D, right panels), indicating a large proportion of CD8 SP cells in ATT thymus were immature single positive (ISP) cells. Moreover, we assessed the positive selection of CD4 and CD8 cells by staining thymocytes for CD69 and TCR (H57 antibody). Both CD69 and TCR expression levels are up-regulated in thymocytes after positive selection, and thereafter, CD69 levels decrease, whereas TCR levels remain high in mature SP cells (23). As demonstrated in Fig. 2(E), the number of CD69⁻TCRˡᵒʷ CD4 cells before positive selection was markedly larger in ATT mice than in wild-type mice (Fig. 2E), supporting the less efficient positive selection of CD4 T cells in ATT mice. The majority of CD8 SP cells belonged to the CD69⁻TCRˡᵒʷ population in ATT mice (Fig. 2E), consistent with the conclusion that the majority of CD8 SP cells were ISP cells in ATT thymus. TCR levels in CD4 and CD8 cells post-positive selection (in the CD69ˡᵒʷ⁻ⁿᵉᵍᵃᵗⁱᵛᵉTCRʰⁱᵍʰ population) were similar in ATT and wild-type mice (Fig. 2E).

ATT and wild-type mice were fed with a HFD, and the accumulation of T cells in adipose tissue and their activation state were analyzed. Interestingly, the proportion of CD4 T cells was larger than that of CD8 T cells in adipose tissue of ATT mice, in contrast to the CD8-dominant profile in the spleen (Fig. 2F). In CD4 T cells (Foxp3⁻ non-T$_{reg}$ CD4 T cells), the proportion of CD4⁺ activated T cells was profoundly larger in ATT than in wild-type adipose tissue, though it was comparable in the spleen in both mice (Fig. 2G). In contrast, most CD8 adipose tissue T cells were CD25-negative in obese ATT adipose tissue, indicating that they were not activated (Fig. 2G). These results suggest that that the Vα5/Vβ8.2 TCR appeared to recognize certain adipose tissue antigen(s) predominantly

in a class II MHC dependent fashion, and CD4 transgenic T cells were specifically activated in obese adipose tissue.

Abrogated anti-inflammatory states in adipose tissue in lean ATT mice

Mathis's group reported that thymus-derived T$_{reg}$ cells are amplified in response to a specific antigen in adipose tissue, thereby suppressing adipose tissue inflammation under lean conditions, and that such T$_{reg}$ cells are decreased with the progression of obesity, contributing to the acceleration of inflammation (13–15). Therefore, we first assessed adipose tissue T$_{reg}$ cells in wild-type and ATT mice fed a normal chow diet. The proportion of Foxp3⁺ T$_{reg}$ cells within all CD4⁺ cells in adipose tissue was similar in young (8 weeks of age) wild-type and ATT mice. In 30-week-old wild-type mice, the proportion of T$_{reg}$ cells was essentially larger than in young mice; in some mice, it increased up to 40% of CD4 T cells (Fig. 3A, left panel). In contrast, in ATT mice, the T$_{reg}$ proportion was even decreased in aged mice compared with young mice (Fig. 3A, left panel). In the spleen, such a profound increase in T$_{reg}$ cells was not observed in aged wild-type mice, and a similar T$_{reg}$ cell population size was found in wild-type and ATT mice (Fig. 3A, right panel). These results support the presence of specific adipose tissue T$_{reg}$ cells that migrate directly from the thymus, and they were not our Vα5/Vβ8.2 cells. Intriguingly, the T$_{reg}$ proportion was varied in aged wild-type mice, and in some mice, it was almost comparable to that in young mice (Fig. 3A). Since the level of *Il33* mRNA in adipose tissue was similar in all aged wild-type mice and comparable with that in ATT mice (Fig. 3B), such an insufficient increase of T$_{reg}$ cells
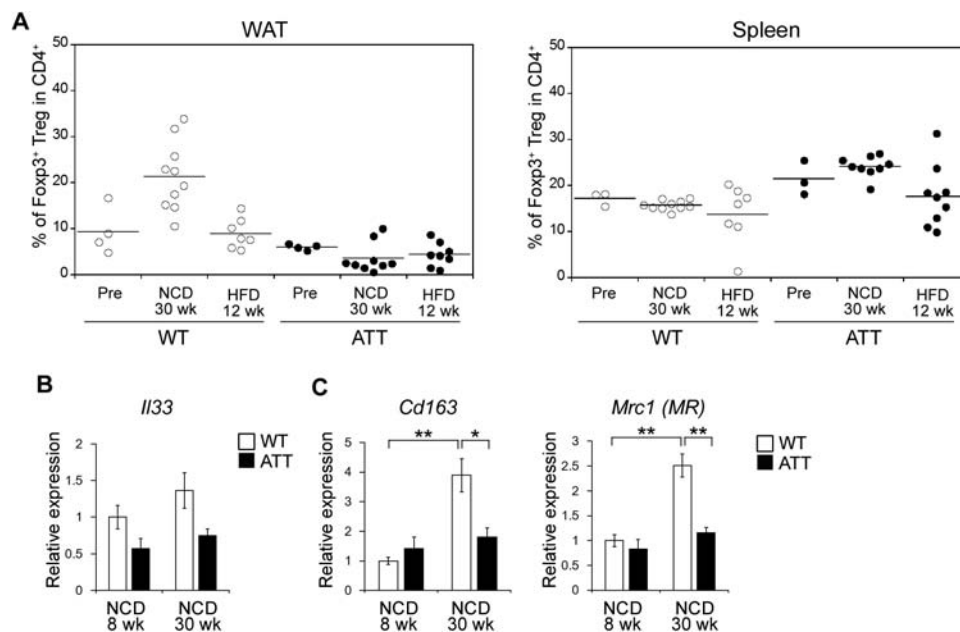

Fig. 3. Abrogated anti-inflammatory environment in adipose tissue of ATT mice. (A) The proportion of Foxp3⁺ T$_{reg}$ cells in whole CD4 T cells in the epididymal white adipose tissue (WAT) or spleen of indicated mice. Pre: 8-week-old mice under a NCD before HFD started. Each dot corresponds to the result from an individual mouse. The averages are indicated by bars. (B,C) qPCR analysis using RNA isolated from epididymal fat obtained from Pre and 30-week-old mice maintained under NCD for mRNA levels of *Il33* (B), *Cd163* and *Mrc1* (MR) (C). n = 3–5 each. Error bar: SEM. *$P < 0.05$; **$P < 0.01$.

in some wild-type mice might be due to inadequate exposure of the specific antigen recognized by the T_{reg} TCR in adipose tissue. Consistent with a previous report, the proportion of Foxp3$^+$ cells in CD4$^+$ cells after a 12-week HFD was reduced compared with that in aged, lean mice, and it was smaller in ATT mice than in wild-type mice (Fig. 3A, left panel).

In addition to T_{reg} cells, anti-inflammatory M2 macrophages increased in aged, lean wild-type mice. As shown in Fig. 3(C), the mRNA levels for M2 macrophage marker genes, *Cd163* and *Mrc1* [encoding mannose receptor (MR)] increased significantly in adipose tissue at 30 weeks of age compared to 8 weeks of age, when assessed by qPCR using RNA from SVF cells. In sharp contrast, no significant increase in the levels of M2 macrophage marker genes was observed in aged, lean ATT mice (Fig. 3C). Thus, the anti-inflammatory state in adipose tissue induced by the accumulation of T_{reg} cells and M2 macrophages was abrogated in ATT mice. Although the link for the expansion of T_{reg} cells and the increase in M2 macrophages is not clear, specific T cells (but not our Vα5/Vβ8.2 cells) appeared to be involved in the development of anti-inflammatory states in aged, lean adipose tissue.

Comparable inflammatory states in adipose tissue in wild-type and ATT mice

We wondered whether adipose tissue inflammation, including M1 macrophage recruitment, was promoted in lean, aged ATT mice, as no accumulation of T_{reg} cells and M2 macrophages was observed in their adipose tissue. In histology, however, no F4/80$^+$ macrophages developed obvious clusters or crown-like structures (CLSs) (24) in both wild-type and ATT mice (Fig. 4A). qPCR analysis showed that the mRNA levels for inflammatory/M1 genes, including *Tnfα*, *Ccl2* (encoding MCP-1), *Nos2* (encoding iNOS) and *Il1b* (encoding IL-1β) increased mildly at 30 weeks of age compared to 8 weeks of age, in both wild-type and ATT mice (Fig. 4B). The proportion of CD25$^+$-activated Foxp3$^-$ non-T_{reg} CD4$^+$ cells in adipose tissue was not different significantly in 30-week-old wild-type and ATT mice (Fig. 4C). Thus, despite the abrogated anti-inflammatory environment, no profound inflammation occurred in adipose tissue in aged, lean ATT mice in terms of M1 macrophage infiltration and/or rigorous accumulation of effector T cells.

Conversely, a large number of macrophages demonstrating CLS formation were observed within visceral fat tissue in both wild-type and ATT mice after a 12-week HFD (Fig. 4D). The mRNA levels of *Adgre1* (encoding F4/80) increased comparably in both mice (Fig. 4E). Thus, the obesity-associated recruitment of M1 macrophages to adipose tissue was induced similarly in wild-type and ATT mice. The mRNA levels for inflammatory/M1 (*Tnfα*, *Ccl2*, *Nos2* and *Il1b*), and regulatory/M2 (*Cd163* and *Mrc1*) genes expressed in macrophages were also essentially similar in obese wild-type and ATT mice (Fig. 4E), indicating no essential difference in macrophage M1/M2 polarity and inflammatory state in adipose tissue in obese wild-type and ATT mice. In addition, whole-body insulin resistance was observed at comparable levels in wild-type and ATT mice fed a HFD for 12 weeks, based on the results of an intra-peritoneal insulin tolerance test (Fig. 4F). Accordingly,

serum glucose and insulin levels increased similarly in both mice after the 12-week HFD (Fig. 4G). Thus, the induction of obesity-associated M1 macrophage recruitment and the following inflammation, as well as insulin resistance, were not influenced under the condition where all T cells expressed a single TCR. Together with the observations in aged ATT mice under a normal chow diet, it is likely that the removal of an anti-inflammatory state is not sufficient, but obesity progression (e.g. by a HFD) is required to induce the rigorous recruitment of M1 macrophages to adipose tissue, resulting in subclinical inflammation.

Discussion

In the current study, we isolated a Vα5/Vβ8.2 T cell that accumulated in adipose tissue at a specific period during the progression of obesity. In addition, we generated transgenic mice expressing this TCR (ATT mice). Various important lessons were obtained by the analysis of ATT mice, which provided new insights into the role of T cells in obesity-associated adipose tissue inflammation. It was curious that both CD4$^+$ and CD8$^+$ T cells harbored the same Vα5/Vβ8.2 TCR in obese adipose tissue. One might argue that this finding was brought about by a cross-contamination during the single-cell RT–PCR. However, we believe it is unlikely, since both CD4$^+$ and CD8$^+$ T cells were positively selected and appeared in the periphery in ATT mice. It is not clear whether both T cells were positively selected by only class I or class II MHC, or both. Nevertheless, the Vα5/Vβ8.2 TCR should recognize a certain adipose tissue antigen presented by class II MHC cells, as CD4 T cells predominantly infiltrated and were activated in adipose tissue in ATT mice. These data confirm the accumulation of the Vα5/Vβ8.2 T cells in adipose tissue of obese wild-type mice presented in Fig. 1 and also implicate their participation in the obesity-associated chronic inflammatory events of adipose tissue, which lead to the insulin resistance.

However, intriguingly, there was no difference in the inflammatory state, including macrophage CLS formation and inflammatory cytokine levels in adipose tissue, as well as in the consequent insulin resistance, between obese wild-type and ATT mice fed a HFD. This is contrast to a marked disease acceleration often observed in various transgenic mouse models for autoimmune diseases, which express a TCR isolated from T cells recognizing pathogenic antigens. For instance, Katz *et al.* identified a set of TCRs from T cells infiltrating to the Langerhans islets in non-obese diabetic (NOD) mice, and produced its TCR transgenic mice on a NOD background. They observed prominently accelerated insulitis and consequent diabetes in the mice (25). Thus, it appeared that T cells might not be involved in the adipose tissue chronic inflammation causing insulin resistance. One might argue that although the Vα5/Vβ8.2 T cells did accumulate and were activated in obese adipose tissue, they might not be responsible T cells for inflammatory events by chance. If so, however, adipose tissue inflammation should have been ameliorated in ATT mice, as no T cells other than Vα5/Vβ8.2 cells were present in the mice. It might be possible that multiple T-cell repertoires are required to be effective to obesity-associated adipose tissue inflammation. If so, the involvement of T_h1 T cells in the progression of inflammation and the requirement
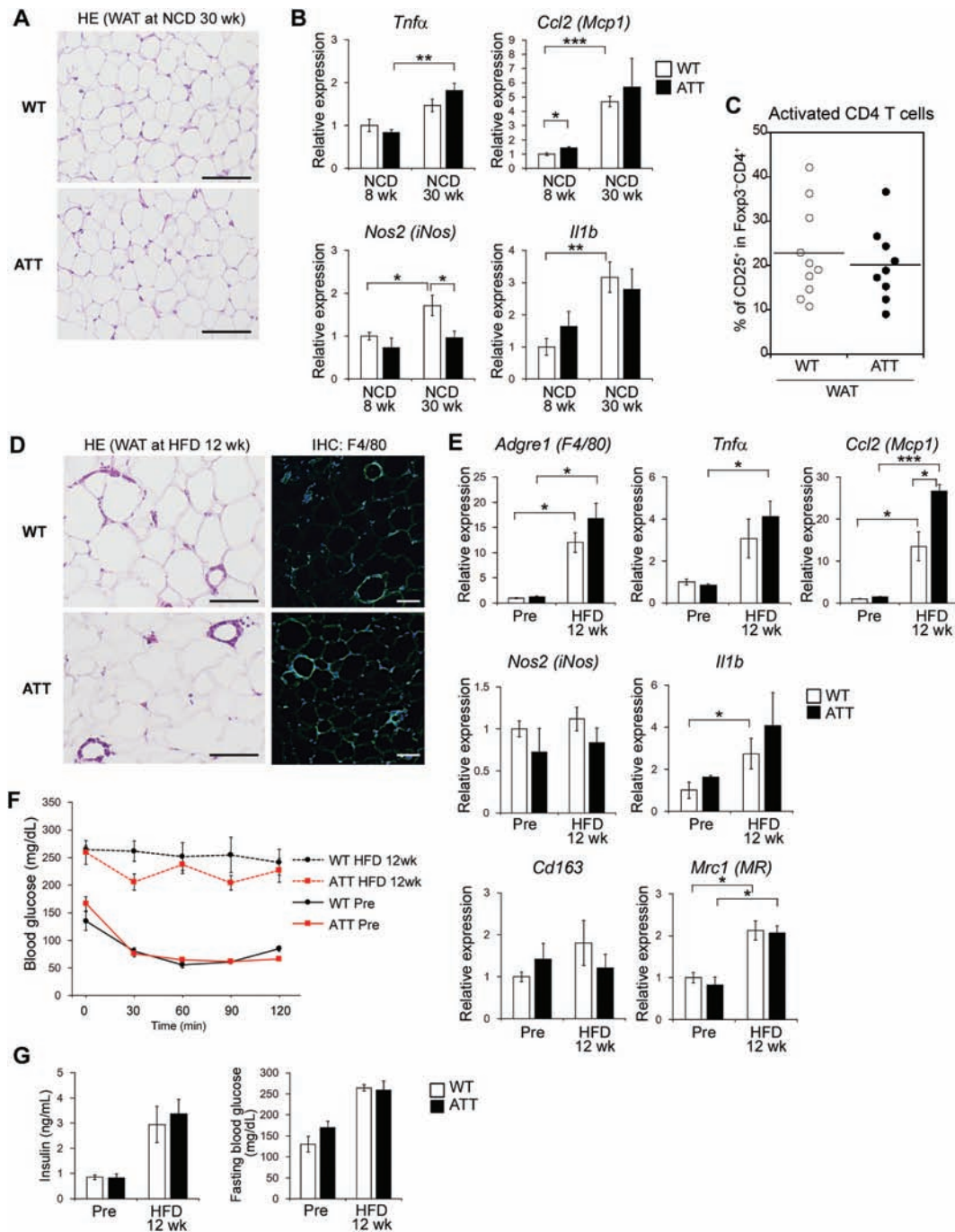
Fig. 4. Comparable inflammatory states in wild-type and ATT adipose tissues. (A) Specimens of the epididymal white adipose tissue (WAT) from 30-week-old wild-type (WT) and ATT mice under a NCD were stained by H&E. No typical CLS was observed in both mice. At least three different areas in three different sections per mouse were analyzed in three mice of each genotype. Scale bar: 100 μm. (B) mRNA levels for *Tnfα*, *Ccl2 (Mcp1)*, *Nos2 (iNos)*, *Il1b* were assessed by qPCR using RNA isolated from epididymal fat obtained from wild-type and ATT mice maintained under NCD at 8 and 30 weeks of age. n = 3–5 for each group. Values were normalized to those of GAPDH and presented as relative expression to those of lean wild-type mice. Error bar: SEM. (C) The proportion of CD25$^+$ cells in CD4$^+$Foxp3$^-$ T cells in the epididymal white adipose tissue in wild-type and ATT mice maintained under a NCD for 30 weeks. Each dot corresponds to the result from an individual mouse. Averages are indicated by bars. (D) Specimens of the epididymal white adipose tissue (WAT) from wild-type and ATT mice fed a HFD for 12 weeks were stained for F4/80 (pan macrophage marker; green) and Hoechst (blue), or by H&E. Scale bar: 100 μm. (E) qPCR analysis for the mRNA levels for *Adgre1 (F4/80)*, *Tnfα*, *Ccl2 (Mcp1)*, *Nos2 (iNos)*, *Il1b* (M1 markers), *Cd163* and *Mrc1 (MR)* (M2 markers) using RNA isolated from epididymal fat obtained from wild-type and ATT mice fed a HFD for 0 (Pre) and 12 weeks. n = 4–6 for each group. Values were normalized to those of GAPDH and presented as relative expression to those of lean wild-type mice. Error bar: SEM. (F) Insulin tolerance test (ITT) performed on wild-type and ATT mice fed a HFD for 0 (Pre) or 12 weeks; n = 3–5 for each group. (G) Insulin levels in mice maintained under NCD or fed a HFD for 12 weeks. n = 4–5 for each genotype. Fasting blood glucose levels in mice fed a HFD for 0 (Pre) or 12 weeks. n = 4 for each genotype.

of CD8 T cells for the initiation of macrophage infiltration demonstrated in previous reports ([16], [17]) might occur in TCR-independent fashions. Certainly, these possibilities need to be evaluated further, such as by analyzing other TCR transgenic mice lines fed a HFD. In addition, the role of Vα5/Vβ8.2 T cells in inflammatory events in adipose tissue needs to be clarified by pursuing additional experiments including the identification of the antigen recognized by this TCR.

In addition, the aged, lean ATT mice (under a NCD) provided important information about the role of T cells in developing an anti-inflammatory state in adipose tissue. We confirmed the increase of adipose tissue T_{reg} cells in wild-type mice along with ageing (although there was large variation among individuals). In contrast, the number of adipose tissue T_{reg} cells was decreased in aged, lean ATT mice. This result re-confirmed the scenario proposing the presence of specific T_{reg} cells that increase in adipose tissue along with ageing ([13]–[15]), but our Vα5/Vβ8.2 T cells are not included in this process. Ideally, however, the generation and analysis of a new transgenic mouse line expressing the TCR from the thymus-derived specific adipose tissue T_{reg} cells would further corroborate Mathis's hypotheses regarding whether T cells only accumulate in adipose tissue and not in lymphoid tissue; whether they decrease in adipose tissue in obese mice and whether adipose tissue inflammation and subsequent insulin resistance are prevented in obese mice.

In addition to T_{reg} cells, we observed that M2 macrophages increased in adipose tissue in aged wild-type mice, but not in ATT mice. However, whether the increase of M2 macrophages was brought about by T_{reg} cells or by other non-T_{reg} cells (certainly different from Vα5/Vβ8.2 T cells) is still unclear. The transgenic mice expressing the TCR from adipose tissue T_{reg} cells might also provide solid information about this issue. Finally, it may be worth re-emphasizing that aged, lean ATT mice, in which the anti-inflammatory environment consisting of T_{reg} and M2 macrophage cells was abrogated, exhibited no spontaneous M1 macrophage infiltration and related inflammation in adipose tissue. Thus, it is likely that the decrease of T_{reg} cells (as well as M2 macrophages) is not solely sufficient for the initiation of M1 macrophage recruitment. Further studies are necessary to clarify the link between these two events precisely.

Supplementary data

Supplementary data are available at *International Immunology* online.

Funding

Acknowledgements

References

1 Hotamisligil, G. S., Shargill, N. S. and Spiegelman, B. M. 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259:87.
2 Arkan, M. C., Hevener, A. L., Greten, F. R. *et al.* 2005. IKK-beta links inflammation to obesity-induced insulin resistance. *Nat. Med.* 11:191.
3 Neels, J. G. and Olefsky, J. M. 2006. Inflamed fat: what starts the fire? *J. Clin. Invest.* 116:33.
4 Shoelson, S. E., Lee, J. and Goldfine, A. B. 2006. Inflammation and insulin resistance. *J. Clin. Invest.* 116:1793.
5 Wellen, K. E. and Hotamisligil, G. S. 2003. Obesity-induced inflammatory changes in adipose tissue. *J. Clin. Invest.* 112:1785.
6 Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L. and Ferrante, A. W. Jr. 2003. Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* 112:1796.
7 Xu, H., Barnes, G. T., Yang, Q. *et al.* 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* 112:1821.
8 Solinas, G., Vilcu, C., Neels, J. G. *et al.* 2007. JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. *Cell Metab.* 6:386.
9 Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* 3:23.
10 Gordon, S. and Taylor, P. R. 2005. Monocyte and macrophage heterogeneity. *Nat. Rev. Immunol.* 5:953.
11 Lumeng, C. N., Bodzin, J. L. and Saltiel, A. R. 2007. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* 117:175.
12 Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. and Locati, M. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25:677.
13 Feuerer, M., Herrero, L., Cipolletta, D. *et al.* 2009. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat. Med.* 15:930.
14 Cipolletta, D., Feuerer, M., Li, A. *et al.* 2012. PPAR-γ is a major driver of the accumulation and phenotype of adipose tissue T_{reg} cells. *Nature* 486:549.
15 Kolodin, D., van Panhuys, N., Li, C. *et al.* 2015. Antigen- and cytokine-driven accumulation of regulatory T cells in visceral adipose tissue of lean mice. *Cell Metab.* 21:543.
16 Winer, S., Chan, Y., Paltser, G. *et al.* 2009. Normalization of obesity-associated insulin resistance through immunotherapy. *Nat. Med.* 15:921.
17 Nishimura, S., Manabe, I., Nagasaki, M. *et al.* 2009. CD8⁺ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat. Med.* 15:914.
18 Kurokawa, J., Nagano, H., Ohara, O. *et al.* 2011. AIM is required for obesity-associated recruitment of inflammatory macrophages into adipose tissue. *Proc. Natl Acad. Sci. USA* 108:12072.
19 Letourneur, F. and Malissen, B. 1989. Derivation of a T cell hybridoma variant deprived of functional T cell receptor alpha and beta chain transcripts reveals a nonfunctional alpha-mRNA of BW5147 origin. *Eur. J. Immunol.* 19:2269.
20 Brake, D. K., Smith, E. O., Mersmann, H., Smith, C. W. and Robker, R. L. 2006. ICAM-1 expression in adipose tissue: effects of diet-induced obesity in mice. *Am. J. Physiol. Cell Physiol.* 291:C1232.
21 Greaves, D. R., Wilson, F. D., Lang, G. and Kioussis, D. 1989. Human CD2 3′-flanking sequences confer high-level, T cell-specific, position-independent gene expression in transgenic mice. *Cell* 56:979.
22 Scobie, L., Hector, R. D., Grant, L. *et al.* 2009. A novel model of SCID-X1 reconstitution reveals predisposition to retrovirus-induced lymphoma but no evidence of gammaC gene oncogenicity. *Mol. Ther.* 17:1031.
23 Miyazaki, T., Wolf, P., Tourne, S. *et al.* 1996. Mice lacking H2-M complexes, enigmatic elements of the MHC class II peptide-loading pathway. *Cell* 84:531.
24 Cinti, S., Mitchell, G., Barbatelli, G. *et al.* 2005. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J. Lipid Res.* 46:2347.
25 Katz, J. D., Wang, B., Haskins, K., Benoist, C. and Mathis, D. 1993. Following a diabetogenic T cell from genesis through pathogenesis. *Cell* 74:1089.