Interferon-γ, a Th1 Cytokine, Regulates Fat Inflammation
A Role for Adaptive Immunity in Obesity

Viviane Zorzaneli Rocha, Eduardo J. Folco, Galina Sukhova, Koichi Shimizu, Israel Gotsman, Ashley H. Vernon, Peter Libby

Abstract—Adipose tissue (AT) can accumulate macrophages and secrete several inflammatory mediators. Despite its pivotal role in the progression of chronic inflammatory processes such as atherosclerosis, the adaptive role of immunity in obesity remains poorly explored. Visceral AT of diet-induced obese C57BL/6 mice had higher numbers of both CD4+ and CD8+ T cells than lean controls, monitored by flow cytometry. When stimulated in vitro, T cells from obese AT produced more interferon (IFN)γ than those from controls. AT from obese animals also had more cells expressing I-Aβ, a mouse class II histocompatibility marker implicated in antigen presentation, as determined by immunostaining. Differentiated 3T3-L1 cells stimulated with recombinant IFNγ or T-helper 1-derived supernatant produced several chemokines and their mRNAs. Obese IFNγ-deficient animals had significantly reduced AT expression of mRNA-encoding inflammatory genes such as tumor necrosis factor-α and monocyte chemoattractant protein-1, decreased AT inflammatory cell accumulation, and better glucose tolerance than control animals consuming the same diet. Obese mice doubly deficient for IFNγ receptor and apolipoprotein (Apo)E on a mixed 129Sve/C57BL/6 (129/B6) genetic background, despite exhibiting similar AT mRNA levels of tumor necrosis factor-α and monocyte chemoattractant protein-1 as 129/B6-ApoE−/− controls, had decreased expression of important T cell–related genes, such as IFNγ-inducible protein-10 and I-Ab, and lower plasma triglycerides and glucose. These results indicate a role for T cells and IFNγ, a prototypical T-helper 1 cytokine, in regulation of the inflammatory response that accompanies obesity.

(Key Words: inflammation ■ obesity ■ adipose tissue ■ T cell ■ IFNγ)

The recent development of a worldwide obesity pandemic has gained wide recognition.1,2 Approximately two-thirds of US adults are either overweight or obese.3 These alarming statistics portend a gigantic health burden, because excess adiposity entrains an array of atherogenic risk factors, including diabetes and dyslipidemia.4 From a mechanistic perspective, both obesity and atherosclerosis involve chronic low-grade inflammation.

Obesity associates with macrophage accumulation in white adipose tissue (AT),5,6 where these infiltrating cells interact with adipocytes and endothelial cells, comprising a local inflammatory network.7–9 This crosstalk results in the production of multiple cytokines and chemokines, such as tumor necrosis factor (TNF)α and monocyte chemoattractant protein (MCP)-1,10 which can activate, propagate, and sustain the local inflammatory response in AT.11

Similarly, the inflammatory process within the arterial wall that characterizes atherosclerosis involves accumulation and activation of macrophages.12–14 These phagocytes in atheroma also interact with local cells and secrete multiple mediators that modulate lesion evolution and complication. Other leukocytes also contribute to atherosclerosis. T cells orchestrate the inflammatory cascade evolution, depending on the set of cytokines they predominantly produce, T helper (Th)1 or Th2.12,13,15 Interferon (IFN)γ, the signature Th1 cytokine, elicits the production of macrophage mediators, induces leukocyte adhesion molecules, chemokines, and class II major histocompatibility antigens and increases antigen-presenting capacity by macrophages and endothelial cells.16–18 The enhanced antigen-presenting capacity of these cells can participate in adaptive immunity, an important contributor to the progression of atherosclerotic lesions.18 Yet, the role of T cells in AT remains poorly explored. A previous study reported a greater number of T cells in white AT of mice fed a high-fat diet,19 but the operation of Th1 cytokines such as IFNγ in the AT inflammatory network remains unknown.

The present study examined the participation of T cells, particularly of the signature Th1 cytokine IFNγ, in the setting of fat inflammation. In vitro data show that IFNγ modulates...
important functions of adipocytes. Our in vivo results affirm that IFNγ regulates the inflammatory response in obese AT and establish a novel mechanism by which mediators of adaptive immunity can contribute to the complications of obesity.

Materials and Methods

Diet-Induced Obesity in Mice

Male C57BL/6 (Taconics) and IFNγ-deficient (IFNγ−/−) (The Jackson Laboratory) mice were fed ad libitum a standard low-fat (LF) diet (PicoLab Rodent Chow 5053; 13% kcal from fat) after weaning until 6 weeks old. Mice were then switched to high-fat (HF) diet (D12108 from Research Diets; 40% kcal from fat, 1.25% cholesterol, 0% cholate) and kept on this diet for 15 or 21 weeks. Lean controls were maintained on the LF diet throughout the experiment.

Apolipoprotein E–deficient (ApoE−/−) (The Jackson Laboratory) and IFNγ receptor (IFNγR)-deficient ApoE−/− (IFNγR−/−ApoE−/−) mice were switched from the LF to HF diet 8 weeks after weaning and kept on this diet for 8 weeks. The ApoE−/− and IFNγR−/−ApoE−/− animals used in the experiments all descended from the initial set of ApoE−/− and IFNγR−/−, in C57BL/6 and 129SvEv (129) genetic backgrounds, respectively. We crossed 129-IFNγR−/− and C57BL/6-ApoE−/− mice to generate F1 offspring heterozygous for both genes with a mixed 129/C57BL/6 background. From F1 interbreeding, we generated the 129/C57BL/6 F2 offspring. We subsequently crossed F2×F2 and obtained 129/C57BL/6-ApoE−/− and 129/C57BL/6-IFNγR−/−ApoE−/−. In our experiments, we used ApoE−/− and IFNγR−/−ApoE−/− animals from the same generation (F3) of siblings in an attempt to homogenize the genetic backgrounds. All genotyping was performed by PCR of DNA extracted from mouse tails.

On the day of harvesting, mice were anesthetized with 2,2,2-tribromoethanol (2.5 mg/10g body weight [BW]) injected IP and received heparin IP. All experiments involving animals were performed according to a protocol approved by the Standing Committee on Animals of the Harvard Medical School.

Analysis of AT-Derived Stromal Vascular Cells by Flow Cytometry

Peripinidylamyld fat from lean and obese C57BL/6 mice (15 weeks of LF or HF diet, respectively) was digested as described in the online data supplement, available at http://circres.ahajournals.org. AT-derived stromal vascular cells were washed with DMEM supplemented with 10% FCS, counted, labeled with conjugated antibodies for F4/80 (Caltag), CD3 (eBioscience), CD4, CD8, CD11c, and B220 (BD Pharmingen), and analyzed with a FACScan.

Analysis of Inflammatory Cells in AT by Immunohistochemistry

Peripinidylamyld AT (from mice on LF or HF diet for 21 weeks) was fixed as described previously and embedded in paraffin. Section staining was performed for rat antimonie CD45, Mac-3, I-Aβ (BD Pharmingen), and CD3 (Abcam) as described in the online data supplement. Positive cells were counted in 10 consecutive visual fields at the same magnification.

Intracellular Staining of AT-Derived Stromal Vascular Cells

Equal numbers of stromal vascular cells from lean and obese mice were incubated at 37°C in media with 10 μg/mL brefeldin A (BD Biosciences) with or without 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma) and 1 μg/mL ionomycin (Sigma). After 4 hours of incubation, cells were labeled with conjugated anti-CD3 (eBioscience), fixed, and permeabilized with BD Cytotoxic/Cytoperm kit (BD Biosciences), labeled with conjugated anti-IFNγ (BD Pharmingen), and analyzed with a FACScan.

Culture and Differentiation of 3T3-L1 Cells

Murine 3T3-L1 preadipocytes were cultivated and differentiated for 11 days as described in the online data supplement. At day 11, cells were stimulated with recombinant mouse IFNγ (Chemicon) at 10, 50, or 100 U/mL and harvested 24 hours after stimulation.

Organ Culture

Peripinidylamyld AT (350 mg) was extracted from C57BL/6 animals on the HF diet, minced, and incubated with media alone or media containing 100 U/mL murine recombinant IFNγ. Media were collected after 6 hours of incubation at 37°C.

Effects of T Cells on Mature Adipocytes

Spleenic CD4+ T cells were positively selected from C57BL/6 male mice and cultured and activated in vitro as described in the online data supplement. After 7 days, conditioned media were used to stimulate differentiated 3T3-L1 cells for 24 hours in the presence or absence of a neutralizing anti-IFNγ antibody at 10 μg/mL.

Cytokine and Metabolic Determinations

IFNγ-inducible protein 10 (IP-10), MIG (monokine induced by IFNγ), and adiponectin were measured using Quantikine ELISA kits (R&D Systems). Plasma levels of leptin were also measured with an ELISA kit (Crystal Chem). MCP-1, TNFα, interleukin (IL)-6, IL-2, and IL-12 were measured by BD Cytometric Bead Array (BD Biosciences). Total plasma cholesterol, triglycerides, and glucose were determined by enzymatic colorimetric methods (Wako).

For glucose-tolerance tests, mice were deprived of food for 12 hours and then injected IP with glucose (1 mg/g of BW). Blood from the tail vein was used for glucose measurement by a blood glucose meter (OneTouch Ultra, LifeScan) at time 0 and 20, 40, 60, 90, and 120 minutes after glucose administration.

Microarray Analysis

Differentially 3T3-L1 cells were treated or not with 100 U/mL IFNγ for 4 and 24 hours. Total RNA was isolated from 5 to 6 different plates with an RNeasy Mini Kit (Qiagen) and used (10 μg) for microarray screening on Mouse Expression Array 430 2.0 Affymetrix chip. Data were captured using the Affymetrix GeneChip Laboratory Information Management System. Criteria for differential regulation by IFNγ treatment were set as t-statistic >2 at a probability value of <0.05. Genes with chemotactic activity were clustered by dChip application and ranked according to their probability values at 24 hours.

Quantification of Gene Expression by Reverse Transcription–Quantitative PCR

Total RNA was isolated from up to 500 mg of AT with RNeasy Lipid Tissue Mini Kit (Qiagen), and equal amounts were reverse-transcribed by Superscript II (Invitrogen) according to the instructions of the manufacturer. Quantitative PCR was performed in a MyQ Single-Color Real-Time PCR Detection System (Bio-Rad). The sequences of mouse primers used are described in the online data supplement. The mRNA levels of the various genes tested were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as an internal control in all experiments.

Results

AT From Obese Mice Contains More T Cells Than That From Their Lean Counterparts

As expected, C57BL/6 mice that consumed a HF diet had higher BW and more peripinidylamyld AT than controls (mean BW after 15 weeks of LF or HF diet: 32.7 g versus 46.3 g, respectively; after 21 weeks: 31.7 g versus 44.4 g, respectively; P<0.01 in both time points). In accord with previous studies, white AT from obese animals contained more macrophages (F4/80-positive cells) than that from lean ones, as
determined by flow cytometry (Figure 1, I). AT from obese animals also contained more T lymphocytes bearing CD3, a pan–T-cell marker (Figure 1, I), including cells of both CD4 and CD8 T-cell subsets (Figure 1, II). The obese group also tended to have more cells bearing CD11c, a dendritic cell marker, but the difference did not reach statistical significance (Figure 1, III). B-cell numbers did not differ between the 2 groups (Figure 1, III).

Quantitative analysis of inflammatory cells by immunohistochemistry in C57BL/6 mice after 21 weeks of HF or LF diet yielded findings compatible with those of flow cytometry. AT from obese mice had more cells bearing CD45 (a pan-
leukocyte marker), Mac-3 (macrophage marker), or CD3 than AT from lean congenic mice (Figure 1A through 1M). The AT of the obese group also contained more cells expressing I-Ab, a mouse major histocompatibility class II antigen (Figure 1N through 1Q), indicating a greater state of immune activation in fat from obese compared to lean mice.

**IFNγ Expression in AT Increases with Diet-Induced Obesity in Mice**

Extracts of AT from obese animals had higher levels of IFNγ mRNA than lean controls after 21 weeks of the HF or LF diet, respectively (Figure 2A). AT stromal cells isolated from lean and obese mice had negligible intracellular levels of IFNγ protein, as shown by flow cytometry after brefeldin A treatment to block secretion. However, after stimulation with PMA and ionomycin, T cells from AT of obese animals produced significantly more IFNγ than those from lean controls (Figure 2B and 2C). The number of other IFNγ-producing cells did not differ between the 2 groups of mice.

**IFNγ Stimulates the Expression of Chemokines by 3T3-L1 Cells In Vitro**

The presence of T cells and IFNγ in AT suggested the operation of T-cell chemoattractants. Our prior studies showed the presence of T cell–tropic chemokines in atherosclerotic tissue. We therefore tested whether differentiated 3T3-L1 adipocytes could produce such mediators in vitro. Supernatants of activated murine CD4 cells stimulated expression of the prototypical T-cell chemoattractant IP-10 from differentiated 3T3-L1 cells (Figure 3A). Blocking antibodies against IFNγ limited IP-10 release from the 3T3-L1 adipocytes (Figure 3A). Adipocytic 3T3-L1 cells stimulated with murine recombinant IFNγ, even at levels as low as 10 U/mL, elaborated significantly increased levels of the T-cell chemoattractants IP-10 and MIG, as well as the monocyte chemoattractant MCP-1 (Figure 3B). IL-6, IL-10, IL-12, and TNFα levels changed little or not at all after IFNγ stimulation of 3T3-L1 adipocytes (data not shown). These results suggested that IFNγ regulates inflammatory gene expression.
selectively in adipocytes. Therefore, we undertook a broader analysis of the effects of IFN\(\gamma\) on gene expression in adipocytic cells by transcriptional profiling of 3T3-L1 cells treated with 100 U/mL IFN\(\gamma\) for 4 and 24 hours. Compared to controls, the IFN\(\gamma\)-stimulated adipocytes significantly increased the production of several chemokines from both the CC and CXC chemokine families (Figure 3C and Table I in the online data supplement). Consistent with our previous results, IFN\(\gamma\) stimulation significantly increased expression not only of the T-cell chemoattractants MIG, IP-10, and I-TAC (IFN-inducible T-cell chemotactic) but also of MCP-1, MCP-2, and RANTES (regulated on activation, normal T cell expressed and secreted).

Similar to the results of 3T3-L1 stimulation with IFN\(\gamma\), incubation of mouse perigonadal AT with this cytokine in culture significantly increased the secretion of IP-10 and MIG (Figure 4A and 4B). Interestingly, media from IFN\(\gamma\)-treated AT had higher TNF\(\alpha\) levels than untreated controls (Figure 4C), in contrast to the experiments with differentiated 3T3-L1. These different results most probably occur because AT contains not only adipocytes but also inflammatory cells such as macrophages, considered the most important source of TNF\(\alpha\) in the AT.

IFN\(\gamma\) Deficiency in Mice Limits Inflammatory Gene Expression in AT and Improves Insulin Sensitivity In Vivo

In vivo studies sought correlates of the in vitro effects described above. C57BL/6 and IFN\(\gamma\)-deficient male mice consumed a LF or HF diet for 15 weeks. The groups on the HF diet had significantly higher BW than those on LF diet, but weight did not differ between wild-type (WT) and IFN\(\gamma\)-deficient animals on the same diet (Figure 5A). Mice on the HF diet had heavier visceral fat pads than those consuming chow (Figure 5B). Interestingly, within the lean group, the IFN\(\gamma\)-deficient animals had significantly smaller peripididymal fat pads than controls.
IFNγ-deficient and WT animals consuming chow diet had similar glucose tolerance curves (Figure 5C). On HF diet, however, IFNγ-deficient animals had better glucose tolerance than controls (Figure 5D through 5E).

As demonstrated by others, diet-induced obesity yielded increased expression of inflammatory genes in AT. For example, mRNAs encoding CD68, a macrophage marker, and TNFα increased in AT of obese WT mice compared to lean controls, as previously shown. Interestingly, the IFNγ-deficient obese group had lower expression of these messages compared to the obese WT group (Figure 6A and 6B), despite their similar BWs. The obese IFNγ-deficient animals had significantly reduced expression of chemokines such as MCP-1 (Figure 6C) compared to obese controls. These animals also had decreased levels of RANTES (Figure 6D), a cytokine that can exert antiinflammatory actions. This observation raises the possibility of a counterregulatory mechanism also operating in obesity. T regulatory cells (T regs) comprise a major source of IL-10 in several inflammatory conditions, including atherosclerosis.

RNA levels of FoxP3, a forkhead family transcription factor and a marker for T regs, did not differ between obese IFNγ-deficient and obese WT animals or between either of these groups and lean WT controls (data not shown), suggesting that local recruitment of T regs does not play a role in the IL-10 increase in obese AT.

STAT (signal transducer and activation of transcription) proteins can regulate transcription in inflammation and host defense. STAT-1, in particular, mediates the effects of IFNs. As expected, our transcriptional profiling experiments showed increased expression of this transcription factor in IFNγ-stimulated adipocytes (data not shown). The AT of obese WT animals also had increased STAT-1 mRNA levels compared to that from lean mice. AT from obese IFNγ-deficient mice, however, did not show elevated levels of STAT-1 mRNA (Figure 6F).

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Figure 5. IFNγ deficiency does not change total body weight but improves glucose tolerance (GT) in obese mice. A and B, Bars represent average numbers of each group, and differences were calculated by Student’s t test. C and D, Average GT curves from both groups under LF diet (C) and from groups under HF diet (D). Area under the GT curve was calculated for each mouse, and the average of each group represented in E. Differences between groups were calculated by Student’s t test. *P≤0.05 vs WT/LF; #P≤0.05 (n=5 to 6).
IFNγ Deficiency Changed Leptin and Total Cholesterol Levels

The various groups of animals had similar plasma levels of adiponectin, except the IFNγ-deficient animals consuming a chow diet, which had lower adiponectin levels than the others (supplemental Figure IA). As expected, the obese mice had significantly higher leptin levels than their lean counterparts. Interestingly, both lean and obese IFNγ-deficient groups had lower plasma leptin levels than WT controls (supplemental Figure IB). Both obese groups had higher plasma total cholesterol levels than lean mice. Although WT and IFNγ-deficient animals had similar total cholesterol levels on a chow diet, obese IFNγ-deficient mice had lower cholesterol levels than WT controls (supplemental Figure IC).

IFNγ Deficiency Reduces Inflammatory Cell Accumulation in White AT

Macrophage infiltration indicates local inflammation in white AT. We, therefore, sought in vivo evidence for a net proinflammatory effect of IFNγ in AT by assessing its macrophage content. Lean animals from both WT and IFNγ-deficient groups had similar low numbers of Mac-3-positive cells in their white AT (Figure 7A and 7B). Obese mice, as demonstrated before, had more macrophages in their AT than the lean ones, but fat from the IFNγ-deficient group showed fewer of these cells than obese controls (Figure 7C and 7D).

Infiltrating inflammatory cells in obese white AT typically assume a crown-like distribution, arrayed around adipocytes. Interestingly, AT from the obese IFNγ-deficient group not only had significantly fewer isolated macrophages than the obese WT group, but also fewer “crown” formations (supplemental Figure II).

IFNγ Deficiency Regulates Visceral Fat Inflammation and Affects Lipid and Glucose Metabolism in Obese ApoE−/− Mice

Abundant evidence links inflammation to atherosclerosis. The foregoing findings favoring a role for IFNγ in the fat inflammatory network in C57BL/6 animals stimulated further exploration of the effects of impaired IFNγ signaling in AT of atherosclerosis-prone mice. Others have analyzed atherosclerotic lesions in mice with impaired IFNγ signaling.26 Mice doubly deficient for IFNγ receptor and ApoE (IFNγR−/−-ApoE−/−) and their matching ApoE−/− controls consumed a HF diet. After 2 months, visceral AT of IFNγR−/−ApoE−/− had lower levels of mRNAs encoding various inflammatory genes compared to single-deficient ApoE−/− controls (Figure 8), despite their similar BWs (data not shown). The visceral fat of those animals had reduced levels of IP-10, MIG, I-Aγ, RANTES, and C-X-C receptor-3 (CXCR3) mRNA (Figure 8A through 8E), each encoding a mediator with important roles in the adaptive immune response of atherosclerosis. The decreased expression of CD3 (Figure 8F) agreed with these findings, indicating a reduced T-cell content in AT of doubly deficient animals compared to controls. Levels of MCP-1, CD68, and TNFa mRNA, on the other hand, did not decrease in the doubly deficient animals compared to controls (data not shown), in contrast to the reduced expression of these genes in obese nonatherosclerotic IFNγ-deficient animals described above. Absence of IFNγ signaling in obese ApoE−/− animals also affected lipid and glucose metabolism. Although the 2 groups had the same total cholesterol levels (supplemental Figure IIIA), IFNγR-deficient animals had significantly reduced plasma triglycerides (supplemental Figure IIIB) and glucose (supplemental Figure IIIIC) in the fed state.
Discussion

Our results demonstrated the importance of the prototypical Th1 cytokine IFN-γ in white AT inflammation in mice with diet-induced obesity. In agreement with a previous study, our work showed that T cells, both CD4 and CD8 subtypes, exist in greater numbers in AT of diet-induced obese mice than in lean controls. Immunolocalization generally showed T lymphocytes in clusters with other T cells and macrophages, forming crown-like structures around adipocytes.

Beyond their mere presence, the magnitude of I-Ab expression by antigen-presenting cells such as dendritic cells and macrophages indicates their level of immune activation. Encountering antigen-presenting cells bearing class II major histocompatibility antigens can trigger adaptive immune responses mediated by T cells. The overall increase of I-Ab expression in visceral AT of obese, but not lean mice, observed here indicates local T-cell activation and operation of IFN-γ, an important regulator of I-Aβ.

Macrophages and dendritic-like cells in AT have recently received considerable attention. Yet, the participation of T cells and their cytokines in obesity and its metabolic consequences remains poorly explored. The local predomi-

Figure 7. IFN-γ deficiency limits inflammatory cell accumulation in obese visceral AT. Fixed and paraffin-embedded AT was stained with anti–Mac-3 antibody, and positive cells were counted in 10 consecutive fields in each slide. A representative image from each group is shown (A through D). Numbers from each group were plotted in E. Differences were calculated by Student’s t test. #P<0.05 vs WT/LF; *P<0.05 (n=5 to 6).

Figure 8. IFN-γ deficiency regulates inflammation in visceral fat of obese ApoE−/− mice. mRNA levels of IP-10, MIG, I-Aβ, RANTES, CXCR3, and CD3 (A through F) in peripipidimal AT were quantified by RT-qPCR and normalized to GAPDH. Fold change was calculated relative to ApoE−/−. *P<0.05 vs ApoE−/−; **P<0.01 vs ApoE−/− (n=5 to 8).
nance of Th1 over Th2 cytokines in other chronic inflammatory conditions such as atherosclerosis\(^\text{12}\) focused our attention on IFN\(\gamma\), a Th1 prototype. Indeed, IFN\(\gamma\) mRNA expression in AT increases in diet-induced obese animals fed a HF diet for 21 weeks compared to lean controls. Interestingly, T cells extracted from fat tissue of obese mice and stimulated in vitro also produced higher amounts of this cytokine than those extracted from lean animals. This result suggests that obesity primes T cells from AT toward a Th1 slant.

IFN\(\gamma\) participates in the progression of atherosclerotic plaque, where it affects importantly all local cell types: endothelial cells, smooth muscle cells, and macrophages.\(^\text{12}\) IFN\(\gamma\) might also play an important role in growing AT, where adipocytes respond to inflammatory products derived from infiltrating macrophages such as TNF\(\alpha\).\(^\text{7}\) Indeed, when stimulated by IFN\(\gamma\), differentiated 3T3-L1 cells secrete various inflammatory mediators, including the T-cell chemoattractants IP-10 and MIG. These results suggest the potential of a positive-feedback loop that amplifies T-cell recruitment to AT during obesity. Our study shows that although quantitatively less prominent than macrophages, T cells may deci-

sion of genes with established roles in T-cell chemotaxis and signaling in those animals significantly reduced the expres-

sion of inflammatory genes, including chemokines and cytokines. The reduced accumulation of leukocytes in AT of IFN\(\gamma\)-deficient animals affirmed the functional consequences in vivo of the increased mRNA levels of these proinflammatory cytokines mediated by IFN\(\gamma\). Interestingly, obese IFN\(\gamma\)-deficient mice also had lower plasma levels of leptin and total cholesterol and had greater glucose tolerance, supporting the systemic consequences of altered adaptive immunity in obesity.

The significant suppression of several inflammatory genes in visceral fat tissue of IFN\(\gamma\)R\(^{-/-}\)ApoE\(^{-/-}\) mice compared to ApoE\(^{-/-}\) agreed with these results. The disruption of IFN\(\gamma\) signaling in those animals significantly reduced the expression of genes with established roles in T-cell chemotaxis and adaptive immune response in atherosclerosis, such as IP-10,\(^\text{22}\) MIG,\(^\text{22}\) RANTES,\(^\text{28,29}\) and I-A\(^\text{b}\).\(^\text{12,13}\) Importantly, IFN\(\gamma\) stimulation augmented expression of all these genes in differentiated adipocytes in our microarray data, again suggesting an interaction between the Th1 arm of the immune response and adipocytes. IFN\(\gamma\)R\(^{-/-}\)ApoE\(^{-/-}\) also had reduced CD3 content in visceral AT compared to ApoE\(^{-/-}\), supporting the operation of an autocrine loop where T cells can perpetuate their own presence in AT through IFN\(\gamma\). The suppressed inflammatory response in visceral AT of doubly deficient mice relative to their controls may also strengthen the link between fat inflammation and atherosclerosis development, because a previous study already demonstrated reduced atherosclerotic lesion size associated with IFN\(\gamma\) receptor deficiency in ApoE null mice.\(^\text{26}\) The abrogation of IFN\(\gamma\) signaling in obese ApoE\(^{-/-}\) mice related to changes in lipid and glucose metabolism, as discussed with C57BL/6 mice. The ApoE\(^{-/-}\) and IFN\(\gamma\)R\(^{-/-}\)ApoE\(^{-/-}\) animals used in our experiments were all F3 siblings derived from the offspring of a single cross between the original pair of 129-IFN\(\gamma\)R\(^{-/-}\) and C57BL/6-ApoE\(^{-/-}\). However, we cannot exclude the possibility of differences in the mixed (129/C57BL/6) genetic background of the final mice and rule out the influence of this possible heterogeneity on our findings.

Previous animal and human studies corroborate the aforementioned effects of IFN\(\gamma\) on the endocrine system.\(^\text{30,31}\) Interestingly, previous in vitro observations supported a direct stimulatory effect of IFN\(\gamma\) on lipolysis and increased supply of nonesterified fatty acids to the liver.\(^\text{32,33}\) The importance of this cytokine in host defenses and its influence on fuel mobilization bear evolutionary plausibility and reaffirm the link between inflammatory responses and metabolic disturbances also observed with other cytokines, such as TNF\(\alpha\).

Recognized for decades as pathogenic partners in other chronic inflammatory diseases including atherosclerosis, our results point to macrophage/T-cell cooperation in obesity, another long-term inflammatory condition. The I-A\(^\text{b}\) positivity of macrophages in AT supports crosstalk between these prototypic cells of the innate and adaptive immune responses.\(^\text{12,13}\) In addition to the known interaction between infiltrating macrophages and adipocytes within the obese AT, T cells also appear to participate in this network. T cells do not exclusively produce IFN\(\gamma\), despite its classification as a classic Th1 cytokine. Further studies, such as conditional disruption of the IFN\(\gamma\) gene in T cells, would be necessary to precisely establish the source of this cytokine in the AT environment.

Although not previously considered a Th1-associated condition, our present data establish that the key cytokine secreted by this T-cell subset, IFN\(\gamma\), can promote inflammation in fat tissue. These results highlight the importance of further exploration of adaptive immunity and the role of T cells and their products in obesity.
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Disclosures

None.

References

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Expanded Materials and Methods

Isolation of AT-derived SVCs and flow cytometry

One gram of peri-epididymal fat from lean and obese C57BL/6J mice (15 weeks of LF or HF diet, respectively) was minced in PBS containing 2% bovine serum albumin (BSA) and 250 U/ml of collagenase type II (Worthington) and incubated at 37°C for 1 h. The digested tissue was passed through a 70-μm cell strainer (BD Biosciences) and the flow-through centrifuged. After aspirating the supernatant, red blood cells were lysed with ACK lysing buffer (Gibco). The remaining cells were washed with DMEM supplemented with 10% FCS, counted and labeled with conjugated antibodies or their respective isotype controls before acquisition by a FACScan.

Analysis of inflammatory cells in AT by immunohistochemistry

Peri-epididymal AT (from mice on LF or HF diet for 21 weeks) was fixed in periodate-lysine-paraformaldehyde fixative as described previously (see reference in the text), and embedded in paraffin. Five-micron sections were stained for rat anti-mouse CD45, Mac-3, I-A^b (BD Pharmingen) and CD3 (Abcam), and then incubated with appropriate biotinylated secondary antibodies followed by incubation with avidin-biotin complex (Vector). Next, the reaction was visualized with 3-amino-9-ethyl carbazole (DAKO). Sections were counterstained with Gill’s hematoxylin solution (Sigma). Positive cells were counted in 10 consecutive visual fields at the same magnification.
**Culture and differentiation of 3T3-L1 cells**

Murine 3T3-L1 pre-adipocytes were cultivated in DMEM high glucose supplemented with 10% FCS. After reaching confluency (day 0), cells were stimulated with DMEM containing 10% FCS, 0.5 mmol/L 3-Isobutyl-1-methylxanthine (Sigma), 1 μmol/L dexamethasone (Sigma), and 10 μg/ml of porcine insulin (Sigma), to induce differentiation. At day 2, media were replaced by DMEM with 10% FCS and 10 μg/ml of insulin, and changed every 48 h. At day 11, cells were stimulated with recombinant mouse IFNγ (Chemicon) at 10, 50, or 100 U/ml, and harvested 24 h after stimulation.

**Culture and activation of T cells in vitro**

Splenic CD4+ T cells were positively selected from C57BL/6J male mice and cultured in vitro with 2 μg/ml of anti-CD28 (Bioexpress) and 10 ng/ml of recombinant mouse IL-12 (R&D Systems) in a plate coated with 5 μg/ml of anti-CD3 (BD Pharmingen). After 48 h of incubation at 37°C, cells were transferred to fresh plates and incubated with 10 U/ml of recombinant mouse IL-2 (R&D Systems) for 72 h. Cells were then washed and again incubated in a plate coated with anti-CD3. After 48 h, conditioned media were used to stimulate differentiated 3T3-L1 cells for 24 h in the presence or absence of a neutralizing anti-IFNγ antibody at 10 μg/ml.

**Sequences of mouse primers**
TNFα, 5’-CTGTAGCCACGTCGAGC-3’ and 5’-TTGAGATCCATGGCGTTG-3’; CD68, 5’-CTCCTCTAAGGCTACAGGCTACAGGCTGCTG-3’ and 5’-TCACGGTTGCAAGAGAAAACA-3’; MCP-1, 5’-GGCTGGAGAGCTACAAGAGG-3’ and 5’-TCTTGAGCTTGGTGACAAAAAC-3’; RANTES, 5’-AGCAGCAAGGTCTCCAATC-3’ and 5’-GGGAAGCGTATACAGGGTC-3’; IL-10, 5’-ACTGCACCCACTTCCCCAGT-3’ and 5’-TGTCGACCTGTCCTTTGTT-3’; STAT-1, 5’-TGAGATGTCCCGGATAGTGG-3’ and 5’-CGCCAGAGAGAAATTTCGTG-3’; IFNγ, 5’-TCTGGAGGAACTGGCAAAAG-3’ and 5’-TTCAAGACTTCCAAGAGTCTGAGG-3’; IP-10, 5’-GCTGCCGTCATTTCGTC-3’ and 5’-TCTCAGTGGCCCCTGCATC-3’; MIG, 5’-CTTTTCCTTTTGGGCATCAT-3’ and 5’-GCATCGTGCATTCTTATCA-3’; CXCR3, 5’-GCCAAGCCATGTACCTTGAG-3’ and 5’-GGAGGGTGCTTGTTTCCAG-3’; I-Ab, 5’-GTGGTGCTGATGGTGCTG-3’ and 5’-CCATGAACACTGGACTGAGAATG-3’; CD3, 5’-TCTCCACCCAGACTGAGC-3’ and 5’-GCATGTCTCTCCATCTTAGAAG-3’; GAPDH, 5’-TGGGTGTGACCATGAGAAG-3’ and 5’-GCTTGGTGTGACCATGAGAAG-3’.

**Supplementary figure 1. Plasma levels of adiponectin, leptin, and total cholesterol.**

Values representing the animals individually and the average in each group are plotted for each measurement. WT/LF, wild type mice/low fat diet; IFNγ-/-LF, IFNγ-deficient mice/low fat diet; WT/HF, wild type mice/high fat diet; IFNγ-/-HF,
IFN-γ-deficient mice/high fat diet. §p<0.05 relative to WT/LF; #p<0.05; n=5-6 in each group.

**Supplementary figure 2. IFNγ deficiency limits the number of crown-like formations in obese visceral AT.**

Peri-epididymal AT was fixed and paraffin-embedded. Sections were stained with anti-CD45 antibody, and “crowns” were counted in 10 consecutive fields in each slide. One crown is the result of positive cells around one single adipocyte. A representative picture from each group is shown (A-D). Numbers from each group were plotted in the graph (E). Differences were calculated by Student’s t test. WT/LF, wild type mice/low fat diet; IFNγ−/−LF, IFN-γ-deficient mice/low fat diet; WT/HF, wild type mice/high fat diet; IFNγ−/−HF, IFN-γ-deficient mice/high fat diet; *p<0.05; n=5-6 in each group.

**Supplementary figure 3. Plasma levels of total cholesterol, triglycerides, and glucose in ApoE−/− and IFNγR−/−ApoE−/−**

Values representing the animals individually and the average in each group are plotted for each measurement. ApoE−/−, apolipoprotein E-deficient mice; IFNγR−/−ApoE−/−, IFNγ-receptor-deficient and ApoE-deficient mice. *p<0.01 vs ApoE−/−; n=9 in each group.
Supplementary table 1. Transcription profiling study
Differentiated 3T3-L1 cells were stimulated with 100 U/ml of recombinant mouse IFNγ or left untreated (controls). After 4 and 24 h, control and treated cells were harvested and mRNA was extracted and used in a microarray screening. The table shows the CC and CXC chemokines and receptors that significantly changed compared to controls, ranked by their p value at 24 h. n=5 for each group at 4 h, and n=6 for each group at 24 h.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Other denominations</th>
<th>4h paired t statistic</th>
<th>4h paired p value</th>
<th>24h paired t statistic</th>
<th>24h paired p value</th>
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<tr>
<td>Chemokine (C-X-C motif) ligand 9</td>
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</table>
Supplementary figure 2

A  
WT/LF

B  
IFN\(_{\gamma}\)/LF

C  
WT/HF

D  
IFN\(_{\gamma}\)/HF

Supplementary figure 2

WT/LF

IFN\(_{\gamma}\)/LF

WT/HF

IFN\(_{\gamma}\)/HF

CD45-positive crowns/10 fields

A B

C D

0 10 20 30 40 50 60

CD45-positive crowns/10 fields

WT LF IFN\(_{\gamma}\)/LF WT HF IFN\(_{\gamma}\)/HF

*