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Fat T_{reg} cells: a liaison between the immune and metabolic systems

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Abstract

Obesity is accompanied by chronic, low-grade inflammation of adipose tissue, which promotes insulin resistance and type-2 diabetes. How does fat inflammation escape the powerful armamentarium of cells and molecules normally responsible for guarding against a run-away immune response? Regulatory CD4$^+$ T cells expressing the transcription factor Foxp3 (termed T_{reg} cells) are a lymphocyte lineage specialized in controlling immunologic reactivity. T_{reg} cells with a unique phenotype were highly enriched in the abdominal fat of normal mice, but were strikingly and specifically reduced at this site in insulin-resistant models of obesity. In loss-of-function and gain-of-function experiments, T_{reg} cells regulated the inflammatory state of adipose tissue and insulin resistance. Cytokines differentially synthesized by fat-resident regulatory and conventional T cells directly impacted on the synthesis of inflammatory mediators and glucose uptake by cultured adipocytes. These findings open the door to harnessing the anti-inflammatory properties of T_{reg} cells to inhibit elements of the metabolic syndrome.

INTRODUCTION

Type-2 diabetes and other elements of the metabolic syndrome have increased at an alarming rate over the past several decades. There has been a parallel augmentation in obesity, now recognized to be a major contributor to the insulin resistance underlying this spectrum of metabolic abnormalities [1]. Just how obesity promotes insulin resistance is not known, but results from clinical, epidemiological and molecular studies have converged to highlight the role of inflammation.

Prolonged nutrient overload results in a state of chronic, low-grade inflammation in adipose tissue [2] and systemically [3], in particular in visceral (abdominal) fat depots [4]. Visceral fat produces a number of inflammatory cytokines and chemokines [leptin, tumor necrosis factor

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(TNF)-α, macrophage chemoattractant protein (MCP)-1, interleukin (IL)-6, etc], whose production can be elevated and expression dysregulated in the obese state, contributing importantly to insulin resistance [5].

At the cellular level, macrophages play a key role. With increasing obesity, they accumulate in visceral fat tissue, sometimes contributing as much as half of the cellularity [6, 7]. The increase in number is accompanied by an evolution in phenotype from the anti-inflammatory ("alternatively" activated, M2) form to the pro-inflammatory ("classically activated", M1) form [8, 9], which correlates with an increase in insulin resistance (e.g. [10]). In the obese state, macrophages and adipocytes make several of the same regulators and mediators, including TNF-α, IL-6, matrix metalloproteinases (MMPs), peroxisome proliferator activated receptor (PPAR)-γ and fatty acid binding protein (FABP)-4 (reviewed in [1, 11]). These findings, along with others, suggest that adipocytes and adipose tissue macrophages (ATMs) may contribute to insulin resistance in concert, both inhibiting and enhancing each other's activities [11].

Like any inflammatory state, the chronic, low-grade inflammation associated with obesity should be subject to the control mechanisms that normally rein in over-exuberant immune responses. These mechanisms encompass a battery of cell-types, which can operate through cell-cell contact via a variety of receptors, or through a diversity of soluble mediators. Cells with a potentially regulatory phenotype have previously been associated with obesity [e.g. natural killer (NK)T cells] [12], and there have been prior reports of anti-inflammatory cytokines being detected in adipose tissue [e.g. IL-10 and transforming growth factor (TGF)-β] [8, 9]. Yet, research on this topic has been very limited, and the influence – or loss of influence – of important control mechanisms remain unaddressed. For example, the role of arguably the most important regulatory cell population, $CD4^+Foxp3^+$ T_{reg} cells, begs to be explored.

A small subset of T lymphocytes, normally constituting only 5–20% of the $CD4^+$ compartment, T_{reg} cells are thought to be one of the body's most critical defenses against inappropriate immune responses – operating in contexts of autoimmunity, allergy, inflammation, infection and tumorigenesis [13, 14]. Typically, they control the behavior of other T cell populations, but can also influence the activities of innate immune system cells [15–17]. T_{reg} cells are quite specifically characterized by high-level expression of the forkhead/winged-helix transcription factor, Foxp3. Deficiencies in this factor cause the lymphoproliferation and multi-organ autoimmunity found in *scurfy* mutant mice and human IPEX patients.

Here, we examined the T_{reg} cells residing in murine adipose tissue, both their representation and phenotype, comparing visceral versus subcutaneous fat depots and lean versus obese mice. Their functional importance was evaluated in loss-of-function, gain-of-function and *in vitro* experiments. Lastly, an analogous population in human adipose tissue samples was sought. Our observations introduce an intriguing fat-resident T lymphocyte population whose products may have applications in treating the metabolic syndrome.

RESULTS

Adipose tissue T_{reg} cells

Adipose tissue is composed of multiple cell-types. Most prominent are adipocytes, but vascular endothelial cells, macrophages [6, 7] and lymphocytes [12, 18] are also found in the stromovascular fraction (SVF). According to multi-parameter flow cytometry, about 10% of SVF cells from the abdominal fat of ~30-week-old C57Bl/6 (B6) animals fell within the lymphocyte gate, close to half of which were of the $CD3^+$ T lineage, split 3:1 between the $CD4^+$ and $CD8^+$ compartments, respectively (Fig. 1a, upper panels). Surprisingly, more than

half of the CD4$^+$ T cells expressed Foxp3 (Fig. 1a, lower panels), a much higher fraction than that normally found in lymphoid [e.g. spleen, lymph node (LN)] or non-lymphoid (lung, liver) tissues (Fig. 1b), including in subcutaneous fat (Fig. 1c). The two types of adipose tissue had similar low levels of T$_{reg}$ cells at birth, with a progressive accumulation over time in the visceral, though not the subcutaneous, depot (Fig. 1c). The dichotomy between visceral and subcutaneous fat is potentially important given the association of the former and not the latter with insulin resistance [4, 19].

Immunohistological examination revealed Foxp3$^+$ cells in the spaces between adipocytes, mainly, but not only, in regions where several of them intersected (Fig. 1d, left panels). Fat tissue, especially from obese individuals, can host substantial numbers of macrophages, which accumulate in so-called "crown-like" structures, replete with dead-adipocyte residues [6, 7, 20]. T$_{reg}$ cells were observed in such structures, in close proximity to macrophages and other leukocyte aggregates (Fig. 1d, right panels).

We estimate that 15,000–20,000 Foxp3$^+$ cells reside in one gram of epididymal adipose tissue in an ~30-week-old B6 mouse. Given their known potency, this value very likely represents a biologically significant number – for example, transferring as few as 5,000–10,000 T$_{reg}$ cells can protect a mouse from autoimmune diabetes (and many of the cells do not even survive the transfer process) [21, 22].

A particular gene-expression profile

We wondered whether the CD25$^+$Foxp3$^+$ cells in abdominal adipose tissue were of typical T$_{reg}$ phenotype. They functioned as effectively as analogous cells isolated from the spleen when introduced into a standard *in vitro* suppression assay (Fig. 2a). [Fat T conventional (T$_{conv}$) cells also performed as expected, ie no suppressive activity and a normal proliferative response (Fig. 2a).] The lability and low recoverable numbers of fat T$_{reg}$ cells have so far prevented us from assaying their activities in *in vivo* suppressor assays. So we turned to the well-established transcriptional "T$_{reg}$ cell signature", derived from the data of multiple groups [23–26], as an indicator of *in vivo* function.

Clearly, the overall transcriptional profile of the T$_{reg}$ cell population from visceral fat differed from the patterns of its spleen and LN counterparts more than the latter two did from each other (Fig. 2b). This observation also held for the T$_{conv}$ cell populations at these sites, though not as strikingly so (Fig. 2c). Focusing specifically on the documented T$_{reg}$ cell signature [23–26], we found the spleen data to show an excellent recapitulation of its major features: as anticipated, most genes known to be up-regulated in T$_{reg}$ cells (red) descended to the right on the p-value vs fold-change (FC) "volcano" plot, while most down-regulated loci (blue) dropped to the left (Fig. 2d, left panel). Fully 93% of the signature was present. In contrast, evidenced by their position at the volcano summit, many of the signature T$_{reg}$ cell genes were not significantly up- or down-regulated in the corresponding population from visceral fat, e.g. CD103 and Gpr83 (Fig. 2d, center and right panels). The data on CD103 (and others) were confirmed by flow cytometric analysis (Fig. 3b). These observations on the T$_{reg}$ cell signature were true whether the comparator was T$_{conv}$ cells from the fat (Fig. 2d, center panel) or the LN (Fig. 2d, right panel), arguing that they reflect special features of adipose tissue T$_{reg}$ cells. Nonetheless, fat-resident CD4$^+$Foxp3$^+$ cells were clearly T$_{reg}$ cells, as much (63%) of the signature was intact, including over-expression of hallmark transcripts like those encoding CD25, GITR, CTLA-4, OX40 and KIrg1, in addition to Foxp3 itself. Confirmation of the elevated expression of several of these signature genes in fat T$_{reg}$ cells was obtained via RT-PCR and flow cytometric quantitation (Fig. 3a, and data not shown). The gene-expression differences observed in T$_{reg}$ cells isolated from the fat versus from the spleen and LN were not a simple reflection of different activation statuses, as a direct

comparison between fat-derived and activated T_{reg} cells showed clearly divergent transcription patterns (Suppl. Fig. 1)

A large set of genes was over-expressed, many of them strikingly so, by the CD4+Foxp3+ T cells residing in abdominal adipose tissue, while not by the corresponding population at other sites examined (highlighted in red on Fig. 2e; listed in Suppl. Table 1). Chief amongst these were loci encoding molecules involved in leukocyte migration and extravasation: Gm1960 (an IL-10-inducible CXCR2 ligand [27]), CCR1, CCR2, CCR9, CCL6, integrin alpha V, Alcam, CXCL2 and CXCL10 (Fig 2e; Suppl. Table 1; Suppl. Fig 2). On the other hand, some molecules of like function, eg CCL5 and CXCR3, were under-expressed in the visceral fat T_{reg} cells (Fig. 2e, left panel). Also remarkable were the extremely high IL-10 transcript levels in CD4+Foxp3+ abdominal adipose cells (Fig. 2e, left vs right panels; Suppl. Fig. 2). A 136-fold augmentation of IL-10 transcripts in fat vs LN T_{reg} cells was estimated from RT-PCR quantitation (Fig. 3c); the increase could also be detected by intracellular staining for IL-10 protein in the T_{reg} cells of fat versus spleen and lung (Fig. 3d). Interestingly, pathway analysis (Suppl. Fig. 3a) suggested that the T_{reg} cells not only produced large amounts of IL-10, but seemed also to be responding to it, as a number of genes downstream of the IL-10R were up-regulated in fat T_{reg} cells compared with LN T_{reg} cells.

Another set of genes was up-regulated specifically in CD4+Foxp3− T cells residing in adipose tissue *vis a vis* their LN counterparts, but not in spleen versus LN (indicated as blue in Fig. 2e; listed in Suppl. Table 1). Some of these loci also coded for molecules implicated in migration and extravasation, including CXCR3 and CCL5. Fat T_{conv} cells appeared to be highly polarized to a T_H1 phenotype as they expressed high levels of Tbet and IFN-γ transcripts (Fig. 2e, left panel; Fig. 3c; and Suppl. Fig. 2), abundant intracellular interferon (IFN)-γ and TNF-α (Fig. 3, d and e), and little if any intracellular IL-4 (Fig. 3d). While the IL-10 effect could also be discerned with fat T_{conv} cells, it was not as striking (Suppl. Fig. 3b and c).

A specific T cell receptor repertoire

The T cell receptor (TCR) repertoire represents another parameter for assessing the degree of similarity of T cell populations: for example, it has been shown that T_{reg} and T_{conv} cell populations have distinct repertoires, with only limited overlap [28–30]. In addition, the TCR repertoire of T_{reg} cells in the abdominal adipose tissue might give an indication of whether their abundance reflects an influx and/or retention of cells of a particular specificity or a local cytokine-induced conversion [31]. To render the repertoire analysis more manageable and interpretable, we exploited the Limited (LTD) mouse line, wherein TCR diversity is restricted to the complementary-determining region (CDR)3α via the combination of a transgenic *TCRα* minilocus and the *TCRα*-knockout mutation [32]. CDR3α sequences were determined from 98 individually sorted visceral fat CD4+CD25+ cells that also expressed Foxp3 RNA, and their distribution was compared with that of CDR3α sequences from fat T_{conv} cells or LN T_{reg} and T_{conv} cells. (Note: We were unable to obtain enough T_{reg} cells from subcutaneous fat to perform a parallel TCR sequence analysis on this depot.)

As expected, the "heat maps" generated from these sequences (Fig. 3f) revealed distinct TCR repertoires for the LN T_{reg} and T_{conv} cell populations, with only limited overlap. Similarly, the fat T_{reg} and T_{conv} cell populations also had different repertoires, rendering it very unlikely that the accumulation of Foxp3+ T_{reg} cells in the abdominal adipose tissue resulted from local conversion of T_{conv} cells. Interestingly, the fat T_{reg} cells had a very restricted distribution of sequences, representing a distinct subset of those normally found in their LN T_{reg} cell counterparts (Fig. 3f and Suppl. Table 2). The CDR3α sequences characteristic of fat T_{reg} cells were often independently generated by different nucleotide

sequences: 50% of sequences found more then three times per individual mouse (3/6) showed such nucleotide variation (Suppl. Table 3). In contrast, none of the fat T_{conv} cells (0/10) did (Suppl. Table 4), suggesting the repeated selection of T_{reg} cells with similar antigen receptors rather than the proliferation of a single clone. The sequences were reproducibly frequent in different mice, again pointing to TCR-driven selection (Suppl. Table 3). These data indicate that the specificity of the TCR may be instrumental in generating the high frequency of T_{reg} cells in visceral fat, perhaps through local recognition of cognate antigen.

Indeed, fat T_{reg} cells displayed unusually high levels of the early activation markers CD69 and Ly6c (Fig. 3g), although it remains possible that such increases instead, or also, reflect cytokine influences. Though TGF-β is readily detectable in adipose tissue [33], and is known to promote T_{reg} cell differentiation/survival [34–36], its effects are an unlikely explanation for the high representation and activation state of T_{reg} cells in fat because the typical changes in gene expression promoted by this growth factor were not observed in this population (Suppl. Fig. 4). For example, CD103 was not up-regulated (Fig. 2d, center and right panels; Fig. 3b). This observation also argues against TGF-β-mediated conversion of CD4$^+$Foxp3$^-$ to CD4$^+$Foxp3$^+$ cells in visceral fat, as has been observed in a few systems [31].

Implication in metabolic control

To learn how this peculiar population of T_{reg} cells responds to excess adiposity, we examined it in three mouse models of obesity: leptin-deficient mice (*Lep$^{ob/ob}$*; commonly referred to as ob/ob) [37], mice heterozygote for the yellow spontaneous mutation ($A^y/_a$) [38] and mice chronically fed a high-fat diet (HFD) [3], all on the B6 genetic background and all displaying insulin resistance (Suppl. Fig. 5). Strikingly, the T_{reg} cell population in abdominal fat was drastically reduced in old ob/ob mice, whether the fraction of T_{reg} cells in the CD4$^+$ compartment or the number of T_{reg} cells per gram of fat was quantitated (Fig. 4, a and b). While five-week-old leptin-deficient animals had somewhat higher (p=0.02) levels of CD4$^+$Foxp3$^+$ T cells in visceral fat (30%) than did wild-type age-matched littermates (10%), this subset progressively declined in the former case and rose in the latter (Fig. 4c) (p=0.0011). As anticipated, ob/ob fat T_{reg} cells were relatively impoverished in IL-10 producers (Suppl. Fig. 6a). The normal representations of T_{reg} cells in the spleen and subcutaneous fat of ob/ob mice (Fig. 4g) argue that the paucity of this subset in visceral fat was not just a reflection of the leptin defect; indeed, the absence of this adipokine was recently reported to foster the proliferation of Tregs [39]. Numbers of conventional CD4+ T cells in the abdominal fat of ob/ob adults were only mildly reduced, and the percentage of these cells making IFN-γ remained very similar (Suppl. Fig. 6, b and c).

There were also reduced levels of CD4$^+$Foxp3$^+$ cells in abdominal fat, but not at other sites, in the A^y/a mice and in HFD-fed animals (Fig. 4, d and e; h and i). The reductions were not as striking as for ob/ob animals, consistent with less insulin resistance in the latter two models (Suppl. Fig. 5). Indeed, we saw a good correlation between insulin resistance and the fraction of T_{reg} cells in abdominal fat (Fig. 4f).

The observed correlation between obesity and insulin resistance on the one hand and a dearth of CD4$^+$Foxp3$^+$ cells in abdominal adipose tissue on the other hand suggests that T_{reg} cells might be implicated in the relationship between the inflammatory and metabolic parameters. To test this notion, we first attempted loss-of-function experiments. Given that it is not currently feasible to ablate T_{reg} cells specifically in the fat, we employed mice expressing the diphtheria toxin (DT) receptor (R) under the control of the *Foxp3* transcriptional regulatory elements, wherein administration of DT results in punctual systemic depletion of T_{reg} cells. We recently developed a line of NOD BAC transgenic mice expressing a DTR-eGFP fusion protein under the dictates of *Foxp3* transcriptional

regulatory elements. Routinely, 85–90% of T_{reg} cells are eliminated in the spleen and LNs two days after DT administration to these animals (unpublished data), similar to what has been described by the Rudensky group with their independent line [40]. Cell death induced by DT is apoptotic, and therefore does not set off a pro-inflammatory immune response [41–44], prompting wide-spread use of this approach to probe diverse immunological issues through specific ablation of particular cell-types, including T_{reg} cells [41, 45–47]. Because T_{reg}-cell-ablated mice develop multi-organ autoimmunity 2 weeks later [40], this strategy required us to look at early indicators of potential T_{reg} cell function, namely alterations in adipose tissue mRNAs encoding inflammatory mediators or upstream changes in metabolic signaling pathways; previous data suggested that two weeks may be too early to see changes in more downstream metabolic parameters, including performance in glucose-tolerance tests (GTTs) [48].

For one set of experiments, we treated 10-week-old male mice with DT every other day for four days, which reduced the T_{reg} cell representation in abdominal fat to about 1/4 the normal (Fig. 5a, bottom panels), while the spleen and lung populations were at about 1/3 the usual (Fig. 5a, top panels and data not shown). The depletion of T_{reg} cells was accompanied by substantial decreases in insulin-stimulated insulin-receptor (IR) tyrosine phosphorylation in epididimal fat and liver, but not in muscle and spleen (Fig. 5, b and c). Parallel results were obtained on AKT phosphorylation (data not shown). At this early time-point, *in vivo* metabolic changes were marginal (not shown), so we conducted a second set of experiments in which mice were treated with DT for longer times. Mice injected with DT every other day for 9 days had a T_{reg} cell fraction about 30% the usual size in the fat, while the spleen, lung and LN populations had bounced back to about 70% the normal (Fig. 5d). [We do not know why there is such a preferential impoverishment of T_{reg} cells in adipose tissue with this protocol (which serves our purpose well) but it is likely to reflect slower repopulation kinetics.] Concomitantly, many of the genes encoding inflammatory mediators [e.g. TNF-α, IL-6, RANTES, Serum Amyloid A (SAA)-3] were induced in the visceral fat depot (Fig. 5e, upper panel), and much less so in the spleen and lung (Fig. 5e, lower panel). Insulin levels were significantly elevated in the T_{reg}-cell-depleted mice, demonstrating insulin resistance (Fig. 5f, upper panel), although fasting blood-glucose levels at this early time-point were unchanged, consistent with adequate β-cell compensation (Fig. 5f, lower panel).

To provide additional evidence that the inflammation promoted by T_{reg} cell depletion in the abdominal adipose tissue was not merely a response to cell death, we used mice [40] wherein most (95%) or only about half of the T_{reg} cells were punctually ablated by DT-treating females carrying a DTR gene knocked into the *Foxp3* locus on the X chromosome, in homozygous vs heterozygous state (Suppl. Fig. 7). In the latter case, random X-chromosome inactivation will protect about half of the T_{reg} cells because the DTR-carrying allele resides within the inactivated chromosome. As expected, when most of the T_{reg} cells were ablated, pro-inflammatory transcripts (eg RANTES, MCP-1) were strongly induced in the fat tissue. In contrast, when only half as many were killed, there was no such induction of pro-inflammatory transcripts, signifying that: 1) substantial DT-induced cell death was not in and of itself the stimulus, and 2) a demi-complement of T_{reg} cells sufficed to protect the fat from inflammation.

As concerns gain-of-function approaches, the lability and low recoverable numbers of visceral fat T_{reg} cells rendered unsuccessful our many attempts at standard adoptive transfer experiments; transfer of more limited numbers of fat T_{reg} cells into lymphodeficient recipients also proved problematic because the resultant homeostatic proliferation altered the phenotype of the introduced population, perhaps most relevantly its profile of cell-surface homing receptors (data not shown). Therefore, as an alternative means to achieve gain-of-function, we turned to *in situ* expansion of T_{reg} cells via injection of a particular

recombinant IL-2:anti-IL-2 monoclonal antibody (mAb) complex demonstrated to selectively elicit T_{reg} cells [49, 50]. Daily injections of the complex for 6 days into mice pre-fed on HFD for fifteen weeks did substantially increase the fraction of T_{reg} cells in the spleen and abdominal fat *vis a vis* PBS-injected controls (averages from multiple experiments 37+/− 4% vs. 21+/−2 % for spleen and 63+/−12% vs. 43+/−17% for abdominal fat) (Fig. 5g). [Note that the reduction in the T_{reg} cell fraction in PBS-injected HFD-fed mice versus mice fed normal chow is less in Fig. 5 than in Fig. 4 because of the shorter time of HFD.] Since the complex-injected mice had been pre-challenged with an HFD, we could assess the influence of an elevated representation of T_{reg} cells on various indicators of insulin resistance. Blood-glucose levels were significantly lower (Fig. 5h) [and, as expected, IL-10 transcript levels were significantly higher (Suppl. Fig. 6d)] in the HFD-fed mice with more T_{reg} cells. While HOMA-IR (Fig. 5i) and glucose tolerance [measured via an intraperitoneal (ip) GTT] (Fig. 5j, left panel) all trended towards lower values in the T_{reg}-cell-enriched HFD-fed animals, these differences fell short of statistical significance, probably due to the greater experimental variability inherent in these assays. In fact, given the very short experimental window that we had to work in to avoid expansion of effector T cells, small differences are not at all surprising. In order to enhance power, we injected a number of additional HFD-fed mice with IL-2:anti-IL-2 complexes vs. PBS under similar conditions, accumulating a total of 11 individuals for each group. The T_{reg} cell fraction in the complex-injected HFD-fed mice ranged from 40–83% (Av= 68+/−13%), while that in the PBS-injected animals spanned 18–70% (Av= 45+/−13%). Both HFD-fed groups were glucose intolerant *vis a vis* control mice fed normal chow (NC); however, the complex-injected group, with the highest levels of T_{reg} cells, showed a significant improvement compared with the PBS-injected group (Fig 5j, right panel). Similar results were obtained in the A^y/a system (Suppl. Fig. 8).

These findings indicate that T_{reg} cells guard against excessive inflammation of the adipose tissue and its downstream systemic consequences, and strongly suggest that T_{reg} cells residing in the fat are responsible. While it is not currently feasible to more definitively establish the latter point, given that no fat-T_{reg}-specific reagent exists and that it has not been possible to successfully isolate and transfer fat T_{reg} cells in the requisite quantities, a role for T_{reg} cells, whatever their source, in metabolic homeostasis and its dysregulation in obesity is an unexpected finding.

In vitro effects on adipocytes

A likely mechanism by which T cells residing in adipose tissue impact on neighboring cells is through soluble mediators. Thus, we explored the influences of the major cytokines differentially produced by T_{reg} and T_{conv} cells in fat *vis a vis* at other sites: according to our gene-expression profiling, IL-10 and IFN-γ, respectively. Fully differentiated, lipid-laden 3T3-L1 adipocytes were pretreated or not for 48h with IL-10, and were subsequently stimulated for 24h with TNF-α, an established method for *in vitro* induction of insulin resistance (Fig. 6, a and b). TNF-α induced changes in adipocyte expression of a number of transcripts encoding inflammatory mediators, for example IL-6, RANTES, SAA-3 and MMP3. Strikingly, IL-10 inhibited the TNF-α-induced expression of all of these mRNAs. TNF-α has also been shown to down-modulate insulin-dependent tyrosine phosphorylation of insulin receptor substrate (IRS)1 and to inhibit Glut4-mediated glucose uptake in 3T3-L1 adipocytes; these effects, too, were reversed by IL-10 [8], indicating that this cytokine reverts insulin resistance by a mechanism directly impinging on adipose tissue cells (i.e. is cell autonomous). In striking contrast to the anti-inflammatory effects of this mediator made by visceral fat T_{reg} cells, a major product of the T_{conv} cells at this site, IFN-γ, was pro-inflammatory in the same *in vitro* assay system: expression of SAA3, RANTES and IL-6 transcripts were all induced, and Glut4 mRNA was down-regulated (Fig. 6c).

Our many attempts to support these conclusions by performing co-cultures of fat T_{reg} cells and 3T3-L1 pre-adipocytes have so far been unsuccessful due to rapid death of the T_{reg} cells during the incubation period, likely due to pro-apoptotic factors present in the medium or produced by the adipocytes, eg TNF-α. However, spleen T_{reg} cells were less fragile in these co-culture conditions, and we could demonstrate that they dampen levels of pro-inflammatory transcripts made by activated adipocytes in culture and, perhaps most relevant, inhibit their down-modulation of Glut-4 transcripts (Fig. 6d and data not shown). The greater fragility of fat vs spleen T_{reg} cells may be explained by the former's higher expression of TNF-Rs (data not shown).

And humans?

Finally, we sought to translate our findings to the human arena. We had access to a set of paired frozen omental and subcutaneous fat tissues from a number of individuals with an average body:mass index (BMI) of 44.85, thus (except for one case) falling within the obese (30–39.9) and morbidly obese (>40) range. Given that the samples were frozen, we could not perform flow cytometric analysis on or purification of lymphocyte populations, but could titer FOXP3 transcript levels by PCR (Fig. 6e, left panel). FOXP3 mRNA was readily detectable in both fat depots. Consistent with the observations on obese mice, we found higher levels of FOXP3 transcripts, presumably an indicator of T_{reg} cells, in the subcutaneous adipose tissue. We did not have access to non-obese controls for these studies due to the rarity of performing bariatric surgery on normal individuals. However, we did find a correlation between BMI and the drop in T_{reg} cells in omental vis-à-vis subcutaneous fat (Fig. 6e, right panel). These data suggest that our findings on mice may be translatable to humans, encouraging for more sophisticated analyses on purified T cell subsets from fresh adipose tissue.

DISCUSSION

Finding a peculiar population of regulatory T lymphocytes specifically in the abdominal adipose tissue of normal, but not obese, mice raises a number of questions. First, why and how do T_{reg} cells accumulate at this site? One factor may be antigen stimulation, as suggested by the imprint of antigenic selection on the TCR repertoire of visceral fat T_{reg} cells and by their unusually high state of activation. Depending on the location(s) of any such antigens, they could stimulate circulating T_{reg} cells, provoking them to exit the lymph nodes and invade the fat, and/or re-stimulate T_{reg} cells filtering through adipose tissue, thereby promoting their retention. As discussed above, neither the sequencing nor the gene-expression profiling data provided support for the notion that T_{conv} cells were induced to convert to T_{reg} cells in the visceral fat. A second factor is almost certainly chemokines, given the unique pattern of chemokine/chemokine receptor gene expression by the T_{reg} cells isolated from adipose tissue. Functional studies will be needed to unravel which of these molecules are indeed involved in cell attraction or access to fat, micro-localization once therein, or their recruitment of other leukocyte subsets. Thirdly, there may be a role for adipokines in nurturing T_{reg} cell survival in adipose tissue. A recent study highlighted the negative effect of leptin on the proliferative capacity of T_{reg} cells [39], which fits well with their opposite representations in normal and obese fat: many T_{reg} cells and little leptin in the former; few T_{reg} cells and much leptin in the latter. Given, in contrast, the parallel high levels of T_{reg} cells and adiponectin in normal fat and low levels in obese fat, this adipokine is an interesting candidate as a positive factor, especially since it induces IL-10 synthesis, at least by macrophages [51, 52].

Next, what function(s) are T_{reg} cells performing in normal abdominal adipose tissue? Recent reports have highlighted the interplay between adipocytes and a population of anti-inflammatory macrophages in this fat depot, suggesting a role for resident macrophages in

promoting tissue repair and angiogenesis, and in maintaining insulin sensitivity [8, 9]. Our results argue that the activities of coincident populations of T_{conv} and T_{reg} cells need to be added to the mix. It may be relevant that the T_{conv} cells appear to be making an ongoing T_H1 response to some stimulus and that their major product, IFN-γ, promotes synthesis of inflammatory mediators by adipocytes. The T_{reg} cells may be keeping this response in check, as well as regulating the activities of their macrophage and adipocyte neighbors – indeed, their physical location in crown-like structures at adipocyte junctures would encourage interaction with both cell-types. IL-10 is one candidate for playing a role in T_{reg}-cell-mediated regulatory activities, given its association with improved insulin sensitivity in a number of contexts in both rodents and humans [53–55], though this cytokine is also produced by the fat-resident anti-inflammatory macrophages [8].

Lastly, what provokes T_{reg} cells to vacate abdominal fat in obesity, or perhaps more likely, to refrain from entering it? It may be a secondary effect. Increasing adiposity has been associated with an influx of macrophages [6, 7], in particular the inflammatory macrophage subset [8, 9], into the abdominal depot; as well as with increased local and systemic concentrations of inflammatory cytokines, such as TNF-α and IL-6. In addition, a reciprocal increase and decrease in the adipokines leptin and adiponectin, respectively, have been reported, fueling speculation of aggressive crosstalk between the two cell-types in the obese condition [11]. One possible scenario, then, is that elements of this changing environment are unfavorable for T_{reg} cell entry, expansion or survival, leading to a secondary decline of this regulatory population; in fact, leptin [39] and IL-6 [56, 57] are already known to have such properties. However, it is also possible that loss of T_{reg} cells from the abdominal fat is the primary effect. While their exaggerated numbers in lean adipose tissue had been sufficient to keep the chronic inflammation under control, reduced T_{reg} cell numbers with increasing adiposity could open the gate to an invasion of inflammatory macrophages and thereby a more robust synthesis of inflammatory cytokines. Whether the influx of macrophages or the efflux (or death) of T_{reg} cells proves upstream, both processes must be downstream of an initiating event, as yet undefined, though suggestions have included local hypoxia [58], increased adipocyte death [20] and adipocyte stress [59].

METHODS

Mice

Male B6 (at different ages and retired breeders 25–35 weeks old), mice homozygous for the obese spontaneous leptin mutation, ($Lep^{ob/ob}$; commonly referred to as ob/ob) and $Lep^{ob/wt}$ mice, mice heterozygous for the yellow spontaneous mutation ($A^y/_a$), $Foxp3^{GFP}$/B6 reporter mice [23], and Limited (LTD) mice [32] were bred in our SPF facility at the Joslin Diabetes Center, or were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice on HFD were fed with a rodent diet of 45 or 60 kcal% fat from Research Diet (New Brunswick, NJ; Cat# D12451). Others were bought from Jackson laboratory, as indicated. Mice expressing DTR under the control of Foxp3 ($Foxp3^{DTR}$) were generated in our facility and will be described in detailed elsewhere. A construct expressing the DTR-eGFP cDNA was a gift from Dr Dan Littman. In brief, a BAC spanning from 150kb upstream to 70 kb downstream of Foxp3 transcription start site was isolated. The DTR-eGFP cDNA with a stop-codon was inserted between the first and second codon of the Foxp3 open reading frame. The recombinant $Foxp3^{DTR}$ BAC was injected into the NOD strain.

Isolation of T cells and cytokine stimulation assay

Abdominal (epidydimal) adipose tissue, subcutaneous adipose tissue, lung and liver were removed after flushing the organs through the portal vein and the heart ventricle, cut into small pieces (or passed through a sieve in the case of the liver) and digested for about 40

minutes with collagenase type II (adipose tissue, Sigma) or collagenase type IV (Sigma). Cell suspensions were then filtered through a sieve (or for the lung smashed through the sieve), and the SVF was harvested after spinning. Cells were stained with: anti-CD4, anti-CD8, anti-CD3, anti-CD25 and anti-B220; and for some experiments with anti-CD103, anti-GITR, anti-CD69 and anti-Ly6c mAb, and were fixed and permeabilized according to the manufacturer's instructions (eBiosciences), followed by intracellular staining of Foxp3 (eBiosciences) and CTLA-4.

For intracellular cytokine staining, cells were stimulated with PMA (50ng/ml) (Sigma) and ionomycin (1nM) (Calbiochem) for 4 hours. Golgistop (BD) was added to the culture at the recommended amount during the last three hours. Cells were stained with: anti-CD4, anti-CD8, anti-CD3, anti-CD25 and anti-B220 antibody, and were fixed and permeabilized according to the manufacturer's instructions (eBiosciences), followed by intracellular staining of Foxp3 (eBiosciences), IFN-γ, TNF-α, IL-10 or IL-4. Cells were then analyzed using a Moflo, Coulter Epics XL or LSRII instruments and FlowJo software.

Microarray analysis

Lymph node and abdominal fat TCR$^+$CD4$^+$ and CD25^{hi} (T$_{reg}$) or TCR$^+$CD4$^+$ and CD25$^-$ (T$_{conv}$) cells were sorted from retired male breeder B6 mice, and spleen T$_{reg}$ and T$_{conv}$ cells were sorted from Foxp3^{GFP}/B6 reporter mice [23]. RNA was extracted with Trizol and amplified for two rounds using the MessageAmp aRNA kit (Ambion), followed by biotin labeling using the BioArray High Yield RNA Transcription Labeling Kit (Enzo Diagnostics), and purified using the RNeasy Mini Kit (Qiagen). The resulting cRNAs (three independent datasets for each sample type) were hybridized to M430 2.0 chips (Affymetrix) according to the manufacturer's protocol. Initial reads were processed through Affymetrix software to obtain raw .cel files. Microarray data were background-corrected and normalized using the RMA algorithm implemented in the GenePattern software package [60], and replicates averaged (full datasets are deposited at NCBI under accession# GSE7852). A consensus T$_{reg}$ signature was compiled from four independent analyses [23, 26]. The color-coding in the figures denoted genes 1.5 fold over- (red) or under- (blue) expressed in T$_{reg}$ cells in all four reference datasets. The fat T$_{reg}$-specific gene set included loci specifically over- or under-expressed in fat T$_{reg}$ cells, and was generated by including genes 2-fold or more over- (red) or under- (blue) expressed in fat T$_{reg}$ vs. fat T$_{conv}$ cells as well as more than 2-fold different between fat T$_{reg}$ and LN T$_{conv}$ cells. To exclude the classical T$_{reg}$-specific genes, LN T$_{reg}$ vs. LN T$_{conv}$ had to be less then 1.25-fold for over- or more then 0.8 for under-represented genes.

In vivo depletion and expansion of Treg cells

For depletion: 10-week-old Foxp3^{DTR+} and Foxp3^{DTR-} control mice were injected ip with DT (Sigma), 40ng/g body weight, every other day for 9 days or, in some experiments, for 4 days. Overnight fasting blood-glucose and-insulin levels were measured and abdominal adipose tissue, lung and spleen were removed for RNA extraction.

For expansion: mice fed 15 weeks on HFD were purchased from Jackson Laboratory (Bar Harbor ME), fed for 12 weeks with HFD in the Jackson facility [60 kcal % fat from Research Diet (New Brunswick, NJ; Cat# D12492)]. Complexes of the anti-IL-2 mAb JES6-5H4 (BD) and mouse IL-2 (PeproTech) were prepared and ip-injected as described [49]. In brief, 5 μg of anti-IL2 and 0.5 μg mIL-2 per mouse were incubated for 20 minutes on ice followed by ip injection. Mice received daily injections for 6 or 9 days, and were analyzed on day 14. Control mice were injected with saline (PBS). In some experiments mice were fed with HFD for 8 weeks [60 kcal % fat from Research Diet (New Brunswick, NJ; Cat# D12492)] and were injected with the complex for 9 days. For glucose tolerance tests (GTT),

glucose (2.0 g/kg) was administered by intraperitoneal injection after an overnight fast. Blood glucose was measured before, 15, 30, 60 and 120 minutes after glucose application.

Additional methodology are described in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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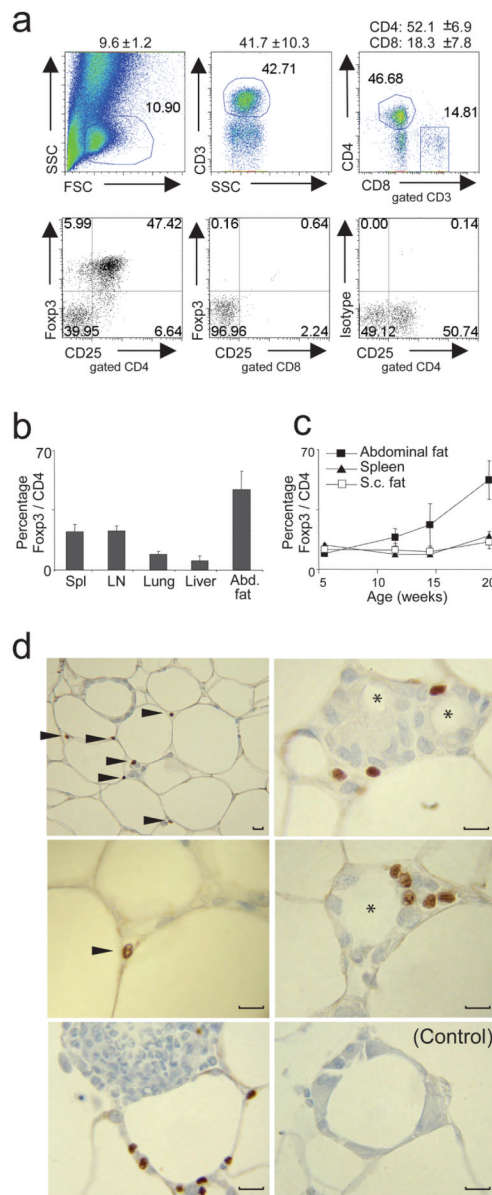
Figure 1.
Abdominal (epidydimal) and subcutaneous (s.c.) fat pads as well as spleen, LN, lung and liver were isolated from retired-breeder B6 male mice, and the SVF fraction was stained for Foxp3, CD3, CD4, CD8 and CD25. (a) Upper panel: T cell distribution in SVF fraction from abdominal fat tissue. Numbers on top indicate the mean and standard deviation (SD) for cells in the lymphocyte gate after fixing and permeabilization, fraction of CD3+ T cells among lymphocyte-gated cells and distribution of CD4+ and CD8+ T cells. Lower panel: Percentage of Foxp3+CD25+ T cells in abdominal fat tissue gated on CD4+ or CD8+ T cells. Organs of 5 mice were pooled. Representative dot plots are shown. (b) Frequency of Foxp3+CD4+ T cells in different organs. Mean and SD from at least three independent experiments are shown, whereas organs from 4–5 mice per experiment were pooled. (c) Kinetic of T$_{reg}$ cell appearance in abdominal and s.c. fat tissue as well as in spleen. (d) Immunohistology of abdominal adipose tissue. Arrow-head indicates Foxp3 staining. Note: Foxp3 expression is restricted to the nucleus. * refers to dead-adipocyte residue surrounded

by a crown-like structure formed by immune cells. Control staining with isotype antibody. Original magnification: left panel, upper picture 400x, all other 1000x.
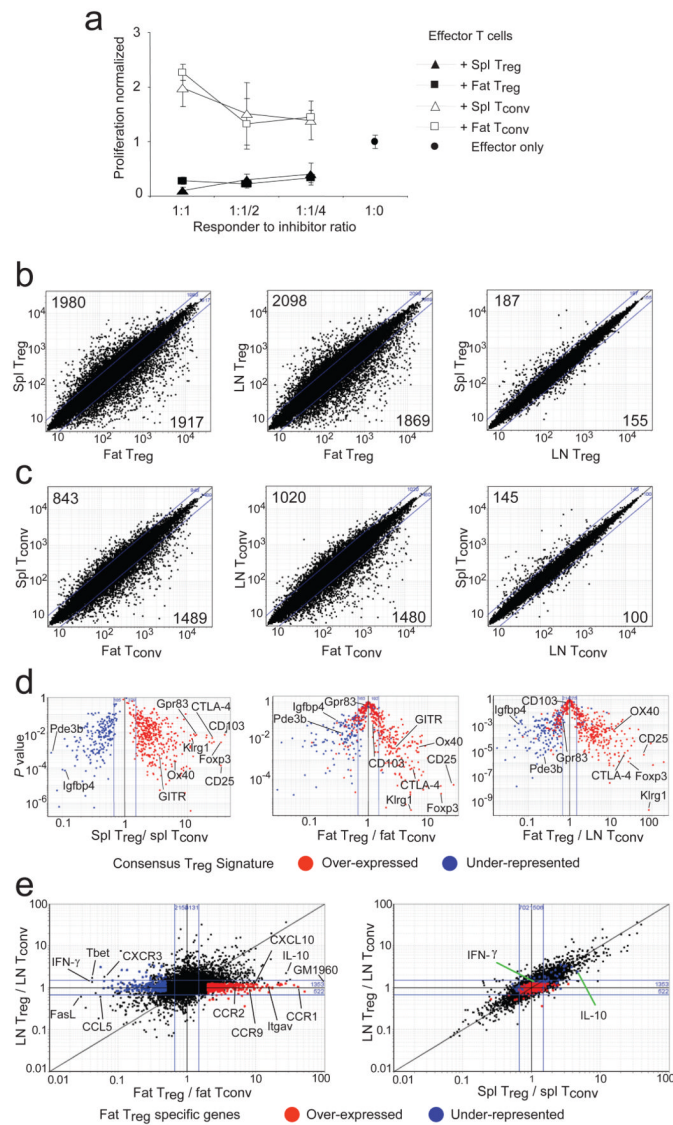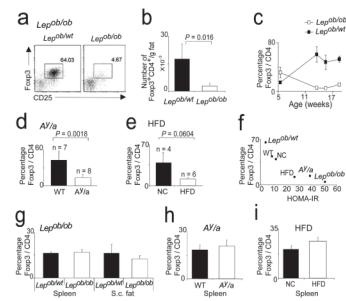
Figure 2.
Functional comparison of T_{reg} and T_{conv} cells from abdominal adipose tissue, LN and spleen. $CD4^+CD25^+$ T_{reg} and T_{conv} cells were isolated from retired-breeder B6 mice. (a) A standard *in vitro* suppression assay was performed. Spleen-derived $CD4^+$ effector T cells (responder cells) were incubated at various ratios with different T cell populations (spl: spleen, Fat: adipose tissue derived T cells). (b–e) Analysis with Affymetrix M430v2.0 chips. Normalized expression values for profiles directly comparing T_{reg} cells (b) between: fat vs spleen (left panel), fat vs LN (center panel), LN vs spleen (right panel). Or for profiles directly comparing T_{conv} (c) between: fat vs spleen (left panel), fat vs LN (center panel), LN vs spleen (right panel). (b and c) Numbers are calculated on the basis of a cut-off of 2-fold from the individual comparisons. (d) "Volcano" plots of gene-expression data comparing *P*-value vs. fold-change for probes from the consensus T_{reg} signature ([23], [26]). Plotted for: spleen T_{reg} vs T_{conv} (left panel); fat T_{reg} vs T_{conv} (center panel); fat T_{reg} vs LN T_{conv} (right panel). (e) Fold-change to fold-change plots comparing T_{reg} expression profiles between: fat T_{reg} (x-axis) and LN T_{reg} (y-axis) (left panel); spleen T_{reg} (x-axis) and LN Treg (y-axis)

(right panel). Genes uniquely up or down regulated in fat T$_{reg}$ cells are highlighted in red and blue.
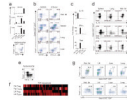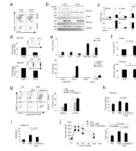
Figure 3.
Phenotypic characterization of T_{reg} cells from abdominal (epidydimal) fat tissue, spleen, lung and liver. (a and b) Cells were isolated from retired-breeder B6 mice, and the SVF fraction was stained for Foxp3, CD3, CD4, CD8, CD25, GITR, CD103 and CTLA-4. (a and c) Relative RNA expression of selected genes from T_{reg} and T_{conv} cells from LN and fat. (d and e) Cytokine-expression profile from T_{reg} and T_{conv} cells from spleen, lung and fat tissue. Profiles for IL-10, IFN-γ and IL-4. Representative dot plots of at least three independent experiments are shown. Organs from 4–6 mice were pooled per experiment. (f) TCR sequences of fat-derived T_{reg} and T_{conv} cells. Abdominal fat and LN T_{reg} and T_{conv} cells were isolated from old male animals from the Limited (LTD) mouse line. The frequency of the CDR3α sequences was analyzed on a single-cell base. Graphic display of the TCR sequences in a heat-map format from T_{reg} and T_{conv} cells. (g) Cells were isolated from abdominal adipose tissue, LN, liver and lung from retired-breeder B6 mice, and the SVF fraction was stained for Foxp3, CD3, CD4, CD8, and for the activation marker CD69 and Ly6c. Representative dot plots are shown.
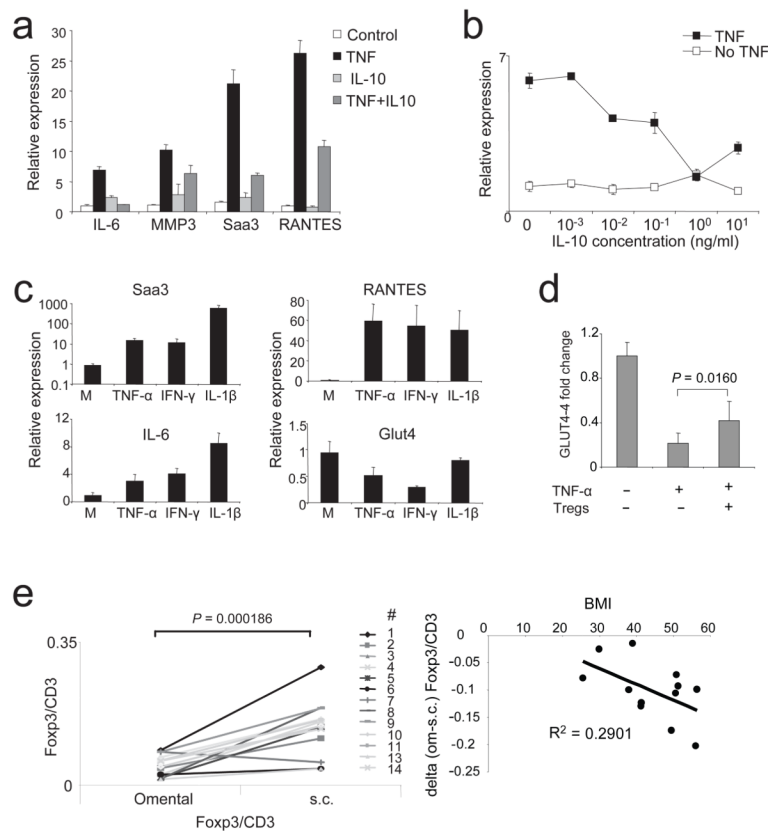
Figure 4.
Three mouse models of obesity: $Lep^{ob/ob}$, A^y/a and HFD. (a–c) Abdominal adipose tissue from $Lep^{ob/ob}$ and heterozygote $Lep^{ob/wt}$ mice was analyzed for T_{reg} cell frequency. (a) Representative dot plots of 13-week-old mice. (b) Total number of T_{reg} cells per one gram fat. (c) Changes of T_{reg} representation over age. Mean and SD are shown. (d) Percentage of T_{reg} cells in abdominal adipose tissue of 24-week-old A^y/a or littermate (WT) mice. (e) Percentage of fat T_{reg} cells in mice fed for 29 weeks with HFD or NC. (f) Correlation of HOMAR-IR and fraction of T_{reg} cells. (g–i) Observed changes of T_{reg} cell proportion in adipose tissue of the three obesity models were not reflected in other organs. (g) $Lep^{ob/ob}$, (h) A^y/a, (i) HFD.

Figure 5.
Loss-of- and gain-of-function experiments. (a–f) Loss-of-function experiment by depleting T$_{reg}$ cells expressing DTR. 10-week-old male mice, either DTR-positive or -negative, were treated every other day for 4 days (a–c) or 9 days (d–f) with DT. (a) Percentage of T$_{reg}$ cells from spleen or the abdominal fat after 4 days of treatment. (b and c) T$_{reg}$ depletion affects insulin signaling in epididymal white adipose tissue (WAT) and liver. Immunoprecipitation and Western blotting of insulin-induced IR shows a decrease in IR phosphorylation (pIR) in epi WAT and liver without differences in muscle and spleen. (b) Blot data. (c) Quantification of pIR normalized by total IR. (N≥4, *p<0.004, T-test); (d) Percentage of T$_{reg}$ cells from the abdominal fat (upper panel) or spleen (lower panel) after 9 days of treatment, with a representative dot plot as an insert. (e) Upper panel: Expression of TNF-α, IL-6, A20, RANTES and SAA3 transcripts in abdominal adipose tissue. Three mice per group, one of two independent experiments is shown. Lower panel: comparison of RANTES and SAA3 transcripts in spleen, lung and abdominal fat (epi fat). (f) Fasting-insulin (upper panel) and -glucose levels (lower panel) after 9 days of treatment every other day. Six mice per group from two independent experiments were pooled. Significance was determined by the Mann-Whitney *U* test. (g–j) Gain-of-function experiment. *In situ* expansion of T$_{reg}$ cells via injection of a mAb specific for IL-2 coupled with recombinant IL-2. (g) Dot plots (left panel) and a summarizing bar graph (right panel) showing T$_{reg}$ cells from spleen and abdominal fat tissue (epi fat) from mice fed normal chow (NC) or with 15 weeks of HFD. Treated with IL-2/anti-IL2 complex or saline for 6 days and analyzed on day 14 (n=6 for each group). Blood glucose (h) HOMA-IR (i), and an i.p. GTT (j) of mice described in (g). (j, right panel), calculated area under the curve (AUC) from all mice tested by GTT (n=11 in each group), including the dataset described in (g). *P*-values were calculated with the T-test.

Figure 6.
Cytokine effects on adipocytes and human correlates. (a and b) IL-10 can reverse TNF-α mediated inflammatory changes in differentiated adipocytes. (a) Expression of IL-6, MMP3, SAA3 and RANTES were measured with qPCR under unmanipulated culture conditions (control); adipocytes were treated with TNF-α (TNF); cells were treated IL-10 (IL-10) alone; or cells were treated with TNF-α and IL-10 (TNF+IL-10). (b) Relative expression of IL-6 in differentiated adipocytes, dose response curve of IL-10. TNF: TNF-α and different concentrations of IL-10. No TNF: only IL-10. Representative experiments are shown. (c) Expression of SAA3, RANTES, IL-6 and Glut4 in differentiated adipocytes, unmanipulated (M) or treated with TNF-α, IFN-γ and IL-1β. Representative experiments are shown. (d) Expression of Glut4 in differentiated adipocytes either unmanipulated or treated with TNF-α in presence or absence of spleen T_{reg} cells. Mean and SD of 3 independent experiments are shown. P-value was calculated with T-test. (e) Paired human omental and s.c. adipose samples from mostly obese individuals (BMI range: 25.5–56.43, average: 44.85). Expression of FOXP3 and CD3 was measured by quantitative PCR. Plotted are the ratios of FOXP3 vs. CD3 for omental and s.c. adipose tissue (left panel). Right panel: the decrease in FOXP3/CD3 ratio in omental versus s.c. adipose tissue was plotted against the BMI for each individual donor from the left panel. Except the positive value of subject #7 (> 2 standard deviations from the mean) was not included. Each dot represents an individual donor.