The outliers become a stampede as immunometabolism reaches a tipping point

Author’s addresses
Barbara S. Nikolajczyk1, Madhumita Jagannathan-Bogdan2
Gerald V. Denis3
1Departments of Microbiology and Medicine, Boston University, Boston, MA, USA.
2Department of Pathology, Boston University, Boston, MA, USA.
3Cancer Research Center, Boston University School of Medicine, Boston, MA, USA.

Correspondence to:
Barbara S. Nikolajczyk
Department of Microbiology
Boston University School of Medicine
72 East Concord Street
Boston, MA 02118, USA
Tel.: +1 617 638 7019
Fax: +1 617 638 4286
e-mail: bnikol@bu.edu

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Summary: Obesity and Type 2 diabetes mellitus (T2D) are characterized by pro-inflammatory alterations in the immune system including shifts in leukocyte subset differentiation and in cytokine/chemokine balance. The chronic, low-grade inflammation resulting largely from changes in T-cell, B-cell, and myeloid compartments promotes and/or exacerbates insulin resistance (IR) that, together with pancreatic islet failure, defines T2D. Animal model studies show that interruption of immune cell-mediated inflammation by any one of several methods almost invariably results in the prevention or delay of obesity and/or IR. However, anti-inflammatory therapies have had a modest impact on established T2D in clinical trials. These seemingly contradictory results indicate that a more comprehensive understanding of human IR/T2D-associated immune cell function is needed to leverage animal studies into clinical treatments. Important outstanding analyses include identifying potential immunological checkpoints in disease etiology, detailing immune cell/adipose tissue cross-talk, and defining strengths/weaknesses of model organism studies to determine whether we can harness the promising new field of immunometabolism to curb the global obesity and T2D epidemics.

Keywords: obesity, type 2 diabetes, T cell, B cell, human, immunometabolism

Introduction
T2D as an inflammatory disease
Type 2 diabetes (T2D) is characterized by hyperglycemia, insulin resistance (IR), pancreatic islet failure, and perhaps most important to immunologists, chronic/unresolved low-level inflammation. The appreciation of IR/T2D as an inflammatory disease began about two decades ago with publication of ‘outlier’ work showing tumor necrosis factor-α (TNF-α) promotes IR (1). This initial work spawned the new field of immunometabolism, which has expanded rapidly to become a major focus for established investigators from the fields of either metabolism or immunology, along with a new breed of scientist trained in both disciplines. This rapid expansion in knowledge has reached a tipping point wherein immunomodulatory drugs are being tested as clinical treatments for T2D.

Inflammation in T2D occurs on multiple levels, as demonstrated by elevated concentrations of pro-inflammatory cytokines in serum and in important metabolic regulatory tissues such as liver and adipose tissue (AT). Chronic inflammation is now appreciated as an integral component driving T2D
pathology and/or complications based on numerous studies. T2D patients have elevated circulating levels of pro-inflammatory cytokines, including interleukin-1β (IL-1β), IL-6, TNF-α, and IL-18 (reviewed in 2). Furthermore, increased inflammatory cytokine levels predict the risk of developing T2D in normoglycemic volunteers (3, 4). Inflammation is also linked to T2D comorbidities, including development of life-threatening complications such as cardiovascular disease (CVD) (5–7). Although the short-term nature of the clinical studies on efficacy of anti-inflammatory drugs for comorbidities has not allowed a rigorous assessment of long-term outcomes, these studies have further emphasized the physiological connection between chronically elevated inflammation and metabolic disease (8–16). Mechanistic links between inflammation and IR are becoming increasingly defined and include c-Jun N-terminal kinase (JNK)- and extracellular signal-regulated kinase (ERK)-mediated insulin receptor substrate-1 phosphorylation/inactivation, inhibition of translation initiation, and lipogenesis/lipolysis imbalance recently described in detail elsewhere (17). Taken together, these studies have established obesity, IR, and T2D as chronic inflammatory diseases.

The dominant sources of inflammatory cytokines in T2D are the immune cells that infiltrate multiple AT depots, pancreatic islets, muscle, and liver (18–23). The relative contribution of AT and AT-associated immune cells to net inflammation in obesity and T2D is disproportionately high because of the great expansion of AT in response to a positive energy balance, the most well understood cause of obesity, IR, and T2D. In combination with expansion of adipocyte size, the absolute numbers of AT-associated immune cells also increase in obesity (18, 19, 24). Multiple types of immune system cells have been shown to drive inflammation systemically likely through postactivation recirculation, or, in the AT, through selective AT infiltration. Comprehensive quantification and functional analysis of immune cell subsets that initiate and/or propagate AT and systemic inflammation is critical to design highly specific therapies to combat T2D-associated inflammation and inflammation-associated comorbidities.

To take advantage of the appreciation of T2D as an inflammatory disease and use this knowledge to design fundamentally new therapies, the field must overcome limitations in both our knowledge of human immunology and the heavy reliance on genetically hypo-variable mouse models. The first steps towards this important goal must be a calculated shift towards in-depth human subject research and an implementation of more rigorous standards for high impact mouse studies that typically include a single subpanel of human material analyses to conclude relevance of comprehensively characterized mouse outcomes.

**Inflammation as a cause and consequence of obesity and T2D**

Numerous rodent model studies implicate inflammation in obesity and/or IR etiology by showing that inactivation of a pro-inflammatory modulator prevents IR. These studies include either naturally occurring mutant or experimental knockout mice, or antibody blocking approaches that target molecules such as Toll-like receptor 2 (TLR2) or TLR4 (25–30). The well-performed studies in this genre match mice for weight gain, given that in the majority of situations mice that are more obese will exhibit IR and elevated levels of pro-inflammatory cytokines. Studies with differences in weight gain caused by gene ablation, for example, often overlook the importance of measuring changes in feeding behavior and hypothalamic responses, or in energy expenditure as root outcomes of gene deletion. Such changes could reveal the bona fide role of a molecule or cell type in whole animal physiology shifts that culminate in obesity and IR independent of inflammation. The importance of ambient temperature for murine analysis is also often overlooked. Mice expend considerable energy to maintain body temperature in typical housing at 20 °C and are considered chronically cold-stressed; they become obese much more readily under temperature-neutral conditions (30 °C).

A minority of pro-inflammatory molecules, including TLR5 and IL-17, appear to prevent obesity and/or IR in the DIO model. In the case of TLR5, a TLR that is not expressed by lymphocytes or adipocytes (31), it appears TLR5 deficiency corresponds with diabetogenic alterations in the gut microbiota (32). These results indicate that chronic low-level sampling of gut-associated commensals is required for metabolic health. In the IL-17 deletion study, genetically altered mice gain more weight than wildtype (WT) controls, making it impossible to determine the role of IL-17 in the whole animal model in the absence of more detailed analysis of, for example, calorie expenditure (33). Despite these exceptions, the animal data are consistent with the interpretation that inflammation can cause IR/T2D by mechanisms mentioned above.

New data indicate that immune cells also respond to the physiological changes that accompany obesity and IR; therefore, immune cells may influence ongoing T2D pathogenesis independent of roles they play in disease etiology. Blood monocytes injected into obese or lean mice assume the phenotype of pro-inflammatory M1 macrophages or
non-inflammatory tissue-repairing M2 macrophages, respectively, in recipient AT. Monocyte characteristics imparted by lean or obese blood donors were irrelevant, indicating that the metabolic environment can dominate macrophage function, at least in AT (34). This study indicates that identifying functions of AT-associated leukocytes is critical and may be more important than identifying blood cell function, at least in some contexts. Taken together, the mouse data indicate the relationship between the immune system and metabolic health is a two way street, with the status of each influencing the other. Therefore, inflammation appears to be both a cause and a consequence of obesity/IR/T2D. Whether these examples predict outcomes in rigorous examination of patients is of paramount importance but remains to be tested.

The linear path to T2D?

Primary care physicians and endocrinologists watch and wait as a high percentage of their patients spiral upward in body mass index (BMI) and downward metabolically, despite valid advice on nutrition and exercise. Treatment choices for these patients are few, with the added obstacle of inadequate insurance coverage that supports behavioral and pharmacological interventions only after T2D diagnosis, largely ignoring an ‘obesity’ diagnosis. Standard measures used to monitor metabolic health in overweight and obese individuals have been used for decades: fasting glucose, glycated hemoglobin (HbA1c), and BMI, with C-reactive protein (CRP), a surrogate measure of inflammation, measured only as an assessment of cardiovascular risk. The worsening of these clinical measures of metabolic health are more or less linear, with the average patient (who maintains or increases BMI) slowly creeping towards numbers that designate him/her as T2D as defined by the American Diabetes Association. Many metabolic parameters change slowly during this period, including increased leptin and decreased adiponectin. This relatively linear pathway to T2D is consistent with results from time course DIO mouse studies, although the rapid induction of IR in response to a lipid bolus in mice argues that non-linear pathways also exist (35). These studies support the conclusion that inflammatory immune cells more or less steadily increase in the expanding visceral AT because of increased infiltration as obesity increases, with IR becoming detectable about 8 weeks after high fat diet (HFD) initiation in relatively young mice (24, 36). In stark contrast to this steady march to IR/T2D, a significant proportion of obese humans, approximately 20–25%, preserve metabolic health. Metabolically healthy obese individuals are characterized by relatively modest systemic inflammation (37, 38). These data raise many clinically critical questions as follows.

1. Are similar progressive immune system changes characteristic of human T2D?
2. Does a blockade of progressively increasing inflammation prevent T2D akin to results from inflammation-compromised DIO mice?
3. Can we identify drug-susceptible checkpoints along the pathogenic continuum from obese/insulin sensitive (IS) to obese/IR to T2D by focusing on immunological changes?
4. Is there an inflammatory ‘point of no return’ in disease pathogenesis?
5. Can we exploit immune cell characteristics as metabolically responsive endpoints in clinical trials for new obesity/IR/T2D medications?
6. Can we rule out the existence of an acute episode that facilitates the transition from obese to obese/T2D?

Additional temporal DIO studies in combination with carefully designed and implemented longitudinal clinical studies aimed at answering these questions are critical for fully exploiting our understanding of T2D as an inflammatory disease.

The role of T cells and T-cell subsets in obesity and T2D

Lymphocyte subsets are altered in obesity and generally skew towards pro-inflammatory phenotypes and functions, thus lymphocytes contribute to local (AT) as well as systemic inflammation in T2D. The importance of lymphocytes in setting the stage for the eventual macrophage-dominated obese AT inflammation is indicated by studies on RAG-null mice lacking both B and T cells, which have an elevated number of macrophages in the AT late in DIO compared with the numbers in lymphocyte-sufficient controls. However, caution is warranted in this multiply immunodeficient model, because RAG-null mice also gain more weight than wildtype counterparts in response to a high fat diet (39). More focused studies have shown that T cells, one of the two major lymphocyte subsets, play major roles in AT and systemic health, as evidenced by a small avalanche of reports over the past few years.

The two main types of T cells shown to regulate metabolic disease express distinct surface molecules, CD4 or CD8, which interact with the hypovariable surfaces of the antigen-presenting moiety, major histocompatibility complex (MHC) class II or class I, respectively. Both subsets can express substantial amounts of cytokines, with CD4+ T cells subclassified based on the ability to secrete IFN-γ (Th1), IL-4/IL-5/IL-13...
Circulating T cells are altered in obesity and T2D patients

Altered function and/or abundance of multiple T-cell subsets has been strongly associated with metabolic disease in mice and humans. Many analyses of T cells in obesity and T2D have focused on circulating cells, partially justified by the appreciation of obesity and T2D as systemic diseases, the high safety profile associated with a blood draw, and the limited availability of other metabolic regulatory tissues (i.e. AT) from lean individuals to enable appropriate matching of patients to a control cohort. One of the earlier (albeit still relatively recent) studies used flow cytometry to show elevated CD4^+ IFN-γ^+ T cells (i.e. Th1 cells) in blood from obese versus lean children. Elevated Th1 cells are important because IFN-γ from Th1 (and CD8^+) T cells promotes inflammation at least in part by stimulating differentiation of the pro-inflammatory macrophages present in large numbers in IR AT. The percentage of Th1s in obese children positively correlated with multiple measures of poor metabolic health and liver disease (41). This report supports the overall conclusion that blood T cells are skewed towards pro-inflammatory subsets in relatively short-term obesity in the absence of T2D.

Our group as well as others has published studies aimed at achieving a more comprehensive understanding of the roles of circulating T-cell subsets in adult obesity and frank T2D. Early studies showed an increased CD4^+/CD8^+ ratio in obesity (42, 43), although the CD4 T-cell subset balance was not reported. An independent study that focused on the CD4^+ T-cell contribution to metabolic health showed serum from obese women contained elevated levels of CD4^+Th17 signature cytokines, including IL-17 and the Th17 supportive cytokine IL-23. This work furthermore showed no increase in circulating Th1 cytokines (IFN-γ and IL-12) (44), although IFN-γ protein can be difficult to detect in serum. In contrast, our analyses showed no difference in the percentage of Th17s cells or in IL-17 secretion from PBMCs of obese/non-diabetic subjects compared to lean healthy age-matched donors (45). This discrepancy between studies could be explained based on the use of different techniques. We focused on specific T cell subsets (45), while the serum studies (44) measured steady state cytokine levels regardless of cellular source. Never the less, our data showed a conceptually similar pro-inflammatory CD4^+ T-cell subset skewing in obese patients with confounding T2D. Additionally, blood from obese/T2D subjects contained a higher percentage of pro-inflammatory Th17 and Th1 subsets compared to obese-only samples, with a concomitant decreased percentage of Tregs. Th2 cells were insignificantly different between samples from obese non-diabetic or T2D patients (45). These findings have been independently confirmed (46). Importantly, our studies showed percentages and/or function of Th17 cells positively associate with clinical parameters, including HbA1c (45). Taken together, these findings support the more comprehensive model that CD4^+ T-cell subsets may be poised for skewing towards pro-inflammatory T cell subsets in obesity, but that pro-inflammatory skewing in combination with Treg decrease is only fully realized in obese patients with T2D. Furthermore, an association with clinical measures of T2D severity established the relevance of circulating T-cell subsets to disease. A comprehensive analysis of circulating T cells from all cohorts (lean/IS, obese/IS, obese/IIR, obese/T2D) in a single study or preferably in a longitudinal study is essential to test the model that CD4^+ pro-inflammatory T-cell subset balance exacerbates as patients progress from obesity to T2D. Overall, these studies raise the possibility that preventing T-cell imbalance may slow or prevent the transition to T2D or may restore insulin sensitivity as has been demonstrated in murine DIO studies (39, 47).

Adipose-associated T cells are altered in obesity and T2D patients

Given the importance of visceral AT inflammation in disrupting metabolic health (48), more recent work has focused on defining T cells in AT from obese and/or T2D patients. Both CD4^+ and CD8^+ T cells are elevated in obese human omental (visceral) and abdominal (subcutaneous) AT, which indicates parallel changes in T-cell AT infiltration and AT inflammation (49). Independent immunohistochemical staining of human
visceral AT from obese subjects confirmed the presence of CD4+ and CD8+ lymphocytes (49, 50). Quantification of T-cell abundance and function (as measured by the mRNA surrogates CD3 and IFN-γ) in AT biopsies from T2D patients showed a significant correlation between mRNA levels and waist circumference, a simple yet powerful indicator of central obesity and IR (50). A comparison of T-cell-associated mRNAs in subcutaneous AT from obese subjects over a well-defined range of insulin sensitivity showed that Th1 and Th17 signatures positively correlate with degree of IR, while Th2 signatures negatively correlate with IR (51). In contrast, mRNA analyses focused on T-cell markers in subcutaneous and visceral AT from morbidly obese individuals led to rather unexpected conclusions: a expansion of protective Tregs and Th2s, which positively correlated with plasma IL-6 and CD68 (macrophage) gene expression (52). This study therefore failed to support a loss of AT-associated protective Tregs in the face of pro-inflammatory T-cell expansion as a major factor in AT inflammation in morbidly obese patients, who, importantly, were not T2D as assessed by fasting blood glucose or use of T2D medication. The unanticipated expansion of Tregs is consistent with more recent analyses of blood from morbidly obese subjects, wherein flow cytometric studies confirmed an increased number of protective Th2s and Tregs compared with lean subject samples. Subjects with overt T2D were also excluded in these latter analyses (53). We speculate that Treg expansion could account for the overall CD4+ T-cell increase in blood from obese women as outlined above (42) and thus may represent an adult adaptation to longer-term obesity compared with the time-frame experienced by obese children (41).

Not all analyses of AT from obesity patients show Treg expansion. Independent analysis of tissue sections of human visceral AT revealed a lower FOXP3 (Treg):Tbet (Th1) ratio, indicative of a pro-inflammatory T-cell balance, is associated with BMI (39). The approach used however could not differentiate between decreased Tregs versus a Treg expansion that was simply outstripped by Th1 expansion. Regardless, these data agree with numerous mouse and human studies that link lower Treg/Th2:Th1/Th17 ratios with obesity and/or IR (45, 47, 49, 54, 55) and our human studies that reached the same conclusion in blood from non-diabetic and T2D subjects (45). These data also raise the possibility that T-cell skewing/expansion is part of a healthy response to increasing obesity and metabolic imbalance and suggest that failure or exhaustion of the anti-inflammatory response may be a critical step on the path to T2D. Taken together, the data support the concept of T2D-associated changes in T-cell balance that may build upon imbalances established during earlier stages of obesity.

Although the finding of expanded Th1/Th17s and decreased Tregs in obese AT is consistent with current dogma, it does not necessarily nullify the findings of increased Tregs in AT (and blood) of morbidly obese individuals (52, 53). The studies that concluded Tregs were elevated in obesity were performed on morbidly obese non-diabetic individuals (avg. BMI>40); the study showing increased pro-inflammatory T cells and decreased Tregs in obesity (51) analyzed samples from significantly less obese individuals (avg. BMI < 35). Our blood analyses also excluded samples from morbidly obese individuals, but importantly, matched obese and obese/T2D donors by BMI (45). Together these data raise the radical possibility that expansion of Tregs in response to obesity imparts metabolic protection. Alternatively, basic physiological differences between people capable of becoming morbidly obese (BMI ≥ 40) and those who are obese with BMIs ≤ 35 may include differential potential for Treg expansion. Additional confusion is perhaps introduced by demonstrations that lipid (palmitate) oxidation preferentially drives (murine) Treg differentiation (56) but that the expanded Th1/Th17:Th2s and increased pro-inflammatory cytokines (51) are not necessarily negated by Treg-promoting lipid-mediated regulation. This speculation assumes that metabolism of T cells from T2D donors is similar to T-cell metabolism in young lean mice that were the focus of the fuel preference studies (56). Clearly additional work is needed to define the metabolic requirements of T cells in obesity and T2D patients.

Th17 cells in obesity and T2D

Human analyses implicating elevated Th1 and decreased Treg cells in IR mirror studies completed in DIO mice; however, studies on roles for Th17 cells have been less consistent across species. Mouse studies that parallel our human T2D T-cell subset analyses showed Th17 cells expand in response to high fat diet feeding in murine DIO. Perhaps surprisingly, this expansion was limited to the subcutaneous, generally less inflammatory AT; Th17 numbers in visceral AT were similar in DIO and chow-fed mice (39). Another DIO study showed IL-17 is produced by CD4+ γδ T cells rather than αβ CD4+ Th17s (33) and raised the possibility that IL-17 may play roles in IR that
are Th17 independent. Despite this possibility, it is perhaps predictable that IL-17-secreting T cells play important roles in T2D-associated inflammation, because their major product, IL-17, promotes many types of pathogenic inflammation (33, 45) through interaction with the widely expressed IL-17 receptor (IL-17R). Activation of the IL-17R triggers NF-κB, thus cytokine production by monocytes, fibroblast, stromal, epithelial, and endothelial cells (57–59). IL-17 also induces mobilization, recruitment, and activation of granulocytes via induction of granulocyte colony-stimulating factor (G-CSF) (60). Importantly, IL-17 activates JNK, one of the kinases responsible for inappropriate phosphorylation of IRS-1 leading to IR (61, 62). IL-17 is thus mechanistically linked with IR. However, definitive evidence for the role of Th17 cells in disease pathogenesis of DIO mouse model of IR has been equivocal. IL-17-null mice are unexpectedly more susceptible to DIO-induced IR, although this study was confounded by increased obesity of the knockout mice. The underlying mechanism accounting for IL-17-mediated suppression of obesity (e.g. shifts in carbohydrate versus fat metabolism, calorie intake) was not reported. A study from the same group that reported DIO-induced Th17 increases in subcutaneous AT (39) showed Th17 expansion in spleen of DIO mice. Furthermore, the obese animals were more susceptible to Th17-mediated autoimmune disease such as colitis and EAE, but the more detailed effect on metabolic health beyond obesity was not reported (63). Despite the confounding weight increase of the IL-17 knockout mouse and the resultant difficulties in interpretation of outcomes, multiple studies confirmed that IL-17 inhibits adipogenesis and/or impairs glucose uptake in 3T3-L1 or human bone marrow mesenchymal cells (33, 64, 65). Once again, this work supports the over-riding concept that the pro-inflammatory T-cell balance identified in IR/T2D human blood and AT (45, 51) is an underlying driver of metabolic imbalance.

Elevation of Th17 in T2D is perhaps expected, given the relationship between Th17 differentiation and IL-1β, a cytokine often associated with T2D (15, 66–70). Lack of IL-1β in DIO mice caused by genetic deletion of IL-1β processing inflammasome proteins corresponds to a decrease in adipose-associated T cells, though Th17 cells were not specifically measured in this study (71). Furthermore, inactivation of surface expression of IL-1R1, a major receptor for IL-1β, predicts the level of IL-17 secretion by CD4+ T cells. Although the stimuli for IL-1R1 upregulation on CD4+ T cells (IL-7, IL-15, and TGF-β) are not cytokines generally associated with obesity or T2D, naive CD4+ T cells respond to IL-1β by secreting IL-17 (72). Furthermore, IL-1R antagonist (in combination with IL-6 blocking antibody) blocks T-cell IL-17 secretion in cells from type 1 diabetes patients (73). Although a clinical trial of IL-1R antagonist (Anakinra) in T2D patients decreases systemic CRP and IL-6 (15), the effects of Anakinra on IL-17 levels were not reported in these studies.

Limitations in the analysis of adipose-associated T cells

Although multiple reports suggest a role for T cells in systemic and AT inflammation, controversy continues as to which T-cell subset (CD4+, a specific CD4+ subset, or CD8+) plays dominant roles in human obesity and T2D. Studies focused on pro-inflammatory functions of CD8+ T cells in DIO mice show these cells infiltrate AT early and produce chemotactic cytokines such as MCP-1/CCL2 and MCP-3/CCL7. Such chemokines induce macrophage activation and recruitment into AT. Thus, CD8+ T cells function, in part, by directing myeloid cell trafficking. Furthermore, genetic depletion of CD8+ T cells can decrease inflammation and improve IR to further demonstrate the importance of CD8+ T cells in adipose-associated inflammation (24). Comprehensive studies identifying the abundance and function of CD8+ T cells in obese/T2D patient blood or AT remain unreported. It will also be important to analyze multiple human AT depots because of the demonstrated differences in visceral and subcutaneous AT (74) (Fig. 1). We predict that both CD4+ and CD8+ T cells will play critical roles in T2D inflammation, although the more tangible advances towards clinically altering CD4+ T-cell subset distribution may yield therapies over the shorter term (75).

It is critical to highlight the fact that many of the studies supporting roles for pro-inflammatory T-cell infiltration and/or cytokine production in obese AT rely exclusively on mRNA levels rather than analysis of cells as functional units. Furthermore, multiple reports that have used flow cytometry to immunophenotype either blood or AT at the cellular level are impossible to evaluate and/or reproduce because of absence of primary flow data. These limitations are easily overcome with existing technologies including sensitive multiplex protein analyses, enzyme-linked immunospot assay, and 6+ color flow cytometry. However, despite these shortcomings, the demonstration of decreased AT T-cell infiltration in response to T2D treatments (76, 77, see below) is consistent with pro-inflammatory T cells supporting IR/T2D in mice or humans and justifies more systematic analyses.

T-cell responses to T2D treatments

Multiple lines of evidence show that changes in T cells parallel treatment-associated improvement in metabolic health.
the comorbidities common to those who are centrally obese. With peripheral AT excess are generally more insulin sensitive and lack protective Treg function, also decreased following weight loss. 

Fig. 1. Pattern of excess adipose tissue deposition associates with metabolic health. Overnutrition is perhaps the best understood cause of obesity and T2D; however, common obesogenic insults such as bisphenol A (BPA) ingestion are also present in human diets. Unlike mice, individual differences in AT deposition in humans are highly variable, but can be grouped based on dominance of central obesity (the ‘apple’ shape) or peripheral obesity (the ‘pear’ shape). Individuals with central obesity are generally metabolically less healthy, with exception of the metabolically healthy obese group, and are characterized by pro-inflammatory immune cell infiltration and macrophage-rich crown-like structures (CLS) in the expanded visceral fat depots (omental, mesenteric, and epiploic). They often have fatty liver disease with or without liver inflammation and immune cell infiltration. Cardiovascular and microvascular disease are common in ‘apples’. Excess lower body fat, as seen in ‘pears’, generally associates with non-inflamed AT. We speculate this because of the need for serial sample collection.

Weight loss caused by gastric banding and calorie restriction decreased the circulating Th1/Th2 ratio in obese individuals. The demonstration that CD25+ T cells, often associated with protective Treg function, also decreased following weight loss indicated that a complete shift from pro-inflammatory to anti-inflammatory CD4+ T-cell subsets may not have been achieved during the time frame of the study. Interestingly, the percentage of naïve T cells (indicated by CD62L surface expression) decreases with hypocaloric/banding-mediated weight loss yet increases following gastric bypass and associated weight loss (78, 79). Apart from changes seen after invasive surgery and weight loss, blood T-cell subsets also shift in response to T2D medications. For example, circulating T cells shifted from activated (CD69+) to protective (Foxp3+) in response to various T2D treatments (76, 77). Studies featuring responses to PPARγ agonists, drugs with known efficacy in T2D, show these drugs also decrease T cells levels in (murine) AT, as measured by CD3 mRNA (76). Careful temporal analysis and serial sampling are needed to discern whether T-cell alterations lead or follow metabolic improvement and/or weight loss.

Utility of blood T cells as an indicator of metabolic health

Because of the similarities in gene expression between obese human visceral/inflammatory AT and blood immune T cells (51, 52) and our data that show T2D associates with an increased ratio of pro- to anti-inflammatory T cells (45), blood may supply accurate surrogate measures for T-cell shifts in the AT. The use of blood rather than AT is clinically important, as blood collection is a minimally invasive procedure that can be successful under modestly clean conditions. However, caution is indicated by the more complete array-based analysis of T cells in DIO mice. These studies showed that AT-associated T cells significantly differ from other cells, including spleen, lymph node, and blood T cells (47). Array analyses of human T cells purified from various AT depots by methods that least alter the cells in parallel with circulating T cells from the same person will fully identify such differences in humans and allow focus on attributes shared by AT-associated and blood T cells. Additionally, whether altered T-cell ratios are a leading or lagging indicator of T2D will require long-term longitudinal studies with careful clinical monitoring. Such studies would be more feasible if focused on blood T-cell characteristics, because of the need for serial sample collection.

Additional T-cell subsets implicated in obesity and T2D

CD4+ and CD8+ T cells are subsets of the most commonly studied T cells, the αβ T cells. However, more ‘innate’ T cells, designated γδ T cells [some of which also express CD8 (80)] have been implicated in obesity and IR. Potential γδ T-cell functions in metabolic disease include influx and pro-inflammatory cytokine production in the AT of DIO mice, including IL-17 as mentioned above (33, 81). Furthermore, the compromised immunosurveillance capability of γδ T cells in obese mice and humans may explain, at least in part, the higher incidence of skin diseases (psoriasis, atopic dermatitis) and wound repair defects in T2D patients (reviewed in 82). Compromised epithelial permeability may also present a chronic supranormal stimulus to γδ T cells, subsequently altering responses to bona fide threats to mucosal surfaces (82). However, in thinking about links between immune cells, wound repair, and mucosal health, careful consideration
has not been given to parsing out the critical influence of elevated blood glucose characterizing some T2D patients, as discussed below.

Another set of less well understood T cells that may play role in obesity and T2D are the natural killer T (NKT) cells, a rare T-cell subset that secretes a variety of cytokines traditionally attributed to more numerous CD+ T-cell subsets (83). However, the experimental data that aims to implicate NKTs in obesity and IR/T2D directly is mixed (84–87), further emphasizing the confusion with regard to this immune cell subset and its importance in T2D. Similarly, the role of non-T natural killer cells in obesity/IR/T2D is not well defined (43).

The role of B cells in obesity and T2D

Temporal pattern of B-cell response to obesity and T2D

In contrast to the strong association between altered T-cell function and T2D, roles for B cells in T2D have been addressed in very few studies. Perhaps the first study to indicate B cells play any role in obesity and IR was completed in New Zealand obese (NZO) mice, a strain naturally prone to obesity and IR. Unlike wildtype NZO, B-cell-null NZO males fail to develop IR. Interestingly, a cross between the obesity-prone NZO and the autoimmune/ genetically related NZB strain accelerated obesity and diabetes onset (88). This result indicated that an autoimmune background exacerbates the polygenically determined tendency for NZO to become IR. This work first indicated that predisposition to IR/T2D and autoimmune disease may be related. Later studies implicated B cells in IR, because B cells infiltrate expanding visceral AT in DIO mice weeks prior to T cells and macrophages (89), although in some studies elevated numbers of F4/80+ cells (presumably macrophages) could be detected in as little as one week following HFD feeding (90). Regardless, B cells are unlikely to be the first immune cell entering the AT in response to obesity. Neutrophil infiltration was reported to occur as early as Day 3 post-HFD initiation (91), consistent with the more standard pattern of cellular infiltrate in an inflammatory response. However, early B-cell infiltration (if independently confirmed) would indicate that the development of AT inflammation involves a fundamentally different order of cellular infiltrate compared with the classical inflammatory process that starts with neutrophils, followed by macrophages, and then (finally) lymphocytes. Importantly, very recent work demonstrated that regardless of when B cells first enter the expanding AT, they are present in the macrophage-rich crown-like structures characteristic of subcutaneous AT from morbidly obese patients (92). It therefore appears that B cells maintain an AT presence over the long term, even if lymphocytes are relatively rare in AT after long-term obesity (18, 92). These studies together indicate cellular AT infiltration is a highly regulated process perhaps orchestrated in part by early B-cell infiltration.

The role of B-cell antibodies in obesity and T2D

One prototypic B-cell product implicated in inflammation, thus in obesity and T2D, is antibody. Antibodies promote inflammatory responses through multiple downstream pathways, including the classical complement pathway (IgM/IgG), mast cell degranulation (IgE), and various types of hypersensitivity reactions (IgM/IgG). Antibodies also play key roles in autoimmune inflammatory diseases, including lupus and type 1 diabetes. We demonstrated that B-cell antibody production is temporally altered by hyperglycemia, a characteristic of uncontrolled T2D (93). This finding is consistent with the idea that some immune system functions are blunted by obesity, as further indicated by modest TLR responses in bone marrow-derived macrophages from obese mice responding to human periodontal pathogens (94). However, links among antibody production, hyperglycemia, and T2D may have clinical utility in only a subset of patients, given that glycemic control is the main goal of T2D treatment, and that the majority of T2D patients are able to adequately control blood glucose levels (as measured by HbA1c) until later stages of disease. In advanced T2D with poorly controlled blood glucose, immunological problems are overshadowed by more pressing maladies including CVD, microvascular disease, and loss of kidney function.

Recent evidence identifies a mechanism by which antibodies (and therefore B cells) may be critical regulators of obesity and T2D, functioning through their ability to protect the intestinal milieu and its associated microbiome. Mice lacking either B cells or IgA have significant alterations in their intestinal epithelium including upregulation of antiviral (interferon-inducible) pathways, lipid malabsorption, and decreased AT deposition under normal chow and high fat diet feeding conditions. The intestinal alterations required unknown ‘inputs’ from the microbiome. A control microbiome from wildtype mice sufficed to induce the epithelial alteration in the absence of B cells or IgA, indicating the unidentified microbial factor is from a typical commensal bug in the intestinal tract. B-cell-null mice had less visceral AT, even upon high fat diet challenge (95), presumably because of indirect effects on intestinal epithelium. We have independently confirmed this result (J. DeFuria et al., manuscript in preparation). The importance of B cells in intestinal epithelial function was...
consistently with analysis of various immunodeficiency/B-cell-compromised patients and their confounding malabsorption issues (95). These intriguing results justify follow-up work to link B-cell defects to lipid malabsorption more rigorously in immunocompromised individuals, including T2D patients.

Another mechanism proposed for antibody-mediated metabolic control is the prospect of auto-antibodies promoting IR/T2D. This seemingly heretical concept has been ignited by work demonstrating a limited T-cell repertoire in DIO mice (39, 47, 49, 96), presumably because of an elevated/inappropriate immune response to internal antigen(s) that activate(s) T cells at some point in disease pathogenesis. Alternatively, limited T-cell repertoires could be a response to an unidentified infectious agent that plays a role in T2D etiology/pathogenesis, although evidence for this possibility remains sparse. Recent results from an auto-antibody array approach demonstrated that obese/IR patients, defined based on steady-state blood glucose levels, have a higher prevalence of serum antibodies to human antigens (and possibly, auto-antibodies) than do obese/IS individuals (97). One feasible origin of auto-antigens may be apoptotic adipocytes in obese AT (98), but the human serum studies did not identify obvious adipocyte-reactive auto-antibodies (97). Regardless, the work convincingly demonstrated that IgG (but not IgM) from DIO mice increased glucose intolerance in B-cell-null mice and concluded that DIO B cells produce pathogenic IgG that promotes metabolic disease. Autoimmune function of the pathogenic IgG was also implied by these studies, although the presence of autoimmune IgGs in DIO mice was not definitively demonstrated (97). Contrary to the inferences of these studies, we have found no evidence of autoimmune activity in DIO mice (J. DeFuria et al., manuscript in preparation), undermining the conclusion that autoreactive antibodies drive obesity and IR/T2D.

Recent work on blood from phenotypic human T2D may explain some of the confusion concerning the autoimmune aspects of obesity/IR/T2D and initiate a better understanding of the so-called ‘type 1.5 diabetes’, also known as latent autoimmune diabetes of adults (LADA)(99). LADA is widely considered to be a T-cell-mediated autoimmune disease with late age onset as compared with type 1 diabetes; however, LADA is often confused with T2D because LADA patients are often obese. A dual characterization of clinically diagnosed ‘T2D’ patients based on the presence of circulating anti-islet antibodies and islet-reactive T cells indicated a significant percentage of T2D diagnoses (>20%) may instead be LADA (100, 101). Although these results indicate that T-cell-mediated islet destruction may be limited to ‘non-T2D’ diabetes patients, they may also call for adding anti-islet T-cell analyses to the clinical differentiation between T1D and T2D patients to begin seriously testing the possibility of T2D as a true autoimmune disease. The paucity of information on the role T cells (and more generally, immune system cells) play in the loss of islet function through autoimmune mechanisms indicates opportunity in this area of inquiry. Importantly, a LADA mouse model has not been reported.

Defining T2D as a true autoimmune/loss of self-tolerance disease would have substantial clinical impact, given the recent development and clinical trials of multiple autoimmune disease drugs. Many of these drugs block various aspects of B-cell function, including the recently approved autoimmune disease agent belimumab that functions by sequestering the B-cell survival factor B lymphocyte stimulator (BLyS) from B cells to decrease B-cell survival (102, 103). Overall, the seemingly contradictory evidence for/against B cells producing autoimmune antibodies in obese/IR individuals indicates that a more thorough analysis of T2D as an autoimmune disease will likely reveal new paradigms for understanding disease pathogenesis. Positive outcomes in such studies may justify clinical trials aimed at determining the efficacy of autoimmune disease drugs in the endocrinology clinic.

The role of B-cell cytokines in obesity and T2D
B cells are demonstrated sources of cytokines both in healthy individuals and those with chronic inflammatory disease, with generally anti-inflammatory IL-10 identified as the protective B-cell cytokine most commonly implicated in unresolved inflammation. Importantly, IL-10 overexpression can prevent IR in the DIO model (104). Attempts to establish a role for immune cell IL-10 in IR through bone marrow transplant of an IL-10-null hematopoietic compartment have been thwarted by compensatory IL-10 production by liver or AT, which rendered relationships between immune cell IL-10 and metabolic health tricky to interpret (105). However, IL-10 blockade in a rat model of DIO/IR increased hepatic inflammatory marker expression, decreased insulin signaling, and stimulated the gluconeogenesis and lipid synthetic pathways (106). IL-10-producing human B cells arise after stimulation through surface Ig alone or in combination with CD40 (107) or upon TLR-mediated stimulation (108). Studies in mice have identified IL-10 producing B cells as a separate B-cell subpopulation, designated B10 cells, or as a more inclusive subset designated regulatory B cells (Bregs) (109–114). Evidence for a human Breg equivalent was initially uncovered in a population of transitional B cells that likely protect against inflammatory disease (115). Additional studies have confirmed the
existence of anti-inflammatory human B-cell populations, although the markers associated with this subset remain controversial (116, 117). Breg cells may be identical to the CD27−IL-10-producing B cells that repopulate multiple sclerosis (MS), rheumatoid arthritis, and lupus patients after B-cell depletion (115, 118–121).

Our published work on B cells from blood of T2D patients support the more global possibility that lack of B-cell-produced IL-10 in T2D patients may compound elevated pro-inflammatory cytokine production from a variety of cell types. B cells from T2D patients, in contrast to B cells from non-diabetic donors, fail to secrete IL-10 in response to stimulation through various TLRs (122). These findings are qualitatively recapitulated in DIO mice (J. DeFuria et al., manuscript in preparation). Thus, the altered IL-10 levels uncovered by genetic analysis of T2D patients (123) may originate, at least in part, from lack of B-cell-produced IL-10. These data justify testing whether B-cell IL-10 deficiencies are physiologically dominant in IR/T2D inflammation, as has been found for other chronic diseases such as experimental autoimmune encephalitis (EAE)/MS and arthritis (118, 124, 125). This possibility is furthermore consistent with demonstrations that IL-10-producing B cells moderate inflammatory disease by blocking pro-inflammatory Th1 differentiation in mice (124, 125), and our demonstration of elevated Th1 function in T2D patients (45).

A second significant change we identified in T2D B-cell cytokine profiles was an unexpected increase in the pro-inflammatory chemokine IL-8. The general increase in IL-8 secretion in B cells from multiple classes of inflammatory disease patients in response to TLR ligands (reviewed in 126) demonstrated that TLR-mediated B-cell IL-8 secretion is a shared feature of B cells from inflammatory disease patients. Serum IL-8 positively associates with BMI and thus is elevated in obese individuals (127, 128). However, our preliminary data aimed at recapitulating these results in DIO mice, using MIP-2 and KC as IL-8 orthologs, showed opposite alterations in these neutrophil chemokines in DIO versus lean mice (authors’ unpublished data), raising caution about using mouse models to understand potential roles of IL-8 in human metabolic disease. Overall, decreased B-cell IL-10 logically links to inflammation and IR/T2D, but the additional importance of elevated B-cell-produced IL-8 remains to be determined.

B cells as master regulators of immunometabolism?

Although much of the published emphasis on the role of B cells in obesity and T2D has focused on B-cell-autonomous functions such as antibody or cytokine production, indirect roles of B cells are suggested by the intestinal epithelium studies outlined above (95) and by demonstrations that B cells are important antigen-presentation cells (APCs). B cells are thought to concentrate antigens to maximize stimulation of their cognate T cells through mechanisms that include linked recognition, cell-cell contact, and even T-cell-regulating cytokines such as IL-10 and IL-8. However, an increasing number of investigations report that B cells indirectly promote inflammatory disease through mechanisms that are likely to be IL-10 independent. For example, B-cell-mediated T-cell regulation plays a key role in diseases that are generally characterized as T-cell dependent, such as EAE/MS (124). Although some reports indicate B cells protect against CVD, one clinically relevant mouse study that used anti-CD20 to deplete B cells (similar to the FDA-approved drug rituxan) in a CVD model indicated instead that B cells support effector T-cell and dendritic cell (DC) activation in DIO mice. B-cell depletion under HFD stress also decreased Th1 function with a concomitant increase in Th17 function, as if DIO-induced loss of Bregs controlled T-cell balance (129). These findings were conceptually reproduced in independent studies, wherein B cell-null mice challenged with DIO had a decreased percentage of IFN-γ CD8+ T cells, and decreased IFN-γ production by cultured visceral AT immune cells. B-cell-null obese mice also had a decreased representation of inflammatory macrophages in visceral AT, thus healthier tissue (97). Our preliminary co-culture data from human PBMCs are consistent with the concept that B cells from T2D samples support pro-inflammatory T-cell function, and although cell-cell contact play some role in this interaction, detailed mechanistic analyses remain ongoing (M. Jagannathan-Bogdan, unpublished data). Overall, our work on human samples, in combination with results from DIO mice, indicate that B cells function through both direct (cell autonomous) and indirect (T-cell-mediated) pathways to regulate inflammation in obesity/T2D. Defining details of B-cell function are particularly critical clinically, given that the B-cell-depleting FDA-approved drug rituxan has a low incidence of major side effects (130–132) and would be ready to move into IR/T2D clinical trials over the short-term.

**Lymphocyte/adipocyte cross-talk in obesity and T2D**

**Adipocyte-mediated regulation of lymphocytes in obesity and T2D**

Adipocytes are the major sources of leptin and adiponectin, pro- and anti-inflammatory adipokines (adipocyte cytokines), respectively, which are altered in obese T2D patients (133, 134). Before T cells and B cells were fully appreciated as major
regulators of IR, multiple investigations showed that leptin, which is generally elevated in obese/IR people, has significant effects on T-cell differentiation and function. This work has been detailed elsewhere in this issue. To the contrary, adiponectin, a second major adipokine with anti-inflammatory properties, blocks antigen-stimulated T-cell proliferation. T-cell stimulation results in upregulation of both adiponectin receptors and cytotoxic T-lymphocyte antigen-4 (CTLA-4), a T-cell surface receptor that moderates the initially strong T-cell response. Thus, adiponectin reinforces the standard T-cell stimulation results in upregulation of both adiponectin receptors and cytotoxic T-lymphocyte antigen-4 (CTLA-4), a T-cell surface receptor that moderates the initially strong T-cell response. Thus, adiponectin reinforces the standard T-cell activation, and source of stimulatory ligands) remains an important field of inquiry (reviewed in 145), with the M1:2 (inflammatory:remodeling/protective macrophage) relationship an active area of investigation. However, from a more classical immunology viewpoint, the obese AT-associated inflammatory immune cells and IR pro-inflammatory adipocytes. A more definitive understanding of this loop along with future detailed time course analyses will be critical for designing experiments that test methods to pharmacologically interrupt the loop to delay or defeat metabolic disease.

Adipokine-mediated immune cell regulation occurs in parallel with regulation of adipocytes by immune cell cytokines. Pro-inflammatory IL-17 and IFN-γ promote human adipocyte IR and lipolysis and inhibit adipogenesis (65, 139). Anti-inflammatory IL-10, mainly from immune cells, protects murine 3T3L1 cells from IR; however, human adipocytes fail to respond to IL-10 because of a lack of surface IL-10 receptor (140). Overall, these results identify likely (if expected) mechanisms that exploit disease-associated adipokine and cytokine imbalance to drive a feed-forward loop between pro-inflammatory immune cells and IR pro-inflammatory adipocytes. A more definitive understanding of this loop along with future detailed time course analyses will be critical for designing experiments that test methods to pharmacologically interrupt the loop to delay or defeat metabolic disease.

The role of myeloid cells in obesity and T2D
The first demonstrations of changes in innate immune cells in response to diet-induced AT expansion were completed almost a decade ago. These studies showed a positive association between macrophages and BMI in obese mice (prior to diet-associated insulin elevation) and in humans (18, 19, 90) and represented landmarks in establishing the role of immune cells in IR/T2D. Subsequent work confirmed elevated AT macrophages prior to onset of IR in mice (90) and supported the conclusion that inflammatory macrophages (and perhaps other immune cells) are drivers rather than responders to IR. The likely multi-functional nature of myeloid cells was indicated by studies showing that macrophages are generally located around dying adipocytes in obese human AT (141), consistent with the interpretation that macrophages both promote and respond to obesity-induced changes in AT physiology. Although longitudinal studies in humans that parallel the mouse work have not been reported, several studies have found an association between visceral AT macrophage presence and obesity, particularly IR-associated central obesity (Fig. 1). Subcutaneous AT macrophages also increase in obesity, though to a lesser extent than visceral AT macrophages, especially in IR patients (142, 143). Preliminary human studies also showed that inflammatory characteristics of AT macrophages receded following weight loss surgery (144).

Although virtually everyone in the field of immunometabolism agrees that macrophages play major roles in IR development, the exact mechanism by which they are activated (including differential AT depot recruitment, in situ differentiation, and source of stimulatory ligands) remains an important field of inquiry (reviewed in 145), with the M1:M2 (inflammatory:remodeling/protective macrophage) relationship an active area of investigation. However, from a more classical immunology viewpoint, the obese AT-associated inflammatory immune cells and IR pro-inflammatory adipocytes. A more definitive understanding of this loop along with future detailed time course analyses will be critical for designing experiments that test methods to pharmacologically interrupt the loop to delay or defeat metabolic disease.

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macrophage subsets in the highly polarized obese AT. However, if even a subset of the F4/80^+CD11b^+CD11c^+ cells instead develop the champion antigen presentation skills of DCs, these cells may significantly shift obese AT physiology, even as they beg the question of the nature and identity of the ‘obesogenic’ antigen. In addition to the lack of knowledge of DCs as important obesogenic or diabetogenic antigen presenting cells (APCs), we also know very little about the possibility that changes in B-cell APC activity play an important role in obesity-associated inflammation through the regulation of T-cell responses. The recent appreciation of adipocytes as APCs and T-cell regulators (149) expands possible roles of classical immunological principles in establishing or maintaining obesity-associated effector T-cell function. The presence of AT APCs introduce the possibility that immune cell cross-talk in the AT may be regulated by contact-dependent, cytokine-independent mechanisms.

In addition to DCs as APCs, unique specializations of DCs also explain why understanding this cell type may be important for defining inflammation in obese AT. DCs, unlike monocytes or macrophages, are well known to support T-cell function by moving around the body in a reconnaissance mission designed to bring foreign antigens to the T-cell-rich lymph nodes and spleen. Macrophages, on the other hand, circulate as precursor monocytes, which can undergo long-term obesity/IR/T2D-associated changes, including enhanced ability to produce inflammatory cytokines in the absence of elevated anti-inflammatory cytokine production (M. Jagannathan-Bogdan, unpublished data). Activated monocytes enter tissue and differentiate into macrophages, often through a CCR2-dependent mechanism (150) and, like mature DCs, likely remain in the target tissue until they die. The understanding of mobile (potentially IR-promoting) DCs in AT physiology may advance the understanding of systemic outcomes of T2D that are not obviously linked to changes in AT macrophage distribution and function.

The importance of myeloid cells for indirect regulation of T2D pathology is highlighted by our data from T2D patients, which showed that pro-inflammatory T cells require monocyte co-culture to achieve T2D-associated levels of Th17 function. Our results furthermore indicated that myeloid cytokines play a minor role in T-cell support: IL-17 secretion by peripheral blood mononuclear cells (PBMCs) was similar regardless of whether the cultures were stimulated through the T-cell receptor (TCR) alone or the TCR in combination with the potent myeloid stimulant lipopolysaccharide (LPS). Furthermore, LPS alone had only a modest ability to activate IL-17 production by PBMCs in the absence of direct T-cell activation (45). Our preliminary work instead suggests that human myeloid cells require close approximation with T cells to boost Th17 function (authors’ unpublished observation), further supporting the conclusion that myeloid cells indirectly regulate T2D inflammation through their effects on T cells. Overall, these studies justify additional focus on function rather than surface phenotype of myeloid cells in human obese/TS, obese/IR, and obese/T2D cohorts to determine if particular subsets are possible targets for therapeutic intervention.

Although much of the work outlined above focuses on the role of one particular cell type in IR/T2D, it is obvious that the reality is more complex than the function of any one cell can explain. The overriding principle supported by currently available immunometabolism studies is that multiple immune cell subsets likely play key roles in each of the many pathways to IR and T2D. We furthermore predict that future work describing the cellular subsets that are critical at each decision point along the way to disease will reveal temporal as well as functional differences in the immune cell contribution to metabolic disease.

**Immunometabolism in the clinic**

**Blood cells as biomarkers for T2D severity and clinical trial outcomes**

Several lines of evidence raise the possibility that immunophenotyping blood will provide a clinically useful assessment of disease pathology/prognosis. This strategy will require researchers to focus on attributes shared between AT-associated and blood-borne immune cells, rather than highlighting differences as in published transcriptome analyses (47). First, lymphocytes likely become activated in the expanding AT, then re-circulate in the blood to promote the chronic systemic inflammation that characterizes obesity and T2D. This trafficking indicates the existence of lymphocyte activation characteristics shared between blood and AT. Second, our preliminary work indicates that in DIO mice, blood immune composition can at least partially reflect the phenotype of AT-associated lymphocytes (unpublished observation). Finally, a comparison between our studies on blood and human AT studies has indicated similarities between immune cells in human blood and AT (39, 45, 47). Taken together, these results focus on a likely subset of characteristics shared by AT and blood immune cells. Comprehensive studies on human immune cells focused on transcriptome (and other) similarities between AT and spleen/lymph nodes/blood will be essential for defining characteristics shared between immune cells from different tissues in T2D. A first step will be a comparison of
immune cell effector functions and surface phenotype from AT and blood of the same individual, a step absolutely required to assess the potential of blood immune cells as a superior diagnostic indicators for, as an example, short-term clinical trial outcomes. The functional flexibility of T2D blood Th17 cells (45) may offer an additional asset to shorten clinical trials in an attempt to control costs without compromising scientific value. To meet these goals, we suggest an approach that includes analysis of (i) multiple immune cell preparation methods (rather than sole reliance on the standard collagenase protocols developed to isolate adipocytes), (ii) variance caused by the anatomical location of the AT depot (Fig. 1), (iii) relationships between blood and AT immunophenotype, and (iv) immune cell function in a comprehensive slice of the obese/IR/T2D population, taking into account common clinical cohorts including those with impaired glucose tolerance and impaired fasting glucose. Such analyses are desperately needed to establish baseline parameters for human immunometabolism and related clinical studies moving forward.

The role of metabolic imbalance in immune cell-mediated pathogen resistance and pathogen tolerance

Long before T2D was recognized as a chronic inflammatory disease, physicians appreciated the differences in immunological processes between T2D and metabolically healthy, lean individuals. Lack of wound resolution, the relationship between CVD and inflammation, and the susceptibility to a specific subset of pathogens, including periodontal pathogens (7, 151), are all immunological processes with high clinical impact in obesity and T2D. Although obesity-associated IR is tightly associated with inflammation, and key pro-inflammatory immune cells from T2D or metabolic syndrome patients produce elevated levels of cytokines in response to standard experimental stimuli (45, 70, 122, 152), the clinical viewpoint is that T2D patients are to at least some degree immunosuppressed. T2D patients have elevated risk of common maladies such as urinary, mucous membrane, and respiratory tract infections (153, 154). However, there is no relationship between such community-acquired infections and HbA1c, indicating that the long term stability of blood glucose levels measured by HbA1c are not the major deciding factor in susceptibility (153, 154). The exceptions to this rule are infections with S. aureus and C. albicans, which are associated with elevated glucose (155, 156).

Vaccine-based studies also shed light on changes in immune system responses in T2D. One study demonstrated attenuated T-cell responses in T2D patients, as evidenced by a reduction in the percentage of IL-2 receptor-positive cells 72 h postvaccination. Other vaccine studies indicated at least the antibody-producing function of B cells are similar between T2D patients and non-diabetic individuals, as indicated by anti-vaccine antibody titers (157,158, reviewed in 159). One caveat to these clinical studies is the biochemical demonstration that unstable or consistently elevated blood glucose levels common in suboptimally controlled T2D patients associate with glycation of IgG, a covalent change that decreases IgG binding activity thus likely compromises immune responses independent of IgG levels (160, 161). Finally, studies in DIO mice show that obese/IR animals are more susceptible to P. gingivalis oral infection, due at least in part to muted TLR2 responses that contrast with the elevated TLR2 responses in B cells from either periodontal disease or T2D patients to artificially synthesized TLR2 ligand (94, 108, 122, 162). These data parallel human studies showing a higher incidence of periodontal disease in T2D patients (163).

Multiple mechanisms may lead to the increased susceptibility of T2D patients to pathogens, most of which involve interactions between metabolic imbalance and the immune system. One possibility is that only pathogens able to withstand the chronic inflammation and elevated immune response potential of T2D-associated immune cells can establish infection. However, strains isolated from infected T2D patients are, if anything, less virulent than strains that infect normoglycemic individuals (164). Perhaps the most likely possibility for perceived immune dysfunction in T2D lies instead in the idea that an appropriately balanced immune response is essential for efficient pathogen clearance. Balance could be defined by ratios among pro- and anti-inflammatory cytokines or in the difference between cytokine production under baseline versus stimulated conditions, with stimulation provided by pathogen ligands. Alternatively, temporal differences in cytokine production, apart from or in addition to quantitative differences, may render the pathogen response less effective in T2D.

Regardless of the validity of the above possible explanations for increased community infections in T2D patients, decades of clinical and experimental evidence supports the conclusion that changes in blood glucose concentration alters the immune response. The simplest studies have been those that measure changes in gene expression in cells treated with high versus low glucose concentrations. Such work has shown isolated monocyte cell lines exposed to high glucose under tissue culture conditions increase expression of pro-inflammatory genes (165), consistent with the possibility that even in well-controlled T2D patients, supra-optimal changes in glucose
concentration could activate gene expression. The importance of elevated blood glucose levels in T2D-associated immune dysfunction are further highlighted by a myriad of reports showing tight glucose regulation is absolutely essential for avoiding serious postsurgical infections in T2D patients. Mechanisms linking infection incidence with the glucose spikes that commonly follow surgery are not well understood. It has been speculated that elevated glucose not only alters immune system function but also provides pathogens with a ready source of fuel (glucose) for successful growth. In this scenario, the question remains as to why elevated glucose is not instead diverted to meet the high-energy requirements of an immune response to prevent infection. Stimulation by lipids or other metabolic products altered in T2D patients have been shown to have pro-inflammatory effects that are similar to glucose-activated inflammation (166), although the mechanisms underlying these effects are controversial. Additionally, high BMI increases the risk of onset and/or severity of MS and autoimmune thyroid disease (167, 168), indicating an improperly tolerized immune system contributes to the perceived elevated immune response, although definitive links to confounding elevated blood glucose or lipid imbalance were not examined. Taken together, these studies are consistent with the idea that the immune system in obesity/T2D is hyper-responsive, perhaps because of elevated levels of endogenous pro-inflammatory ligands such as glucose or lipids. This interpretation of an elevated immune response contrasts with the clinical observations of increased incidence of community-acquired infections in T2D, potentially explained by a hypo-functional immune system.

Rather than label T2D patients as immunocompromised or immunologically hyper-reactive as the above studies have, one option for better capturing the relationships of the immune system to T2D may be to define the imbalances among pro-inflammatory, anti-inflammatory, anti-pathogenic, and pathogen tolerance [the ability to ignore pathogen onslaught while remaining healthy (169)] mechanisms. Perhaps the most remarkable report of the dynamic relationships between immunological responses and metabolic status used a personalized ‘omics’ approach to track temporal changes in a generally healthy individual’s blood transcriptome, proteome, and metabolome over a 14 month period. This time course included two naturally acquired viral infections and auto-antibody analysis of a lean middle-aged male. The germane points of this analysis from an immunometabolism perspective included the astonishing elevation in blood glucose (from approximately 100 to 150 mg/dL) for months following a respiratory syncytial virus (RSV) infection. Equally interesting was the development of autoantibodies to an insulin receptor-binding protein, DOK6, during this infection. Less surprisingly, serum inflammatory cytokines and CRP spiked over a more compressed time frame postinfection with maxima shortly prior to maximum changes in metabolomics parameters (12 versus 18 days postinfection) (170). All of these changes were absent after a human rhinovirus (HRV) infection that occurred approximately 300 days prior to RSV detection. Although this analysis does not attempt to define underlying mechanisms responsible for these changes, it irrefutably highlights (i) the relationships among metabolic homeostasis and immune system function and (ii) the importance of thorough screening and follow-up on human subject studies. The prevalence of sub-clinical infections makes the latter a formidable task even for a seasoned clinical research team.

The general failure of anti-inflammatory therapies for T2D: possible explanations

Perhaps surprisingly, published and ongoing clinical trials to interrupt the adipocyte/immune cell inflammatory loop with anti-inflammatory drugs have had less than spectacular results, with modest decreases (0.5%) in HbA1c, a long-term measure of glucose regulation and an accepted clinical endpoint for T2D studies. Concomitant decreases in pro-inflammatory serum cytokines have been more dramatic (15, 171). Some of these reports represent molecular tweaking of drugs such as salicylic acid, first tested for efficacy in T2D blood glucose control in the late 1800s (172, 173). Although the historic studies resulted in the low-dose aspirin regimen prescribed for almost all T2D patients in the US today, this standard of care has effectively re-set baseline blood glucose levels. However, widespread aspirin use has failed to curb the T2D epidemic in recent times. One possible explanation for the seeming disconnect between anti-inflammatory drug efficacy and T2D as a chronic feed-forward inflammatory disease is that T2D drug trials generally recruit poorly controlled patients (avg. HbAc1 approximately 8.0%) with a multitude of comorbidities and diabetes medications and a diagnosis of T2D for an average of 5+ years (13, 15, 16, 171, 174, 175). Curiously, T2D resolution caused by bariatric surgery is most common in patients diagnosed with T2D <5 years (176), consistent with the possibility that the physiological characteristics of T2D becomes increasingly rigid over time. It is entirely possible that these long-term patients are refractory to anti-inflammatory drugs just as they become non-responsive to T2D medications that controlled their blood
glucose levels earlier in their disease. Whether there are disease stages characterized by weak links in the feed-forward inflammatory loop that are vulnerable to anti-inflammatory or immunomodulatory drugs is an important direction for future work.

Use of mouse models to understand human metabolic disease

Research into causes and treatments of obesity and T2D justifiably utilizes animals, and recent work continues to exploit the multiple strengths of mouse models. Such studies are absolutely required to test hypotheses in complex in vivo settings that cannot be recapitulated by purified cells or other ex vivo disease models. Mouse models have many benefits, including the ability to understand whole animal responses to over-nutrition, a common underlying cause of metabolic disease. Work on numerous genetically manipulated mice has also allowed investigators to pinpoint the function of many genes in the etiology of diseases. By their nature, animal model studies also move forward at a pace that easily outstrips progress attainable by clinical trials, suggesting new drug targets for the treatment of T2D and other obesity-related diseases at an ever accelerating pace.

A discussion of the role immune cells play in obesity must include seminal information from animal studies. However, it is important for both researchers and clinicians to bear in mind some of the immunological and metabolic limitations of translating hypotheses generated in animal models to patient treatments. Mice used for metabolic studies are often rendered IR because of genetic manipulation, such as a gene knockout. Gene knockouts can be mistaken as models for differential gene function/expression attributed to polymorphisms in human association studies. Although many genetic polymorphisms have been linked to obesity and/or T2D (reviewed in 177) and some have obvious connections with metabolic regulation, such as insulin receptor substrate-1 (178), the functional outcome of most polymorphisms is untested. Most difficult to understand is the functional significance of the many single nucleotide polymorphisms (SNPs) located outside protein coding regions that are assumed (but not usually demonstrated) to regulate gene expression. Overall, clean results from mouse knockout studies that delete the ortholog of a human SNP-regulated gene may or may not be relevant to association studies linking the SNP with obesity or T2D. Furthermore, more ‘naturally’ obese mice, such as the widely used ob/ob and db/db strains that are leptin or leptin receptor-deficient, respectively, should not be used for immunometabolism studies because of the importance of leptin signaling in normal lymphocyte development and function as outlined elsewhere in this issue. Finally, epistatic factors and compensatory mechanisms that come into play in mouse knockout strains may be irrelevant to whole animal physiological responses or human biology, although they may explain the relatively common loss of obese phenotypes following long-term breeding of genetically altered mice. Multi-gene mouse models of T2D, such as the TallyHo (TH) mouse, selectively bred to closely recapitulate many characteristics of T2D patients (179), may solve some of the artifact introduced by single gene mutation approaches, especially if stringent validation shows concordance between pathology-associated genetic variations in TH mice and patients. Alternatively, studies in purposefully outbred ’Collaborative Cross’ or ’Diversity Outbred’ mice may increase the translational potential of future animal studies.

Further differences between DIO mouse studies and human physiology indicate caution in labeling standard DIO studies as translational. In the DIO model, mice are fed chow in which 40–60% of the calories are from fat. Feeding generally continues for 12–16 weeks, although some investigators analyze outcomes after a year or more of high fat diet (HFD) (49). The pitfalls of this approach are many. First, shorter term feeding protocols cannot recapitulate the decades of over-nutrition and disease experienced by patients, although the percentage of fat in the less extreme diets (40% calories from fat), is near the range of what some people eat. Second, animals are usually housed in largely pathogen-free environments, with the hope of increasing signal to noise ratio of outcomes and eliminating the influences of confounding infections. Humans are constantly exposed to pathogens and are thus always mounting sub-clinical immune responses. Third, the immunological differences between mouse DIO and human disease abound. For example, murine AT houses many more macrophage-rich ‘crown-like structures’ than human AT, and about 50% of obese people lack crowns, at least in the subcutaneous AT (20, 142, 180). Part of these differences may be due to timing of the AT sample, as macrophage presence, at least in mouse epididymal AT, is an extremely dynamic process that waxes and wanes as AT expands, then remodels (20). In contrast to mice, which have an exceedingly crown-rich area in one defined part of the epididymal AT depot (the testis-distal portion), the various areas of human AT depots will likely never be systematically analyzed because of practical constraints. Furthermore, approximately 90% of DIO mice (typically on a C57BL/6 background or a knockout bred onto that background) become obese and IR, with elevated fasting blood glucose and glucose
intolerance. The naturally low insulin response of this strain likely explains this almost uniform experimental outcome. Humans, in contrast, exist in any possible combination of the above characteristics, including less numerous lean/IR and obese/IS phenotypes. Additionally, adipose distribution varies widely in humans but not in mice, with a ‘pear-shaped’ person generally more metabolically healthy and less inflamed than a centrally obese individual known colloquially as ‘apple-shaped’ (181–183) (Fig. 1). This diversity undoubtedly reflects broad genetic variation as well as food choices and other environmental factors that humans experience. In light of recent evidence for an autoimmune component of IR, it is important to realize that the C57BL/6 mouse strain most often used for DIO studies is not only susceptible to IR but it is also resistant to autoimmune disease. In fact, because of their low level of spontaneous autoimmunity, C57BL/6 mice have been used as an ideal strain for mouse knockout studies aimed at implicating particular genes in autoimmunity. Finally, human research is held to different (and arguably higher) standards than mouse DIO research, which may additionally complicate translation from mouse to human. Human T2D studies are expected to match subjects based on BMI to separate out obesity and T2D as two diseases, a reasonable goal that largely eludes murine analyses with the exception of a handful of obese but IS murine knockouts. Overall, these differences indicate that extrapolating from animal studies to humans is a high-risk endeavor.

Despite the shortcomings in mouse models of both obesity/IR and immunological responses outlined above, many of the immunological findings in the DIO model accurately recapitulate changes in blood immune system cells in long-term T2D patients as reported by our group and others (45, 122). More limited studies have also indicated similarities between mouse and human obese visceral AT immune systems (24, 39, 47, 49, 97). A more complete immunophenotyping of obese/T2D patient AT and rigorous comparison to murine results will allow more accurate comparison and define similarities that may be exploited for clinical research.

A working model for the role of immune system cells in obesity and T2D

Data from multiple studies in combination with our results indicate important relationships among immune cells that differ among lean/non-diabetic, obese, and T2D individuals. These data must be interpreted in the context established by murine DIO studies: immune cells can cause IR/T2D, and immune cells from healthy donors can also be significantly influenced by ongoing disease. Furthermore, published work demonstrates the likelihood of a pro-inflammatory feedforward loop between adipocytes and immune system cells in obesity and T2D. Our working model describing these interactions is shown in Fig. 2.

In non-diabetic (ND) lean individuals, monocytes, T cells, and B cells secrete relatively low to undetectable levels of cytokines that insignificantly affect AT and vice versa in a non-pathogenic homeostasis (Fig. 2A, black arrow below bracket). T cells and T-cell cytokines are likely regulated indirectly by B cells through B-cell induction of Treg expansion, indicated by the small black arrow. With increasing obesity (large black arrow), circulating free fatty acids (FFAs) and endotoxin increase (184) (Fig. 2B,C, stars). We speculate that despite these changes, a subset of individuals, those who eventually become morbidly obese, further expand their Treg percentages or numbers (Fig. 2C), and that this demonstrated Treg expansion allows continued storage of calories as fat with less dramatic metabolic changes, less inflammation, and avoidance of frank T2D. Metabolically healthy obese individuals may also fall into this physiological group, although this possibility has not been tested. We speculate immune cell ligands activate monocytes and B cells in all obese individuals through an undefined mechanism, inducing inflammatory cytokine production (185) (Fig. 2B,C, black arrows from stars). Increasing obesity also induces necrosis of adipocytes leading to recruitment of B cells, T cells, and monocytes into the AT, where monocytes become activated M1-like macrophages and all immune cells secrete a pro-inflammatory cytokine balance (Fig. 2B, large red arrow) (89, 186). Cross-talk between monocytes and T cells induces IL-17 secretion (Fig. 2B, double-headed arrow between monocytes and T cells), although this interaction is not T2D-specific and may not be responsible for elevated IFN-γ. B cells from T2D patients produce elevated IL-8 and very low levels of IL-10 (122), which defines the pro-inflammatory status of these cells. It is yet unclear whether these changes in B cells from T2D patients play a role in the pathogenic interaction with T cells either through cytokines, changes in surface co-stimulatory molecules or, potentially, the loss in their ability to induce Treg expansion (Fig. 2B, black ‘X’ between B cells and Tregs). Importantly, possible interactions between monocytes and B cells remain unknown (Fig. 2B, dashed arrow between monocytes and B cells), though our data indicate that monocytes could serve as additional B-cell activators prior to B-cell–T-cell interaction. Whether T cells from T2D patients have a low activation threshold because of the pro-inflammatory milieu triggering T-cell-intrinsic changes or because of other
physiological changes in metabolic balance is unknown. Although immune cell changes in morbidly obese but metabolically healthy individuals (Fig. 2C) have not been reported in detail, many changes may be similar to those shown in T2D, with the magnitude of changes moderated by increased Tregs (or, not shown, Th2 cells). Additional work on the specific roles of immune cells and their interactions along with their interactions with adipocytes to exacerbate chronical inflammation are essential to further our understanding of the complex interplay between these two seemingly unrelated systems in T2D.

Important outstanding questions in human immunometabolism

The evidence from DIO mice clearly shows that almost any interruption in inflammation prevents obesity and/or IR, thus
strongly supports the importance of inflammation in the etiology of metabolic disease. In contrast, most human immunometabolism studies to date have been snapshots from variably matched populations representing lean/IS, obese/IS, obese/IR, and/or T2D. Some of these human studies test the effects of diabetes drugs on immune cell distribution or function in ongoing disease. However, one intrinsic characteristic of these human sample studies is a focus on disease pathogenesis, rather than on disease etiology that can be studied only as subjects transition from obese/IS to obese/IR/T2D. In other words, there are no compelling anti-inflammatory clinical studies that are directly comparable to the etiological DIO mouse studies. Clinical trials thus far have been limited to relatively short-term inflammation blockade with a variety of drugs, all of which have had modest efficacy (at best) in restoring metabolic health. It is possible that the generally moderately to poorly controlled T2D patients recruited for these trials with disease duration of ≥5 years cannot benefit from anti-inflammatory therapies; it may simply be too late in disease pathogenesis. Alternatively, the lack of anti-inflammatory efficacy in T2D is consistent with the possibility that longer-term treatment is required to assess the promise of immunotherapy in stopping the metabolic deterioration that has developed over decades. It will be critical to determine on the one hand whether there are several immunological routes to T2D in obese individuals, or on the other hand, one main progression of serially dependent insults that produce metabolic disease. Similarly, it will be important to determine whether there is one main therapeutic route or several equally valid possibilities from the immunological point of view. Studies to determine whether relieving inflammation will prevent T2D comorbidities will require a significant long-term investment of time and dollars.

Part of the explanation for the lack of focus on halting the transition from obese/IS to obese/IR/T2D is the perceived ‘danger’ of immunomodulatory drugs, although some drugs, such as the B-cell depletion drug rituxan, have high safety profiles and do not result in general immunosuppression in adults (130). Although these drugs may not be as safe as the age-old advice on diet and exercise, decades of clinical research indicate that this safest option is failing miserably to make a dent in the obesity/T2D epidemic. Longitudinal analyses aimed at understanding critical tipping points in obesity-induced inflammation may identify immune-based therapeutics aimed at preventing the permanent transition from obese/IS to obese/IR or T2D. Relatively simple studies, such as understanding whether cell-intrinsic or cell-extrinsic factors play dominant roles in human disease, are hinting that cell-extrinsic factors matter for some cellular subsets (45). However, these studies are in their infancy. Such analyses will also answer the important question of whether we can identify an optimal time (either with the calendar or by using physiological measures of disease progression) for effective immunomodulatory treatment. Given the extended time course of disease etiology, researchers aiming to complete these analyses must plan (and find funding for) rigorous comprehensive clinical projects that last a decade or more.

In the end, the immunometabolism field must come to some conclusion as to the overarching goal of this newly defined research area. Is it to contribute to cutting edge science in a burgeoning discipline, publishing papers, and funding our research? Is it to justify use of approved and new immunomodulatory drugs to curb the global obesity and T2D epidemics? Hopefully a little bit of both as basic immunometabolism concepts are defined in humans and take a justified place in the toolbox of obesity and diabetes clinicians.

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