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Th17 cytokines differentiate obesity from obesity-associated type 2 diabetes and promote TNFα production

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Abstract

Objective—T cell inflammation plays pivotal roles in obesity-associated type 2 diabetes (T2DM). The identification of dominant sources of T cell inflammation in humans remains a significant gap in understanding disease pathogenesis. We hypothesized that cytokine profiles from circulating T cells identify T cell subsets and T cell cytokines that define T2DM-associated inflammation.

Methods—We used multiplex analyses to quantify T cell-associated cytokines in αCD3/αCD28-stimulated PBMCs, or B cell-depleted PBMCs, from subjects with T2DM or BMI-matched controls. We subjected cytokine measurements to multivariate (principal component and partial least squares) analyses. Flow cytometry detected intracellular TNFα in multiple immune cells subsets in the presence/absence of antibodies that neutralize T cell cytokines.

Results—T cell cytokines were generally higher in T2DM samples, but Th17 cytokines are specifically important for classifying individuals correctly as T2DM. Multivariate analyses indicated that B cells support Th17 inflammation in T2DM but not control samples, while monocytes supported Th17 inflammation regardless of T2DM status. Partial least squares regression analysis indicated that both Th17 and Th1 cytokines impact %HbA1c.

Conclusions—Among various T cell subsets, Th17 cells are major contributors to inflammation and hyperglycemia, and are uniquely supported by B cells in obesity-associated T2DM.

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Keywords

Obesity; type 2 diabetes mellitus; inflammation; T cells; Th17; B cells; lymphocyte; cytokines; principal components analysis; partial least squares discriminant analysis

Introduction

T cells play critical roles in obesity-associated inflammation and insulin resistance (IR) through production of cytokines that induce glucose intolerance and IR in adipocytes and hepatocytes ([1, 2, 3, 4, 5, 6]). Both $CD4^+$ and $CD8^+$ T cells are implicated in IR/type 2 diabetes mellitus (T2DM), but the clinical usefulness of these observations is limited by risks that accompany T cell manipulation ([7, 8]). Known discrepancies between murine and human immune cell function further undermine translatability of studies highlighting critical roles for $CD4^+$ Th1, Th2, Th17, regulatory T cells (Tregs) or $CD8^+$ T cells in murine IR ([1, 2, 6]). An unbiased analysis of human T cell inflammation in T2DM and the mechanistic links between T cells and classical inflammatory mediators of T2DM like TNFα are urgently needed to focus the field on inflammatory sources with high impact on disease pathogenesis. Identification of dominant players in obesity-associated inflammation also promises to address the modest metabolic improvements in clinical trials of anti-inflammatory drugs in IR/T2DM patients ([9]).

An imbalance amongst $CD4^+$ T cell subsets characterizes IR/T2DM in humans and mice ([1, 3, 10]), and various T cell cytokines, including IL-17 and IL-22, induce IR in cultured adipocytes, hepatocytes and muscle cells ([4, 6, 14]). However, definitive conclusions as to whether T cell cytokines, including IL-17A, IL-17RA, IL-21 and IL-22, promote or protect against obesity-associated IR/T2DM are undermined by discrepancies among knockout mouse studies ([6, 15, 16]). Similarly, although T cell inflammation in T2DM requires support from B cells, as measured by IL-17A or IFNγ production ([11, 12]) and parallel decreases in anti-inflammatory Tregs ([2, 11, 13]), mechanisms that link physiological changes in obesity/T2DM to the pro-inflammatory T cell balance remain poorly understood. A comprehensive analysis of T cell cytokines and the underlying cellular support systems are absolutely essential to identify key drivers of T2DM inflammation. Outcomes herein unify claims of the importance of single T cell cytokines and justify a shift towards emphasis on Th17 cells as major contributors to T2DM inflammation.

Methods

Human Subjects/Samples

The Boston University School of Medicine Institutional Review Board approved this study in accordance with the Declaration of Helsinki. Subjects (Table 1) were recruited from the Center for Endocrinology, Diabetes and Nutrition at Boston University Medical Center. Subjects with T2DM were (i) diagnosed with T2DM; (ii) taking T2DM medications; and (iii) under Diabetes Center care. Subjects with obesity but not T2DM were identified by % HbA1c≤5.7 and no T2DM diagnosis. Exclusions were severe comorbidities (renal failure, stroke, severe micro- or macro-vascular disease, blindness), common infections (colds, flu)

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≤2 wks before donation, and/or smoking. Although most subjects were obese (BMI>30), a minority of subjects from both groups was classified as overweight (N=2 or 4 for ND or T2D subjects, respectively). The analyses cannot account for the effects of the extensive list of drugs taken by subjects.

Cytokine analysis

Culture supernatant (25μl) was analyzed using the Th17 Milliplex kit (Millipore, Billerica, MA) and a Bioplex 200 instrument (Biorad). Internal standards were used to confirm plate-to-plate variability at <7%. Percent CV was ≤10%.

Cell culture

Blood was collected by venous puncture into acid/citrate/dextrose-containing tubes and processed as described ([11]). $CD19^-$ or $CD14^-$ PBMCs were prepared by positively selecting out $CD19^+$ B cells or $CD14^+$ cells, respectively, with magnetic beads (Miltenyi), and were confirmed as >97% depleted by flow cytometry. A cells were cultured at 1×10^6 cells/mL in "U" bottom wells in RPMI media supplemented with 10% FCS and antibiotics, and treated with α-CD3/α-CD28 for 40 hrs ([3]).

Blocking Antibody Studies

Neutralizing antibodies specific to IL-17A (100 ng-1μg) and IL-17F (1 μg) (R&D Systems or LifeSpan BioSciences) or IgG isotype controls (0.1–20μg) were added at the same time as stimuli.

Flow Cytometry

Stimulated PBMCs were cultured with Brefeldin A (3μg/ml; eBioscience) added for the last 4hrs. Cells were collected into RPMI/10% FBS with attached cells lifted with Cellstripper (Corning). All reagents were from Biolegend unless otherwise stated. Cells were washed and stained with Zombie Aqua fixable dye, CD19 BUV395 (BD Biosciences), CD4 PE, CD8 PerCP-Cy5.5, CD3 APC-Cy7 and/or CD11b PE-Cy7. Cells were washed, fixed with Fixation Buffer and treated with Permeabilization Buffer, then stained with IL-17F eFluor 660 (eBioscience) and IL-17A BV421 or TNFα APC. Data were acquired on a BD Biosciences LSR II then analyzed with FlowJo 10.0.7. Gating was based on fluorescence minus one and isotype controls. Fig. S1 details gating strategy.

Data Analyses

Data are means ± SEM unless otherwise indicated. Student's *t*-test and two-way ANOVA analysis with Tukey adjustments were performed in SAS 9.4 or Prism (GraphPad). $P < 0.05$ defined significant differences. For multivariate approaches, cytokine data were log transformed, variance scaled, and mean centered (z-scored). Hierarchical clustering was generated using the Euclidean distance between pairs of objects and the shortest distance between clusters. Unsupervised analysis included principal component analysis (PCA), which separates the data into principal components (linear combinations of variables) that best explain data variance. Supervised analyses included partial least-squares regression (PLSR) and partial least-squares discriminant analysis (PLSDA), which use linear

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combinations of cytokines to predict the variance in the dependent variables ([17], [18], [19]). PLSDA-determined cytokine profiles that separated discriminatory classes (e.g. ND vs. T2DM or PBMCs vs. $CD19^-$ PBMCs vs. $CD14^-$ PBMCs) were validated using a leave one out methodology ([18], [20]). PLSR determined the cytokine profiles that separated patients based on HbA1c, and prediction error was calculated using root mean-square error. Orthogonal signal correction, via direct orthogonalization or orthogonal projection to latent structures, was used to pre-process PLS models and aid in model interpretability ([21]). Multivariate analyses were carried out in MATLAB R2014b (Mathworks, Natick, MA).

Results

Cytokine production is higher in T cell-stimulated PBMCs from subjects with T2DM-confounded compared to non-T2DM confounded obesity

Multiple T cell cytokines and subsets have been proposed to play dominant roles in systemic and adipose tissue-associated inflammation in obesity-associated IR/T2DM ([1], [3], [13]). To understand the relative influence of a wide array of T cell cytokines in human T2DM, we stimulated PBMCs from all subjects with the T cell-targeted stimuli αCD3/αCD28, and used multiplex protein assays to quantitate secretion of 25 T cell-associated cytokines. We first ascertained minimum and maximum detection limits for each cytokine and individual plates based on inflection points of the 5-parameter logistic curves, with the lower limit of detection at or above the manufacturer's limit. We used these values to normalize the log10 of cytokine concentrations between 0 and 1 for each plate to allow high fidelity comparison across plates (Fig. S2) and defined cytokines as "interpretably expressed" if the median value from all samples was >5% or <95% of the detectable range (non-colored areas of Fig. S2). By these criteria, we continued analyses on IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-17F, IL-21, IL-22, MIP3α/CCL20, GM-CSF, TNFα and TNFβ.

Initial univariate analysis compared amounts of each cytokine in supernatants of cultures using values derived from the standard curve and plotted without further manipulation. Cells from subjects with T2DM compared to those without T2DM produced higher amounts of multiple Th17-associated cytokines including IL-17A, IL-17F and IL-21, although IL-22 was similarly produced (Fig. 1A). Th2 cytokines, including IL-4, IL-5, IL-10 and IL-13, were also higher in T2DM cultures (Fig. 1B), as were most Th1 cytokines, some of which also support and/or are produced by Th17 cells (IL-2, IL-6, MIP3α/CCL20, TNFα and TNF-β; GM-CSF trended towards significance P=0.06; Fig. 1C; ([22], [23], [24], [25]). IL-9, a Th9 cytokine also produced by Th17 cells ([26]) was higher in T2DM cultures (Fig. 1D). IL-1β, generally from myeloid cells, was produced similarly by PBMCs from people with and without T2DM (Fig. 1D), indicating that indirect myeloid activation following T cell stimulation was insufficient to achieve T2DM-associated increases in IL-1β expression following direct stimulation ([3]). Re-analysis of a subset of subjects that were age-matched (N=13 ND and 13 T2DM) and analysis of cytokine production by PBMCs from T2DM subjects in the younger (<56yrs) vs. older (>56yrs) half of the cohort showed no differences ($p>0.05$), consistent with the interpretation that the age difference between the two groups does not explain differences in cytokine production. Similarly, analysis of samples from females only (not shown) indicated the preponderance of females in the cohort without

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T2DM does not explain outcomes. Overall, numerous $CD4^+$ T cell cytokines are produced at higher amounts by PBMCs from subjects with T2DM.

T cell cytokines differentiate inflammatory profiles in samples from subjects with and without T2DM

To test the ability of cytokine profiles to differentiate PBMCs from individuals with and without T2DM, and to rank the importance of $CD4^+$ T cell subsets in T2DM, we analyzed cytokine production by αCD3/CD28-stimulated PBMCs with a variety of approaches. Hierarchal clustering, which finds single dimension associations, identified no obvious difference in cytokine patterns of samples from the two clinical groups (Fig. S3); we therefore focused on analyses that integrate multi-dimensional cytokine interactions.

We first analyzed cytokine profiles with PCA, an unsupervised approach to determine whether cytokines alone can distinguish samples according to group. Principal component 1 (PC1, X axis) explained ~48% of the difference between “compendium” cytokine profiles from subjects with and without T2DM (Fig. 2A), although overlap in clusters (grey) indicates modest separation of groups by cytokine profiles. Loading analysis, which ranked cytokines from most to least important for group separation along PC1, showed that multiple cytokines characteristic of Th17, Th1 and/or Th2 cells (ranging from IL-17A to IL-17F on Fig. 2B, with Th17 cytokines highlighted by black fill) were similarly important for differentiating cytokine profiles from the clinical groups, as indicated by loading values of 0.27–0.30 (Fig. 2B). We conclude multiple $CD4^+$ T cell subsets are similarly important for differentiating inflammatory profiles generated by samples from subjects with and without T2DM, although the relatively low rank of the Th1 cytokines (TNFβ, IL-6, TNFα, IL-12p70) suggests that Th1s contribute less.

T cell cytokines accurately predict clinical groups

PLSDA, a modeling approach that determines whether cytokine profiles from each subject “fit” that subject into the appropriate clinical group, classified samples from Fig. 1 into the correct clinical donor group 89% of the time (i.e. an 89% calibration accuracy; Fig. 2C). The clean separation of clinical groups (grey fill) indicates that PLSDA-generated latent variables (LVs) will more convincingly identify a T cell subset signature important for correct clinical classification. Loading analyses (to generate LVs) showed the Th17 signature cytokines IL-21, IL-22 and IL-17F are important for classifying a subject as T2DM (LV1; Fig. 2D, black bars). The Th1-associated cytokines IL-12p70 and IL-2 are similarly important to Th17 signature cytokines for classifying subjects as T2DM (Fig. 2D). In contrast, negative loadings for IL-13, IL-10, and IL-4 indicated that Th2 cytokines are important to classify a subject as ND. For cross-validation, we generated the model excluding data from one subject, then asked the likelihood of that model to place the subject into the correct clinical group. Cross-validation accuracy was 75%, much greater than the 50% accuracy expected by chance. We conclude that Th17 cytokines are at least as important as Th1 cytokines for a T2DM classification, while Th2 cytokines negatively associate with T2DM.

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T cell cytokines predict clinical measures of glycemic control

To determine the ability of cytokines to predict glycemic control, we analyzed Fig. 1 data by PLSR, regressed against %HbA1c. Loading analysis of PLSR-generated LV1, which explained 31% of HbA1c variation, indicated that the Th1 cytokines IL-2 and TNFβ, the Th17 cytokines IL-17F and IL-17A (in black), and cytokines made by both Th1 and Th17 cells (MIP3α/CCL20, GM-CSF) are important for explaining HbA1c variance (Figs. 2E–F). Th2 cytokines, with the exception of IL-5, were not impactful. We conclude Th1 and Th17 cytokines are candidate determinants of glycemic control in obesity.

B cells selectively support T cell cytokines in T2DM

B cells promote T cell IL-17A production in T2DM ([11]). To assess the role B cells play in our expanded appreciation of T cell inflammation in T2DM, we stimulated T cells with αCD3/αCD28 in the context of $CD19^+$ B cell-depleted PBMCs. B cell depletion decreased production of the Th17 cytokines IL-17A and IL-22 (Fig. 3A), the Th2 cytokines IL-4 and IL-10 (Fig. 3B), the Th1 cytokine IL-2 (Fig. 3C), and the Th9/Th17 cytokine IL-9 (Fig. 3D). B cell depletion had no impact on these cytokines in samples from subjects without T2DM (Figs. 3A–C). These findings indicate B cells support inflammation in T2DM through selective support of Th17 and non-Th17 T cell responses (Table 2).

$CD14^+$ cells support T cell cytokines independent of disease

$CD14^+$ cells (predominantly monocytes) promote IL-17A production by T cells from both clinical cohorts ([3], [11]). Consistent with our demonstration that B cell-T cell contact is required to maximize IL-17A in T2DM ([11]), studies with monocytes showed maximal T cell IL-17A production by cells from subjects without T2DM required monocyte-T cell contact, with results from T2DM cultures trending towards significance (P=0.06, Fig. S4). To further assess the role $CD14^+$ cells play in our expanded appreciation of T cell inflammation in T2DM, we stimulated T cells in the context of $CD14^+$ cell-depleted PBMCs. CD14 depletion significantly decreased production of the Th17 signature cytokines IL-17A, IL-17F and IL-22 in cultures from both clinical cohorts (Fig. 4A). In contrast, CD14 depletion decreased production of the Th2 cytokines IL-4 and IL-10, the Th1 cytokines IL-2 and MIP3α/CCL20, and the Th9/Th17 cytokine IL-9 only in cultures from subjects with T2DM (Figs. 4B–D). Given that myeloid cells are the major sources of IL-1β in PBMCs, CD14 depletion predictably decreased IL-1β production (Fig. 4D; Table 2). We conclude that $CD14^+$ cells support Th17 cytokines regardless of T2DM status, but that monocytes and B cells overlap in the ability to support non-Th17 arms of T cell cytokine production in T2DM.

Multivariate analysis highlights the differential impact of B cells and $CD14^+$ cells in samples from subjects with and without T2DM

For a more integrated understanding of cellular mechanisms of T cell-mediated inflammation in T2DM, we subjected depleted samples to PLSDA to identify cytokine profiles that classify samples into three distinct groups: total PBMCs, B cell-depleted PBMCs, or CD14-depleted PBMCs.

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PLSDA of cytokines from subjects with T2DM demonstrated that LV1 separated total PBMCs and B-cell depleted PBMCs from CD14-depleted cultures, while LV2 separated total PBMCs from B-cell depleted PBMCs (Fig. 5A). Loading analysis of LV1 identified two Th17 cytokines, IL-17F and IL-17A, as important for distinguishing CD14-depleted PBMCs from other groups (Fig. 5B, top). Unsurprisingly, IL-1β (predominantly a monocyte product) also highly impacted LV1, as did TNFα, a cytokine made by monocytes (Fig. S5) and T cells (see below) in T2DM. IL-6, a Th1 and Th17-supporting cytokine ([24]) was also important for distinguishing cytokine profiles from CD14-depleted PBMCs. In contrast, loadings on LV2 (Fig. 5B, bottom) demonstrated that IL-10 (Th2), IL-22 (Th17), IL-4 (Th2), and MIP3α/CCL20 (Th17/Th1) differentiate PBMCs from B cell depleted PBMCs. Previous work showing B cells from T2DM subjects produce little IL-4 and IL-10 ([27]) counter the theoretical possibility that B cell depletion removed a significant source of these cytokines. Furthermore, inverse association of MIP3α/CCL20 highlighted by PLSDA (Fig. 5B, bottom) contrasts with the lack of change in univariate analyses (Table 2), emphasizing the power of constrained multivariate analyses to identify impactful cytokines by incorporating complex cytokine interactions. We conclude that B cells likely use both direct and indirect mechanisms, perhaps including regulation of Th2 cytokines (IL-4 and IL-10), to regulate Th17s.

To further test whether B cells and monocytes differentially support T cell inflammation in samples from subjects with and without T2DM, we expanded analysis to variously depleted PBMCs from subjects without T2DM. PLSDA separated CD14-depleted PBMCs from total PBMCs and B cell-depleted PBMCs along LV1, but did not separate total PBMCs from B cell-depleted PBMCs (Fig. 5C). Thus total PBMCs and B cell-depleted PBMCs from subjects without T2DM secrete similar cytokine profiles. LV1 loadings showed that IL-17A, IL-1β, TNFα and IL-6 differentiate CD14-depleted PBMCs (Fig. 5D, top). Loadings on LV2 were uninformative (Fig. 5D, bottom). We conclude monocytes support a broad spectrum of T cell inflammation in subjects with or without T2DM, but B cells support T cell cytokines exclusively in subjects with T2DM.

IL-17 regulates TNF-α in T2DM

To independently query the importance of Th17 cytokines in T2DM inflammation, we quantified the ability of IL-17 to stimulate TNFα, the first cytokine implicated in obesity-associated IR ([28]). We stimulated PBMCs in the presence or absence of IL-17A and/or IL-17F blocking antibodies, then measured TNFα production in multiple PBMC cell types (Fig. S1) by intracellular staining. IL-17F, but not IL-17A, neutralization decreased TNFα production by $CD4^+$ and $CD8^+$ T cells (Figs. 6A–C), but had no impact on TNFα production by $CD11b^+$ cells (Fig. 6D), perhaps due to the ability of IL-6 and/or IL-1β, which activate monocyte TNFα. Neutralization of IL-17A plus IL-17F decreased modest B cell TNFα production (Fig. 6E). IL-17A/F neutralization also decreased IL-17A/F production by $CD4^+$ T cells from subjects with T2DM (Fig. 6F–G). Furthermore, the frequency of $TNFα^+CD4^+$ T cells rival the frequency of $TNFα^+CD11b^+$ myeloid cells under these conditions (Fig. 6A). IL-17A/F production by human non-T cells was minimal. Isotype controls for αIL-17A/F did not impact TNFα production (data not shown). These data definitively link the Th17 cytokine signature highlighted by PLSDA to TNFα production in T2DM.

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Discussion

Our data identify a T2DM-associated Th17 signature that regulates downstream TNFα production. Importantly, the Th17 cytokine signature is at least as influential in T2DM inflammation as Th1 or Th2 cytokine signatures that have been highlighted by published studies ([1], [5], [29], [30]), with the unreliability of IFNγ measurements (not shown) a moderating factor in this interpretation. Our approach also clarifies results from conflicting studies on whether a limited array of Th17 cytokines are increased in individuals with obesity-associated IR/T2DM, and addresses technical challenges in attempts to test associations between Th17 cells and T2DM in animal models ([3], [4], [10], [14], [16]). In addition to overall support for a role of Th17 cytokines in human T2DM, the flexibility of the Th17 signature defined herein and in our previous work ([3], [11]), and the significant correlation between frequency of all T cells subsets in human blood and adipose depots ([31]) highlights the possibility that the Th17 signature is a valuable biomarker for T2DM drug trials.

We have used multiple analytical tools to highlight the importance of Th17 cytokines for inflammation in T2DM; however, the ranking of different Th17 cytokines relative to each other and to non-Th17-associated cytokines varies by analysis type. The lesser relative contribution of IL-17A in the PLSDA compared to PCA may derive from greater involvement of IL-17A in physiological facets that cannot be definitively classified as “T2DM” or “ND”. Similarly, the PLS models regressed against HbA1c could be significantly influenced by the action of T2DM drugs developed to normalize HbA1c. However, taken in context with our other multivariate analyses, the result that Th1-related cytokines are at least as important in predicting HbA1c as Th17 cytokines suggests that T2DM-associated Th17 signatures function in part to exacerbate the Th1 response, which causes the downstream changes in adipose, muscle, and hepatic tissue.

The multi-cellular network that regulates inflammation in T2DM is reminiscent of demonstrations that B cells regulate T cells in autoimmune diseases like type 1 diabetes and multiple sclerosis ([32], [33], [34]). Similarly, B cells from both T2DM and rheumatoid arthritis patients use contact-dependent mechanisms to regulate Th17 cells, and inflammation may be further reinforced by the blunted ability of B cells to secrete IL-10 ([27], [35], [36]). The inability of B cells to produce significant IL-10 in T2DM may, in part, explain the disease-dependent outcomes of $CD19^+$ B cell depletion on cultures.

The multi-factorial basis of chronic inflammation in T2DM, including the adaptive immune cell mechanisms identified herein plus innate immune responses ([37], [38], [39]), predicts that anti-inflammatory drugs aimed at a single pro-inflammatory modulator, such as TNFα, will have modest efficacy in improving measures of T2DM inflammation and IR. This prediction has been accurate for clinical trials with anti-inflammatory drugs, although impacts of anti-inflammatory drugs on IL-17-associated comorbidities like hypertension ([40]) remain underexplored. Our data instead raise the possibility that FDA-approved B cell depletion drugs, such as anti-CD20, can be administered at some “sweet spot” in disease progression to prevent support of pro-inflammatory Th17s thus ameliorate an important driver of inflammation-associated IR. This possibility has not been tested, although short-term B cell depletion in T2DM mice increased glucose sensitivity ([12]). Overall, the Th17 signature

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represents a new logical starting point towards leveraging our appreciation of T2DM-associated inflammation into effective clinical approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

T2DM	obesity-associated type 2 diabetes mellitus
PCA	principal components analysis
PLSDA	partial least squares discriminant analysis
PLSR	partial least squares regression
IR	insulin resistance
ND	individuals with obesity but not type 2 diabetes
LV	latent variable
HbA1c	hemoglobin A1c
AT	adipose tissue

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Bullet Points

What is known

- Multiple T cell subsets and their signature cytokines have been implicated in the inflammation that promotes the development of type 2 diabetes in mice and humans with obesity
- B cells support the Th17 T cell cytokine IL-17A in samples from subjects with type 2 diabetes, but monocytes promote IL-17A through disease-independent mechanisms
- IL-17A triggers production of multiple immune cell cytokines by cells from lean subjects, and some of these downstream cytokines have been implicated in diabetogenic inflammation

What this study adds

- Signature cytokines for the Th17 T cell subset unexpectedly dominate cytokines from other T cell subsets in distinguishing inflammatory profiles from people with and without obesity-associated type 2 diabetes, and for predicting type 2 diabetes in subjects with obesity
- B cells support the predictive Th17 cytokine signature specifically in type 2 diabetes, while monocytes support inflammation from all T cell subsets in obese subjects irrespective of type 2 diabetes status
- Th17 cytokines support TNFα production in type 2 diabetes, linking a dominant source of T cell inflammation to a classical mediator of type 2 diabetes

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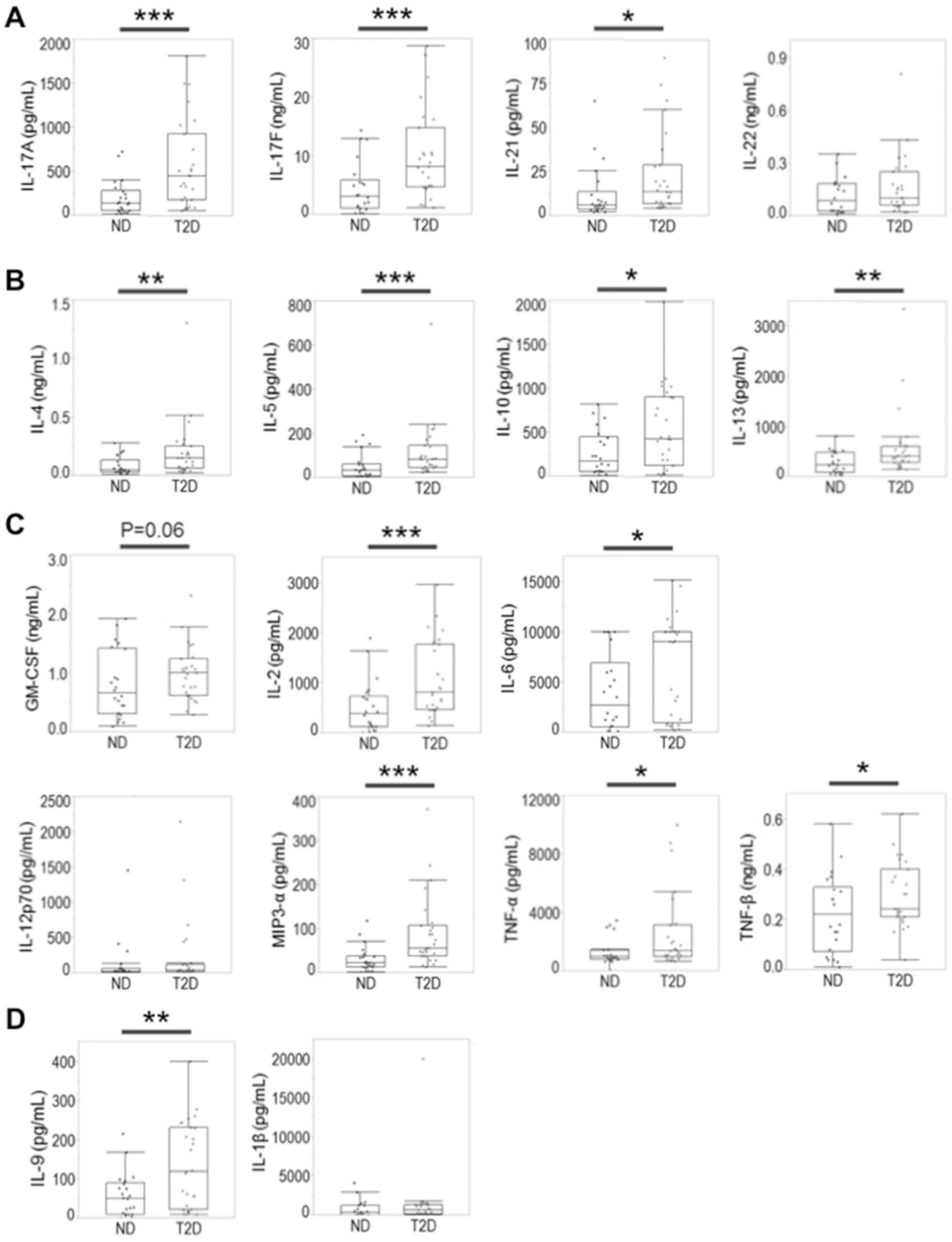

Figure 1. Multiple T cell-associated cytokines are produced in higher amounts by PBMCs from T2DM subjects

PBMCs from subjects in the ND or T2DM clinical groups were stimulated with αCD3/αCD28 before quantification of T cell-associated cytokines by a 25-cytokine bioplex. Cytokines are grouped by T cell subset that predominantly associates with each cytokine as **(A)** Th17; **(B)** Th2; **(C)** Th1 (with GM-CSF and MIP3α also made by Th17); **(D)** Th9/Th17/monocyte. Cytokines shown passed quality assurance steps described in the main text. For

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all panels, *, **, *** indicate differences at $p<0.05$, $p<0.01$ or $p<0.001$ by Student t test. N=22 T2DM or 29 ND.

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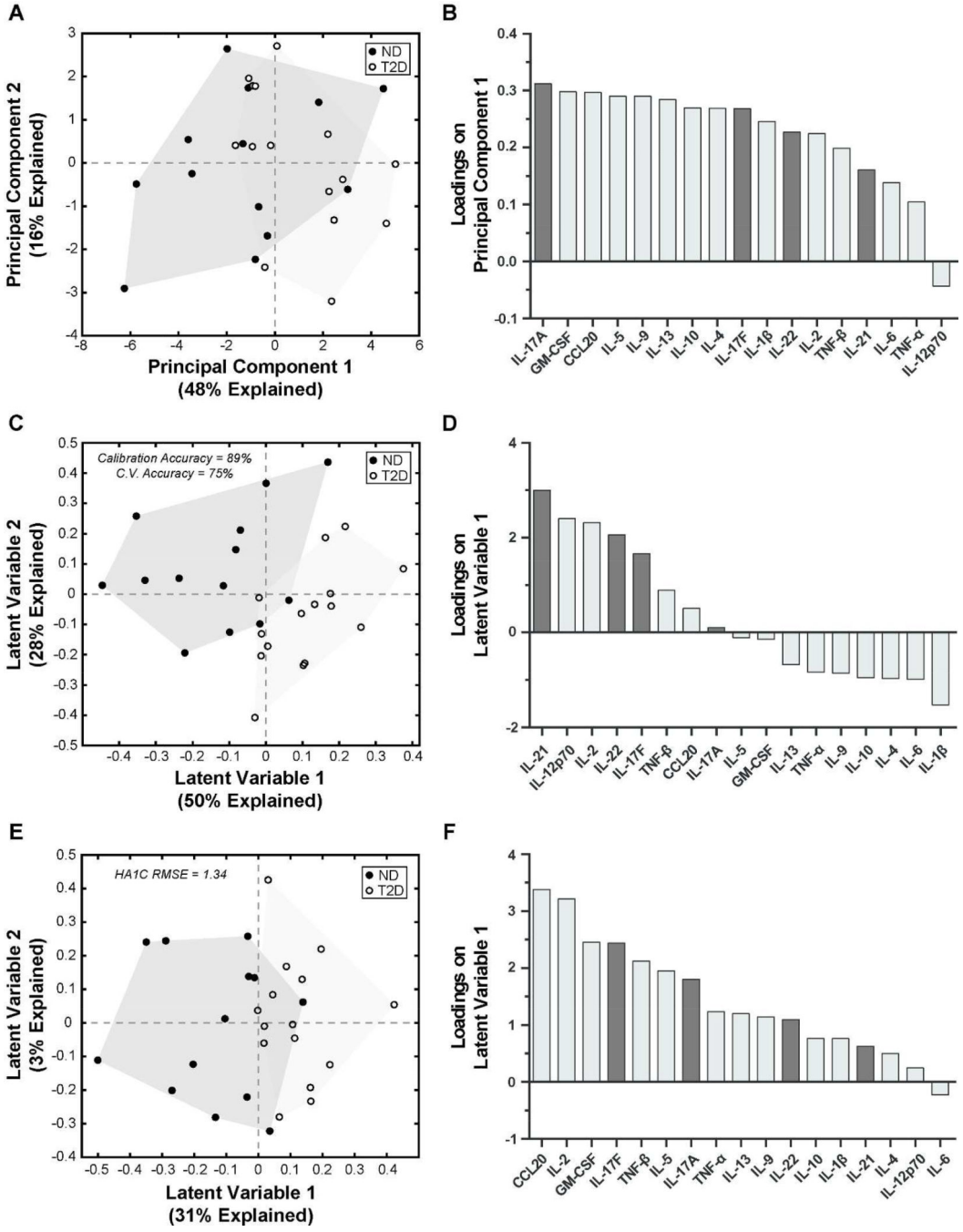

Figure 2. An inflammatory T cell cytokine signature differentiates T2DM from ND

(A) PCA of cytokines produced by PBMCs from T2DM (open circles) or ND (filled circles) subjects. **(B)** PCA loading analysis of principal component 1 (PC1). **(C)** Partial least squares discriminant analysis (PLSDA) of cytokine profiles used in panel A, analyzed for fit into clinical group. **(D)** PLSDA loading analysis of latent variable (LV) 1. Leftmost cytokines have highest influence on classifying subjects at T2DM; rightmost cytokines have highest influence on classifying subjects at ND. **(E)** Partial least squares regression (PLSR) analysis of cytokines, regressed against HbA1c, a measure of T2DM control. **(F)** PLSR loading

analysis of LV1. For panels B, D, and F, darker bars highlight Th17 signature cytokines. N=15 T2DM or 13 ND.

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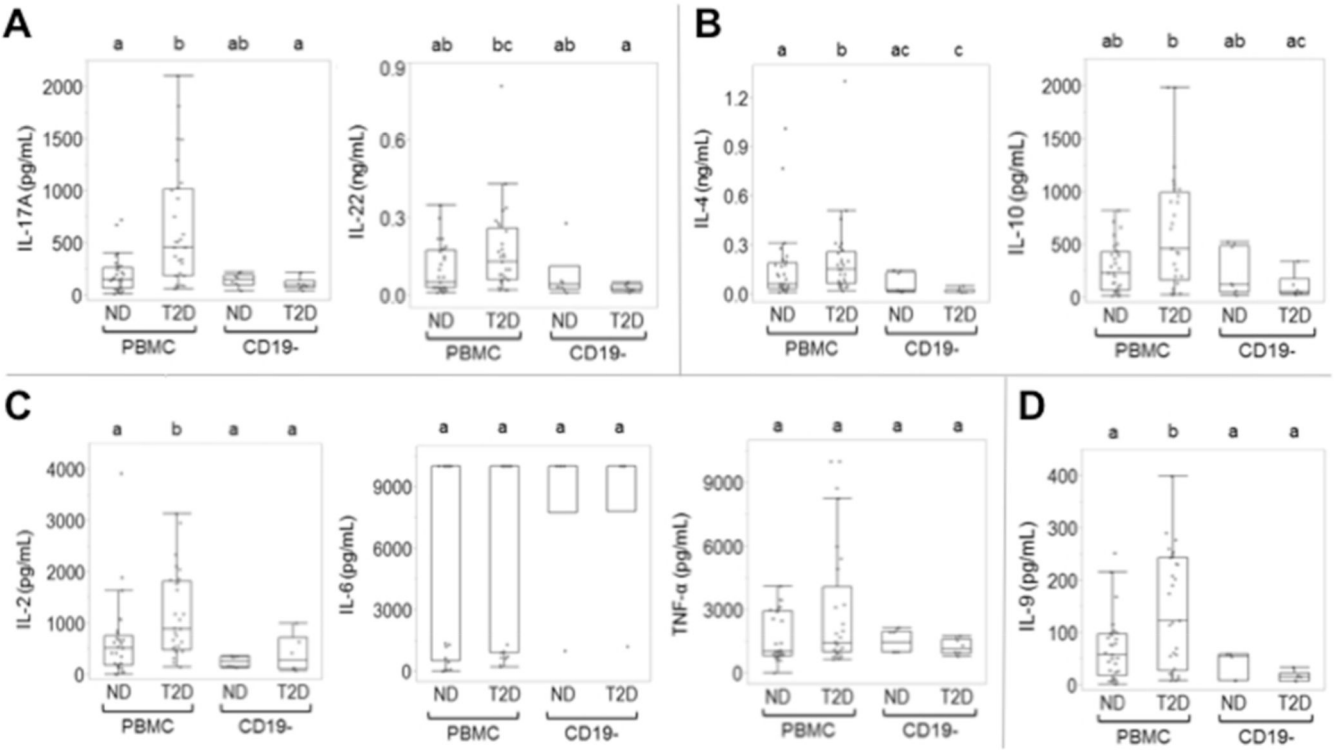

Figure 3. B cells support T cell inflammation in T2DM

Cytokine production in response to αCD3/αCD28 stimulation of PBMCs or B cell-depleted PBMCs ($CD19^{-}$) from subjects with or without T2DM. Cytokines are grouped by T cell subset that predominantly associates with each cytokine as **(A)** Th17; **(B)** Th2; **(C)** Th1; **(D)** Th9/Th17. Cytokines not shown were not significantly different (see Table 2). Box plots in the same sub-panel with different letters are significantly different ($p<0.05$, two-way ANOVA with Tukey's HSD *post-hoc* test). N=22 T2DM or 29 ND for PBMCs; N=6 for CD19-depleted PBMCs.

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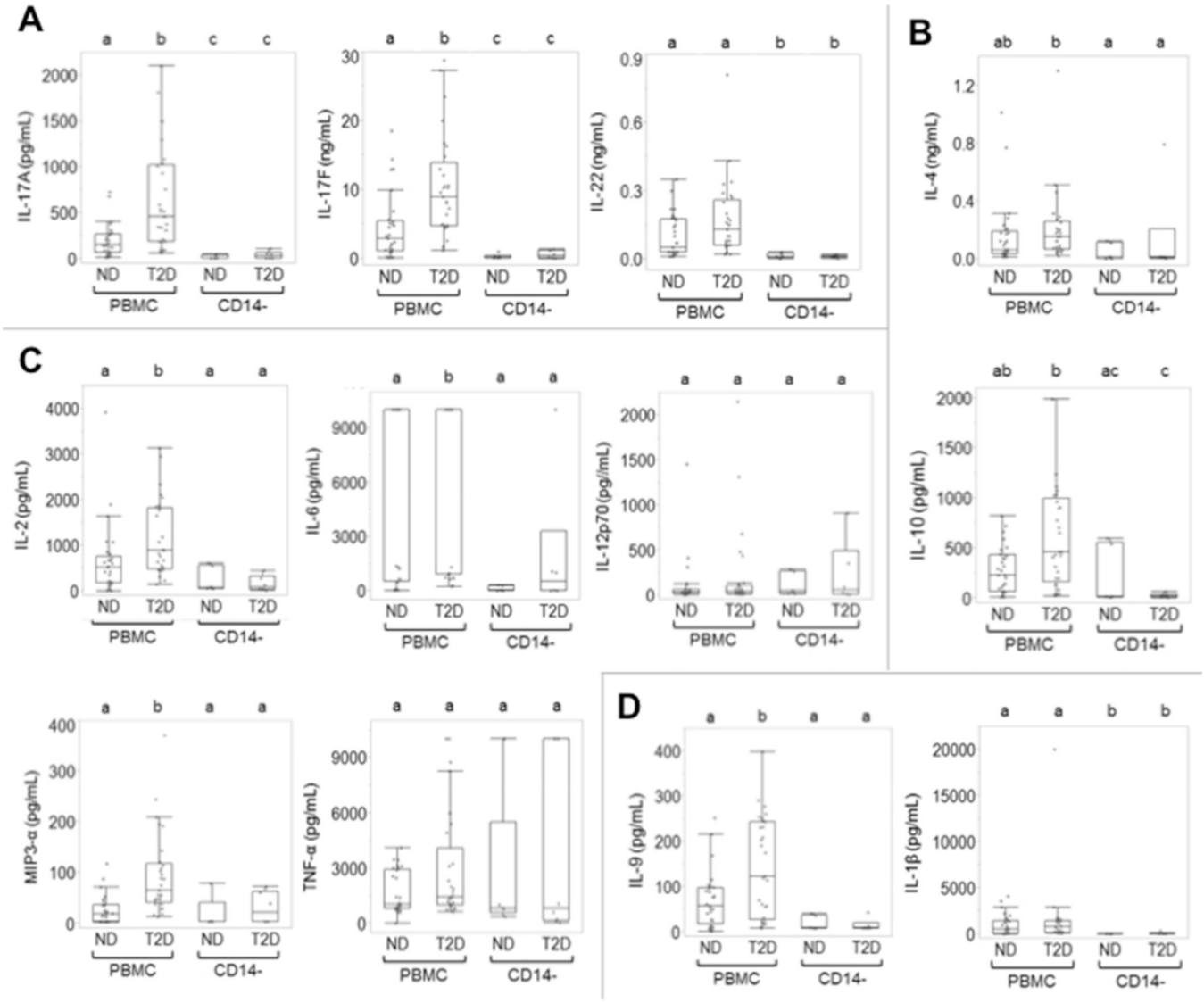

Figure 4. CD14$^+$ myeloid cells support Th17 inflammation in all subjects

Cytokine production in response to αCD3/αCD28 stimulation of PBMCs or CD14$^+$ cell (monocyte)-depleted PBMCs (CD14$^-$) from subjects with or without T2DM. Cytokines are grouped by T cell subset that predominantly associates with each cytokine as **(A)** Th17; **(B)** Th2; **(C)** Th1; **(D)** Th9/Th17/monocyte. Cytokines not shown were not significantly different (see Table 2). Box plots in the same sub-panel with different letters are significantly different as in Fig. 3. N=22 T2DM or 29 ND for PBMCs; N=6 for CD14-depleted PBMCs.

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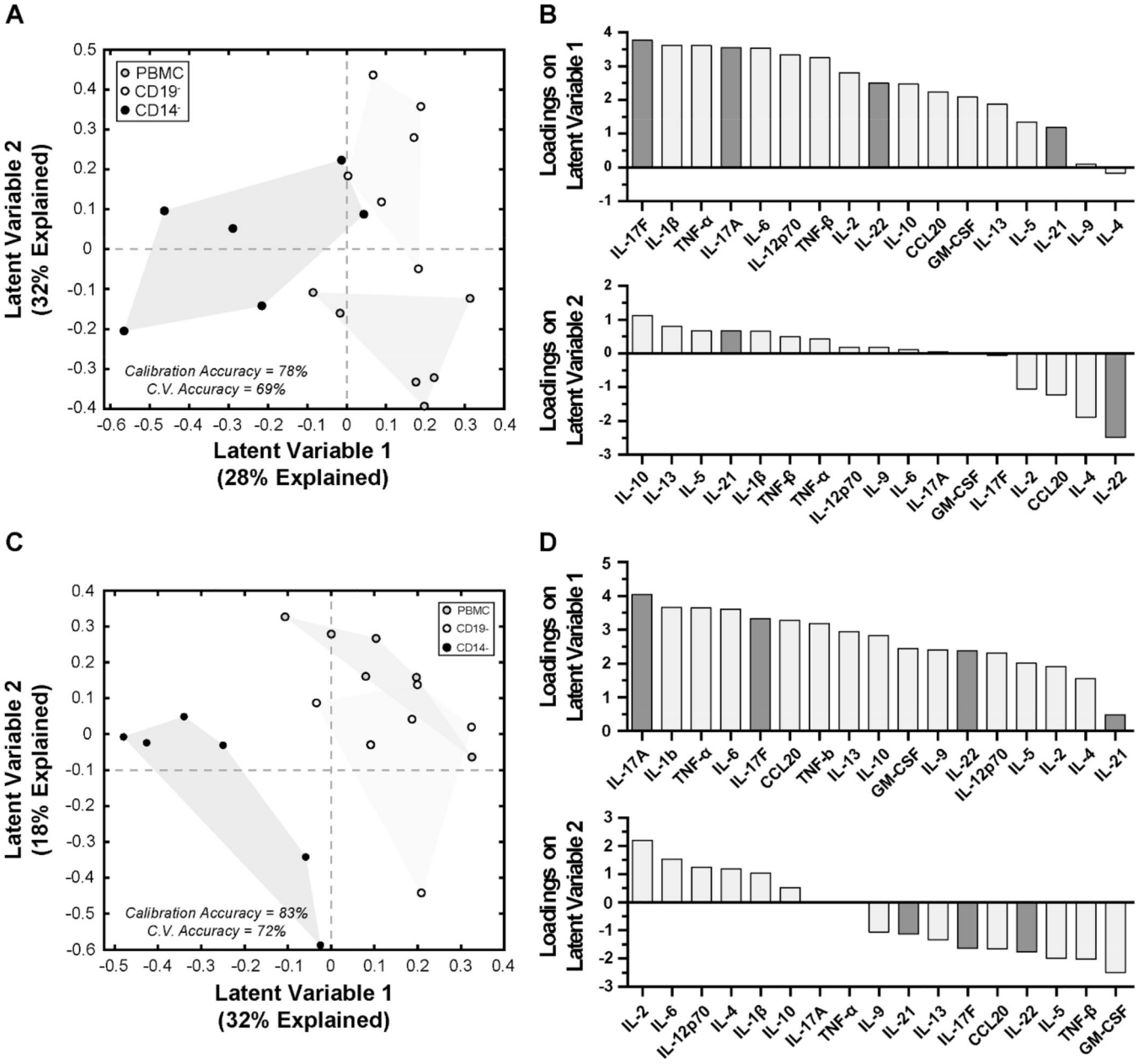

Figure 5. PLSDA analyses indicate B cells and CD14$^+$ myeloid cells support T cell inflammation through T2DM-associated or T2DM-independent mechanisms, respectively

PLSDA of PBMCs, CD14$^+$ cell-depleted PBMCs and B cell-depleted PBMCs from subjects with **(A–B)** or without **(C–D)** T2DM. PLSDA loading analysis on LV1 **(top panel in B, D)** or LV2 **(bottom panel in B, D)** using cytokine profiles from subjects with **(B)** or without **(D)** T2DM. N=6.

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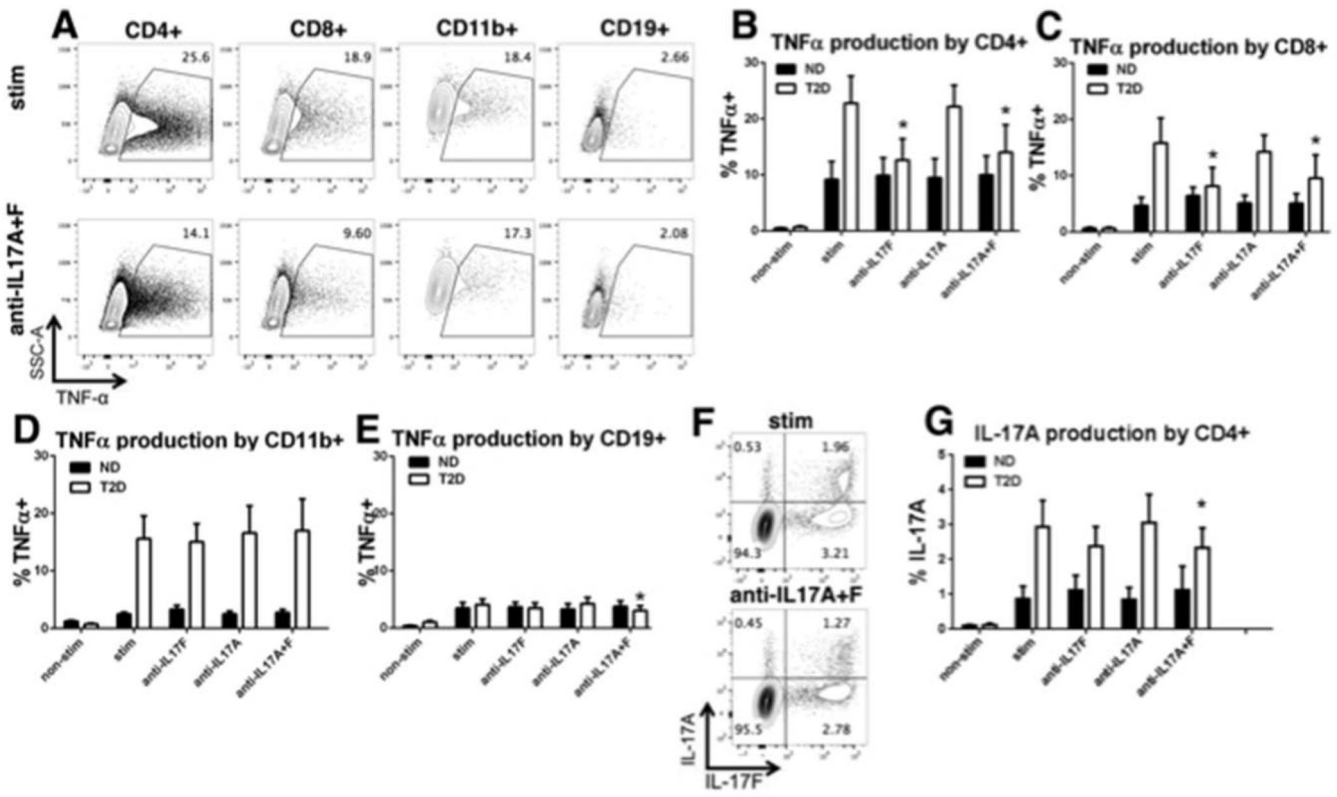

Figure 6. IL-17 blockade decreases T cell TNFα production in T2DM

(A) Percent intracellular TNFα-positive cells with surface marker expression as indicated in the absence (top panels) or presence (bottom panels) of IL-17 blocking antibody, following stimulation of PBMCs with αCD3/αCD28 (stim). Average percentage and SE of **(B)** $CD4^+$ T cells **(C)** $CD8^+$ T cells **(D)** $CD11b^+$ myeloid cells or **(E)** $CD19^+$ B cells that stain for TNFα in the presence of stim +/− blocking antibodies as indicated. **(F)** Representative and **(G)** average (+/−SE) percentage of IL-17A and IL-17F staining in $CD4^+$ T cells from panel B. "Non-stim" indicates PBMCs cultured in the absence of stimuli. "*" indicates significant differences ($p<0.05$) between αCD3/αCD28 stimulation alone or in combination with antibodies listed on the X axis. N=4.

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Table 1

Description of subjects

	Non-T2D	T2D
Age, *y [median (range)]*	41 (22–61)	52 (24–79) *
A1c, % *[median (range)]*	5.3 (4.8–5.7)	7.3 (5.7–12.2) *
A1c, *(mmol/mol) [median (range)]*	34.4 (29.0–38.8)	56.3 (38.8–110) *
BMI, *kg/m^2 [median (range)]*	34 (28–46)	34 (25–41)
Glucose, *mg/dL [median (range)]*	94 (62–202)	143 (90–256) *
Total N	29	22
Females	22	10
Males	7	12
Race		
African American	8	12
African American/White	1	0
Asian	1	0
Hispanic	7	3
Indian Sub-Continent	0	1
White/non-Hispanic	12	6

* indicates a significant different by two-tailed t test

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Table 2

Summary of cytokine production by B cell or monocyte-depleted PBMCs

Cytokine	T2D B cell-depleted	T2D monocyte-depleted	ND B cell-depleted	ND monocyte-depleted	
Th17					
IL-17A	↓	↓	-	↓	*,**
IL-17F	-	↓	-	↓	**
IL-21	-	-	-	-	
IL-22	↓	↓	-	↓	*,**
Th2					
IL-4	↓	↓	-	-	*
IL-5	-	-	-	-	
IL-10	↓	↓	-	-	*
IL-13	-	-	-	-	
Th1					
GM-CSF	-	-	-	-	
IL-2	↓	↓	-	-	*
IL-6	↓	↓		↓	*,**
IL-12p70	-	↑	-	-	*,**
MIP3α	-	↓	-	-	*,**
TNFα	-	-	-	-	
TNFβ	-	-	-	-	
Other					
IL-9	↓	↓	-	-	**
IL-1β	-	↓	-	↓	**,***

Shown in black arrows is direction of a statistically significant change in cytokine amounts in the indicated cultures compared to non-depleted PBMCs. Cytokines are grouped by the dominant CD4+ T cell subset shown to produce the set of cytokines.

"*" indicates outcomes that differ based on presence/absence of T2D;

"**" indicates outcomes that differ based on identity of the cell type depleted.

IL-1β, highlighted by ***, is decreased to at or below the level of detection by depletion of CD14+ cells (predominantly monocytes), consistent with demonstrations that monocytes produce IL-1β.

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