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Impaired CD4+ and T-helper 17 cell memory response to *Streptococcus pneumoniae* is associated with elevated glucose and percent glycated hemoglobin A1c in Mexican Americans with type 2 diabetes mellitus

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Abstract

Individuals with type 2 diabetes are significantly more susceptible to pneumococcal infections than healthy individuals of the same age. Increased susceptibility is the result of impairments in both innate and adaptive immune systems. Given the central role of T-helper 17 (Th17) and T-regulatory (Treg) cells in pneumococcal infection and their altered phenotype in diabetes, this study was designed to analyze the Th17 and Treg cell responses to a whole heat-killed capsular type 2 strain of *Streptococcus pneumoniae*. Patients with diabetes demonstrated a lower frequency of total CD+T-cells, which showed a significant inverse association with elevated fasting blood glucose. Measurement of specific subsets indicated that those with diabetes had, low intracellular levels of interleukin (IL)-17, and lower pathogen-specific memory CD4+ and IL-17+ cell numbers. No significant difference was observed in the frequency of CD4+ and Th17 cells between those with and without diabetes. However, stratification of data by obesity indicated a significant increase in frequency of CD4+ and Th17 cells in obese individuals with diabetes compared with nonobese individual with diabetes. The memory CD+T-cell response was associated inversely with both fasting blood glucose and percent glycated hemoglobin A1c. This study demonstrated that those with type 2 diabetes have a diminished pathogen-specific memory CD4+ and Th17 response, and low percentages of CD+T-cells in response to *S. pneumoniae* stimulation.

Streptococcus pneumoniae is the most frequently identified pathogen in community-acquired pneumonia in the United States in the elderly. It is also an important cause of infection in individuals with underlying medical conditions such as type 2 diabetes and heart disease.[1] Epidemiologic studies show that pneumococcal infections are more severe and associated with more complications in individuals with diabetes than in healthy adults.[2,3]

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Diabetes increases the risk of pneumococcal bacteremia and mortality 1.5-fold and 3- to 4-fold respectively.[3,4] Hospitalization rates for individuals with diabetes and pneumococcal pneumonia are significantly higher than for healthy individuals of similar age (19% vs 15%).[5] The incidence of invasive pneumococcal disease is also higher in those with type 2 diabetes (25.2–39.39 cases/100,000/y) compared with individuals without diabetes (7.5–9.3cases/100,000/y).[3] These clinical observations suggest that individuals with diabetes have an immune dysfunction that limits control of *S. pneumoniae* infection.

Elimination of pneumococci requires effective innate and adaptive immune responses. Innate responses require an influx of neutrophils at the site of infection, deposition of complement factor C3 on pneumococci, and subsequent opsonophagocytic killing of complement-coated pneumococci by neutrophils and macrophages.[6–9] Recently, the importance of T-helper 17 cells (Th17) cells in prevention of carriage or early pneumonia has been reported.[10] Interleukin (IL)-17, the effector cytokine secreted by Th17 cells has proinflammatory functions that enhance pneumococcal clearance by recruiting and priming neutrophils for secretion of antibacterial proteins and peptides such as beta defensins, and by promoting interferon γ (IFN-γ) production to enhance macrophage function for enhanced phagocytosis and intracellular pneumococcal killing.[11,12]

Diabetes was recently shown to be associated with an imbalance in the ratio of T-regulatory (Treg)/Th1/Th17 cells, with the preferential differentiation of CD4+ and Th17 cells as opposed to Th1 or Treg cell populations.[13–15] It was further shown that an increase in the number of Th17 cells and its signature cytokine IL-17 exacerbated inflammation. Given the importance of CD4+ and Th17 cells in an effective immune response to pneumococcal infections, it is likely that alterations in CD4+Th-cell response may, in part, play a role in the observed increase in the susceptibility of patients with diabetes to pneumococcal infection and disease.

MATERIALS AND METHODS

Subjects

The study was conducted using participants from Cameron County Hispanic Cohort (CCHC). The CCHC is a community-based cohort with more than 2000 participants with high rates of obesity and diabetes.[16,17] The rates of diabetes among participants of the CCHC were found to be twice the national rates of diabetes among all Americans and nearly twice as high as previously established rates among Mexican Americans.[16,17] For this study, individuals with diabetes were defined based on American Diabetes Association 2006 criteria, which include a diagnosis of diabetes and on medication for diabetes, or those with a fasting blood glucose (FBG) level of more than 126 mg/dL or an hemoglobin A1c level of more than 6.5. Those with FBG values of less than or equal to 126 mg/dL and no history of diabetes or diabetes medication were classified as patients without diabetes. In the study samples, 20 individuals were identified with diabetes compared with 16 without diabetes. For analysis, the American Diabetes Association 2010 and 2006 criteria for defining diabetes were used. However, observations did not find any differences in analysis based on the two criteria; therefore, results presented are based on the 2003 diagnosis criteria for diabetes.

Ethical approval

Collection of samples and the research described in this manuscript was approved by the University of Texas Houston Health Science Center, School of Public Health, Ethics Committee and the institutional review board (reference no. 069996; title: Innate immune

responses in chronically hyperglycemic patients and association between chronic hyperglycemia and infection control).

Sample collection and processing

A total of 30 mL blood was collected in citrate-treated tubes. Peripheral blood mononuclear cells (PBMC) were purified by density-gradient centrifugation (Polymorphprep; AxisShield). Blood was layered over Polymorphprep in 15-mL polypropylene conical tubes and centrifuged for 35 minutes at 500*g* at room temperature. Cells were washed twice with Roswell Park Memorial Institute medium (RPMI) (Sigma-Aldrich) and centrifuged at 400*g* for 10 minutes. Trypan blue exclusion was used to determine viability and cell concentration. A viability greater than 95% was considered for subsequent cryopreservation. Cells were frozen at a concentration of 10^7 cells/mL in RPMI, 40% fetal bovine serum (FBS), and 10% dimethylsulfoxide at a cooling rate of −1°C/min. Cells were stored at −80°C until used.

T-cell stimulation

Stimulations were carried out using whole heat-killed capsular type 2 pneumococci (*Streptococcus pneumoniae* [D39]). Pneumococci were grown from freezer stocks until cultures reached an optical density of 0.4 (approximately 1×10^7 cfu). Cells were collected by centrifugation, washed, resuspended in saline, and incubated at 65°C for 15 minutes to kill pneumococci. Heat-killed pneumococci were pelleted, and the pellet was resuspended in cell culture media containing RPMI and 10% fetal calf serum (c-RPMI). Phytohemagglutinin was used as a positive control for T-cell stimulation. T-cell stimulation and intracellular cytokine profiling were standardized as described previously, with minor modifications.[18] PBMCs were thawed quickly in a 37°C water bath and washed twice with c-RPMI (cell culture RPMI containing RPMI and 10% FBS) at 700*g* for 7 minutes. Cell concentration was adjusted at 1×10^6 cells/mL using trypan blue exclusion with a viability of 90%–95%. PBMCs were stimulated with 10^8 cfu *S. pneumoniae*, phytohemagglutinin (3 μg/mL) (Remel; Fisher Scientific), or c-RPMI media for 15 hours. Brefeldin A (BD Bioscience) was added for the last 7 hours of incubation. After stimulation, cells were held at 4°C overnight followed by cell-surface staining as follows.

Cell-surface staining

Stimulated PBMCs were centrifuged at 250*g* for 10 minutes and resuspended in BD stain buffer (BD Biosciences) for stain with the antibodies. Cells were labeled with antibodies for cell-surface markers as follows. For identification of Th17, cells were incubated with fluorescein isothiocyanate anti-CD4, and phycoerythrin-Cy7 anti-CCR6 for T memory/T naive phycoerythrin-anti-CCR7 and antigen presenting cell anti-CD45RA. To detect Treg cells, fluorescein isothiocyanate anti-CD4 and antigen presenting cell anti-CD25 antibodies were used. To detect intracellular cytokines, cells were fixed and permeabilized with permeabilization buffer (BD Biosciences), then cells were stained with either PerCP-Cy5.5 anti-IL-17 A or PE anti-Foxp3 and resuspended in BD stain Buffer for flow cytometry.

Sample acquisition was performed on a FASCSCanto II Flow cytometer with FACSDiva 6.1.2 software (BD Biosciences). Instrument setup, automated compensation, and cytometer quality control were completed with BD FACS 7-Color Setup Beads (BD Biosciences). Acquisition gates were set using a purified lymphocyte population, and gates were set on a purified CD+T-cells population. At least 50,000 lymphocytes and 10,000 CD+T-cells events were recorded. Fluorescence-minus-one controls were used to determine positive/negative boundaries. Samples were analyzed using FlowJo (TreeStar) software. Total CD4+ cells were first adjusted to 10,000 and then analyzed subsequently for intracellular cytokine expression. Expression of cell-surface markers are presented as a percentage of positive

cells. Intracellular expression of either IL-17 or Foxp3 is presented as mean fluorescence intensity.

Whole-blood stimulation and measurement of IL-17, IL-6, IFN-γ, and tumor necrosis factor-γ

Whole blood was diluted 1:10 in RPMI and 10% FBS, and incubated with 1×10^7 cfu heat-killed capsular type 2 strain D39 for 72 hours at 37°C. After incubation, cells were centrifuged and supernatants were collected and saved at −80°C for later analysis. Measurement of IL-17, and IFN-γ was performed using a multiplex enzyme-linked immunosorbent assay (Millipore Map, Millipore, Calif) kit per manufacturer's instructions and as described previously.[19] Undiluted supernatants were thawed on ice and incubated with antibody-coated beads, and were read using the Luminex 200 system (Luminex Corporation, Austin, Tex).

Statistical analysis

Univariate analyses of baseline variables found distributions to be nonnormal and skewed. As a result, nonparametric methods were used for data analysis. Differences in median values of baseline characteristics between individuals with or without diabetes were compared using nonparametric Wilcoxon 2-sample *t* tests for continuous variables or χ^2 tests for dichotomized variables.

Differences in percent positive CD4+, Th17, T-naive, T-memory, and Treg cells were compared among participants with and without diabetes using nonparametric Wilcoxon 2-sample tests. Differences in percent positive CD4 cells were compared between participants with and without diabetes, those who were obese (body mass index [BMI] greater than or equal to 30 kg/m^2 vs BMI less than 30 kg/m^2), those with FBG levels greater than or equal to 110 mmol/L vs less than 110 mmol/L, and those with percent glycated hemoglobin A1c (% A1c) greater than or equal to 6.5 vs less than 6.5 by non-parametric Wilcoxon 2-sample tests. Differences in percent positive Th17 cells, and ratios of Th17/Treg cells, were also compared among participants with and without diabetes, those who were obese, and those with metabolic syndrome using Wilcoxon 2-sample tests. Metabolic syndrome was defined as having a BMI greater than or equal to 30 kg/m^2 plus at least 3 of the following: FBG level greater than or equal to 100 mmol/L or diagnosis of diabetes, serum triglycerides level greater than or equal to 150 mg/dL, high-density lipoprotein cholesterol level less than 40 mg/dL (males) or less than 50 mg/dL (females), or hypertension (more than 130 mmHg systolic or more than 85 mmHg diastolic blood pressure). All analyses were considered significant when *P* values were less than 0.05. All analyses were run using SAS v. 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

Demographic characteristics of participants

A total of 36 participants were included in the study (20 with diabetes and 16 without). Information regarding pneumococcal vaccines was not collected from these participants. However, given the low level of health insurance (11.9%) and poor access to health care services,[17] it is highly unlikely that the majority would have received the pneumococcal vaccine. Nonetheless, given the widespread incidence of *S. pneumoniae*, the population is assumed to have been exposed to multiple different strains of pneumococci. We used the capsular type 2 strain of pneumococci for our studies for the following reasons: (1) this strain is sequenced previously and, therefore, information on genes and gene functions is available; and (2) it has been used previously in mouse studies, and data are available on *in vivo* responses to this strain in pneumonia, carriage, and sepsis. This study takes previous

results to the next level by determining the human CD+T-cell response to this strain of pneumococci in those with and without diabetes. Diabetes was self-reported, and 18 of 22 reported patients with diabetes were taking diabetes medication. Close to half of the participants (18 of 44) were overweight or obese, with BMIs ranging between 30 and 53.

Median values (interquartile ranges) for gender, age, BMI, and mean fasting blood glucose (MFBG) were compared, and the results are presented in Table I. Comparison of gender showed a significantly higher percentage of females in the group with diabetes compared with patients without diabetes ($P = 0.02$). Comparison of baseline characteristics showed significantly higher values of FBG and %A1c in patients with diabetes compared with patients without diabetes. Mean values for FBG and %A1c were significantly higher ($P = 0.001$) in subjects with diabetes compared with those without. Triglycerides were higher in the group with diabetes compared with the group without. No significant differences were observed in age of participants, blood pressure, C-reactive protein level, homeostasis model of assessment-insulin resistance, or BMI between patients with diabetes and patients without.

Diabetes is associated with a lower percentage of CD1T-cells

CD+T-cells are critical for an effective immune response to pneumococcal infections. With the observed compromised immunity of patients with diabetes against pneumococcal infections, the percentage of the CD+T-cell population was determined on stimulation with heat-killed capsular type 2 strain of pneumococci. The CD+T-cell data were stratified according to diabetes status, BMI, MFBG, and %A1c (Table II). A significant decrease in relative abundance of CD+T-cells was observed in the group with diabetes compared with the group without. In addition, the percentage of CD+T-cells was also decreased in patients with high MFBG levels and high %A1c compared with those with low MFBG levels and low %A1c. No significant differences in percent CD+T-cells were observed when participants were stratified by BMI status (Table II).

Lower CD4 T central memory (T_{CM}) and Treg response to *S. pneumoniae* in PBMCs from patients with diabetes

Natural infection by *S. pneumoniae* is characterized by nasopharyngeal carriage. Carriage of pneumococci in the nose can persist from several months to a year. Although asymptomatic, the carriage state results in a robust, pathogen-specific memory CD+T-cell response, and, on reinfection, this memory response is crucial for the elimination of pneumococci.[20,21] With the prevalence of *S. pneumoniae* infections in the general public, an assumption was made that the participants of this study had been exposed previously to *S. pneumoniae* and carry memory response. The purpose of these experiments was to determine whether participants with diabetes have alterations in their memory responses compared with control subjects without diabetes. As a definition for this study, memory T-cell subsets are determined by expression of CCR7 and CD45RA; central memory cells (T_{CM}) are CCR7+ and CD45RA−, and effector memory cells are CCR7− and CD45RA−. Naive T cells are designated as CD45RA+.

Cells from participants with diabetes stimulated with whole heat-killed *S. pneumoniae* demonstrated decreased central memory cells producing IL-17 compared with control subjects (Fig 1A). No significant difference was observed in IL-17 production from the naive CD+T-cell population between groups with and without diabetes (Fig 1B). Comparison of the ratio of T_{CM} cells vs naive CD+T-cells that produced IL-17 on stimulation with pneumococci indicated a significant decrease ($P < 0.05$) in the group with diabetes compared with the control group without diabetes (Fig 1C). In addition, there is a significant inverse association of IL-17-producing CD4+ T_{CM} cells with FBG levels and

%A1c, with higher FBG levels and higher %A1c associated with a lower relative abundance of IL-17-producing T_{CM} cells (Fig 2A and B). No difference was observed in relative frequencies of IL producing CD4+Th17-cells between diabetes and non-diabetes (Fig 3A).

Treg cells, defined as CD4+ and Foxp3+ cells, play an important role in controlling the inflammatory response to pneumococcal infection. Thus, a balanced response between Treg cells and effector T cells is important for efficient protection against pneumococcal infections. Analysis of the percentage of Treg cells present during stimulation with pneumococcal antigens showed no differences between the groups with and without diabetes (Fig 3B).

Altered Th17 response to *S. pneumoniae* associated with obesity and fasting blood sugar

Presence of memory CD+T-cells resulted in the expansion of effector CD+T-cells, which in this case were the Th17 cells. Therefore, it was next determined whether having a smaller pool of memory CD+T-cells would have an effect on CD4+ and Th17 cells in samples stimulated with whole heat-killed pneumococci. Among the CD+T-cell-helper subsets, Th17 has been shown recently to play an important role in preventing mucosal carriage and invasive infections by *S. pneumoniae*.[10–12] Th17 cells recruit neutrophils to the site of infection and enhance phagocytosis of invading pneumococci.[11,22] To investigate the presence of Th17 cells during pneumococci stimulation, analysis of CD4+, CCR6+, and IL-17+ cells were examined by flow cytometry. No differences in percentage of IL-17-producing Th17 cells were observed between patients with diabetes and without. A significant increase in percentage of IL-17-positive Th17 cells was found when data were stratified by BMI, suggesting that the combination of obesity and diabetes has a higher impact on Th17 than diabetes alone (Table III). Although patients with diabetes did not differ from patients without diabetes when percentages of Th17 cells were compared, there was a significant inverse correlation in percent Th17-positive CD+T-cells and FBG in diabetic samples when stimulated with pneumococci (Fig 4B), whereas a nonsignificant positive correlation was found between percent Th17 cells and FBG in patients without diabetes (Fig 4A).

To determine whether the number of cells found positive for Th17 in those with and without diabetes produces the same amount of IL-17, geometric means of fluorescent intensities were compared. Comparisons demonstrated a significant difference in levels of IL-17 between those with and without diabetes. Levels of IL-17 were also found to be significantly lower in participants with FBG values of 110 mg/dL or less compared with individuals with FBG levels greater than 110 mg/dL. Intracellular IL-17 was higher in participants older than 60 years of age compared with younger participants (Table IV).

Elevated levels of extracellular IL-17 and IFN-γ in response to *S. pneumoniae*

To determine whether the intracellular levels of IL-17 correlated with the extracellular protein concentration in those with diabetes, and to determine activation of the Th2 subset of CD+T-cells, the extracellular levels of both IL-17 and IFN-γ were assessed. Measurements were made in those with and without diabetes using Luminex technology. A significantly higher concentration of both IL-17 and IFN-γ was observed in supernatants of activated whole blood (Fig 5A and 5B). To correlate with the previously observed higher percentage of Th17-positive CD+T-cells in obese individuals, levels of IL-17 were compared between obese participants with and without diabetes. No significant difference was observed in the concentration of IL-17 between obese patients with diabetes and patients without diabetes (Table V).

DISCUSSION

This study is the first to demonstrate that patients with diabetes have deficiencies in memory CD+T-cell response and in relative abundance of CD+T-cells responding to *S. pneumoniae*. Diabetes is a known risk factor for pneumococcal pneumonia, in that pneumonia in those with diabetes is severe and often fatal.[2,4] An effective immune response to *S. pneumoniae* involves both T-cell dependent[12] and independent[23–25] responses. T-cell-independent protection occurs via generation of anticapsular antibodies whereas T-cell-dependent protection is mediated via a CD4+Th17 cell subset.[1] We have reported previously 2 significant observations related to humoral responses: (1) low levels of antibodies to protein antigens in those with diabetes compared with control subjects without diabetes and (2) antibodies from those with diabetes were less efficient in fixing complement on the surface of pneumococci compared with antibodies from control subjects without diabetes.[26] Data presented herein show alterations in cell-mediated immunity; an altered memory CD4+Th17 response in those with diabetes was observed. These alterations included reduced frequencies of IL-17-producing memory CD+T-cells as well as total CD+T-cells in patients with diabetes compared with patients without diabetes, suggesting a possible explanation for impaired antibody response. These observations are supported by recent studies suggesting impairments in antigen presenting cells and neutrophils, and alterations in CD4/CD8 ratios.[27–29] The observed immune cell dysfunction has been attributed to hyperglycemia and its associated advance glycation end products.[30,31] Reports from several studies provide evidence demonstrating that accumulation of advanced glycation end products and other ligands of receptors for advanced glycation end products (RAGE) results in upregulation of RAGE on immune cells such as dendritic cells, neutrophils, and T lymphocytes in a feed-forward mechanism.[32,33] Expression of RAGE on CD4+T lymphocytes has been shown to modulate adaptive immune responses in diabetes, most likely by altering proliferation rates of CD+T-cells in response to antigen and through changes in CD3 and CD28 costimulatory molecule expression.[30,32,34] Therefore, the observed lower frequency of CD+T-cells and memory CD4+Th17 cells is likely the result of hyperglycemia and expression of RAGE, and subsequent failure of response. Although the presence of RAGE on CD+T-cells was not assessed specifically here, the data indicate a strong negative association between memory CD4+Th17 cells, FBG levels, and %A1c, indicating that hyperglycemia does play a role in modulating responses and phenotypes of CD+T-cells. Another possibility for the observed low frequency of CD+T-cells could be impairment in antigen presenting cells, which play a significant role in recognition of pneumococcal antigens and subsequent activation of naive CD+T-cells.[35]

In contrast to previous reports in which an increase in Th17 cells has been observed, no significant differences in percentages of Th17-positive CD+T-cells in patients with and with diabetes was observed. However, a significant difference in the levels of secreted IL-17 was noted; concentrations of IL-17 were higher in those with diabetes compared with those without. This is in line with previous observations of higher IL-17 levels in those with diabetes.[13] Higher levels of secreted IL-17 in the presence of fewer Th17 cells suggests increased activity of those fewer cells to produce IL-17. Moreover, there are cells in whole blood such as γδ-T cells that produce IL-17 in addition to CD4+Th17.[36,37] Therefore, it is possible that, in diabetes, the level of IL-17 either represents production of IL-17 from both γδ-T and CD+T-cells or it could be that Th17 cells are the only source and that these cells are hyperactive in those with diabetes compared with those without. Further explanation for this observation comes from previous studies from our laboratory and from laboratories of other investigators demonstrating elevated levels of IL-6 in individuals with type 2 diabetes.[19,38,39] The pleiotropic cytokine IL-6 is known to affect differentiation of both Th17 and Treg simultaneously by regulating their respective transcription factors. It has been shown to activate the expression of transcription factor RAR-Orphan Related Receptor

Gamma, resulting in differentiation of CD+T-cells into Th17 cells while inhibiting the expression of Foxp3 and preventing differentiation of Treg cells.[40–42] Therefore, higher levels of cytokine IL-17 in diabetes could be the result of a proinflammatory milieu with higher levels of IL-6. Interestingly, a difference in the percentage of Foxp3-positive CD4+, CD25+, and Treg cells was observed between patients with and with diabetes, suggesting that proinflammatory conditions can affect cytokine production without having an impact on the absolute numbers of CD+T-cells.

Last, when obesity was used as the only factor as a control for diabetes, a significant difference was observed in the percentages of CD+T-cells that were positive for intracellular IL-17. This suggests a larger role for obesity in differentiation of Th17 cells compared with diabetes. This could also explain observations made previously in which an increase in Th17 and subsequent IL-17 levels was shown in diabetes.[13]

Natural infection by *S. pneumoniae* results in a robust pathogen-specific memory CD+T-cell response to *S. pneumoniae* and has been shown to facilitate clearance. The response is a cumulative response to the surface proteins of *S. pneumoniae*. Pneumococcal surface proteins are highly conserved between different serotypes, and therefore response generated against 1 serotype can cross-protect against a different serotype. Alternatively, carriage by 1 serotype can generate a memory response that would provide protection against invading pneumococci of different serotypes. A lower memory CD4+ and Th17 response, as observed, could explain the inability of patients with diabetes to control pneumococcal infections. Therefore, it is imperative to design future studies that continue to investigate and understand mechanistic details modulating the observed discrepancies in differentiation of naive CD+T-cells in this patient population.

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Abbreviations

%A1c	percent glycated hemoglobin A1c
BMI	body mass index
CCHC	Cameron County Hispanic Cohort
CM	central memory
c-RPMI	fetal calf serum
FBG	fasting blood glucose
FBS	fetal bovine serum
IFN-γ	interferon γ

IL	interleukin
MFBG	mean

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AT A GLANCE COMMENTARY

Martinez PJ, et al

Background

Type 2-diabetes and its associated inflammation results in alteration of immune system and increase is susceptibility to infections by pathogens such as Streptococcus pneumoniae. The study therefore investigated the CD4+ and T-helper 17 response in those with and without diabetes.

Translational Significance

These studies were performed using specimen from human subjects and therefore the results can be directly correlated with the disease status in human. Results from these studies will be used to further explore these pathways in animals models of diabetes to determine specific defects which results in increase susceptibility to pneumococcal infections in those with diabetes.

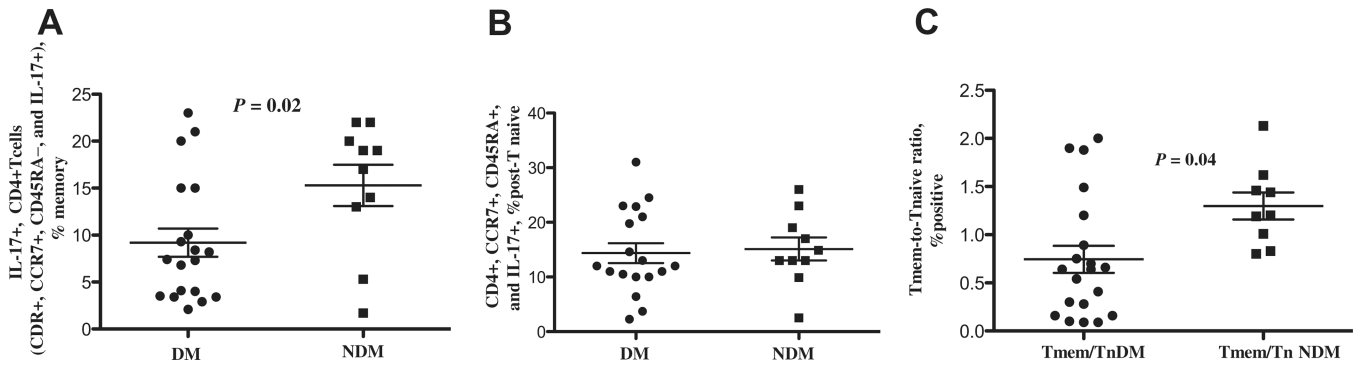

Fig 1.

Phenotypic characterization of CD4+, CCR7+, and CD45RA−, and interleukin (IL)-17+memory (mem); and CD4+, CCR7+, CD45RA+, and IL-17+ naive cells in patients with and without diabetes. Peripheral blood mononuclear cells were stimulated with a whole-heat-killed capsular type 2 strain of pneumococci (D39) for 18 hours, followed by staining for cell surface markers. A total of 10,000 CD+T-cells were gated. Fluorescent minus one (FMO) was used for background staining intensities. (**A**) Diabetes was associated with significantly a relatively low abundance of pathogen-specific memory CD4+ and IL-17 positive cells. (**B**) No significant differences were observed between percentage of naive cells of those with diabetes mellitus (DM) and without diabetes mellitus (NDM). (**C**) A significantly greater ratio of Tmem/Tnaive was observed in NDM patients compared with DM patients. Each dot represents an individual.

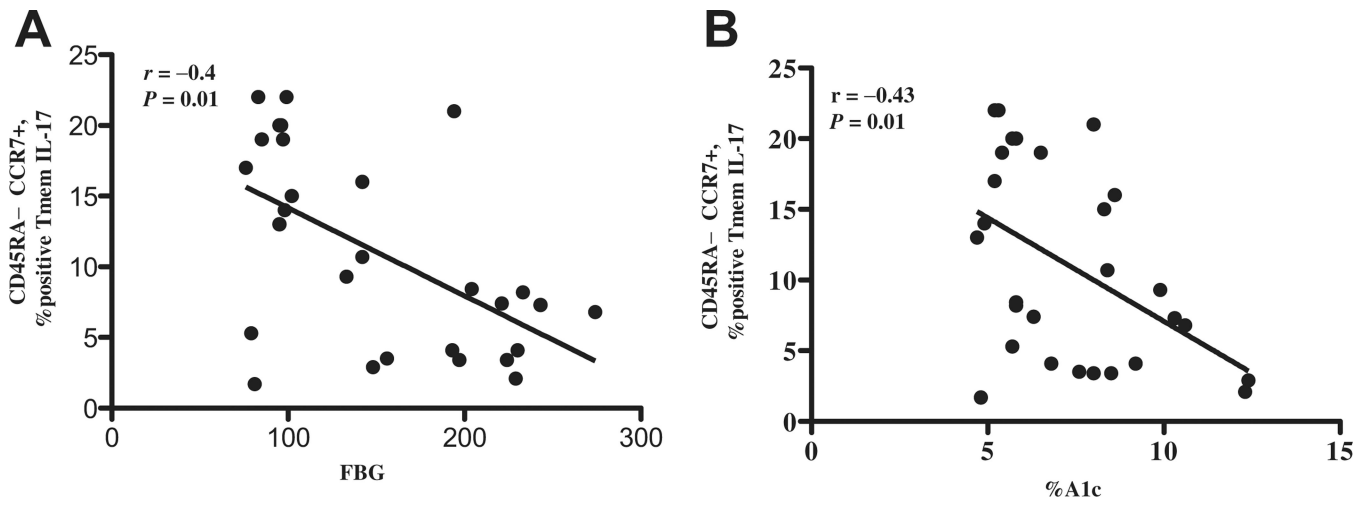

Fig 2.

Relationship of fasting blood glucose (FBG) and percent glycated hemoglobin A1c (%A1c) with positive memory CD4+ and T-helper 17 (Th17) cells. (**A, B**) Both FBG (**A**) and %A1c (**B**) showed a significant inverse relationship with memory CD4+ and Th17 cells. Correlations were calculated using the Spearman nonparametric test.

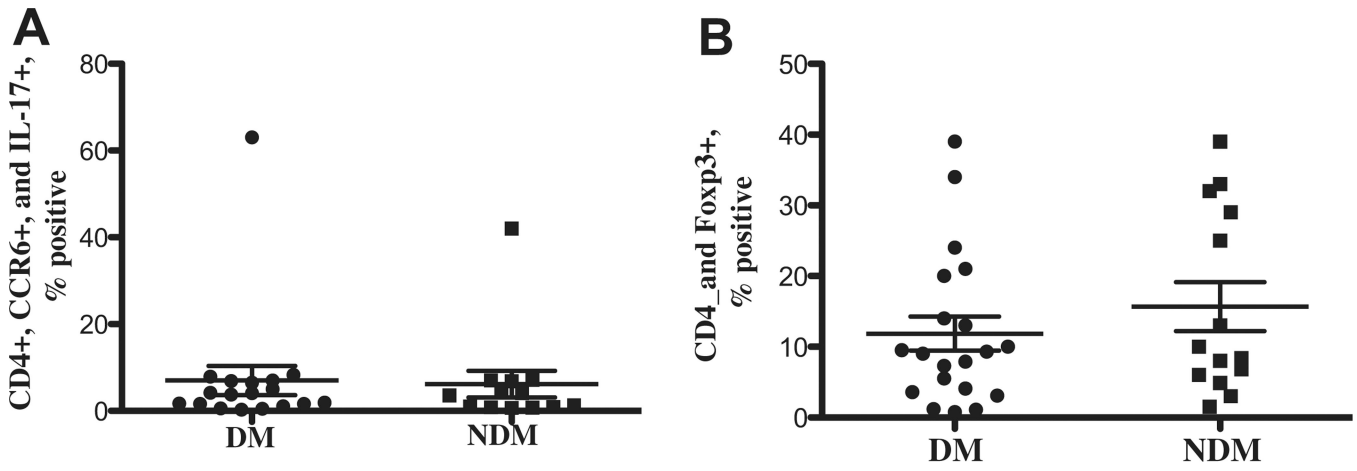

Fig 3.

(**A, B**) Phenotypic characterization of CD4+, CCR6+, and interleukin (IL)-17+ cells (**A**); and CD4+ and Foxp3+ cells (**B**) in patients with diabetes mellitus (DM) and patients without diabetes mellitus (NDM). No differences were observed in CD4+, CCR6+, IL-17+, and T-helper cell 17 cells between those with and without diabetes. No significant differences were observed between patients with diabetes and patients without diabetes with regard to percentage of positive CD4+, Foxp3+, and T regulatory cells. Comparisons were made using an unpaired *t* test, and *P* values less than 0.05 were considered significant for this and all subsequent assays unless otherwise stated.

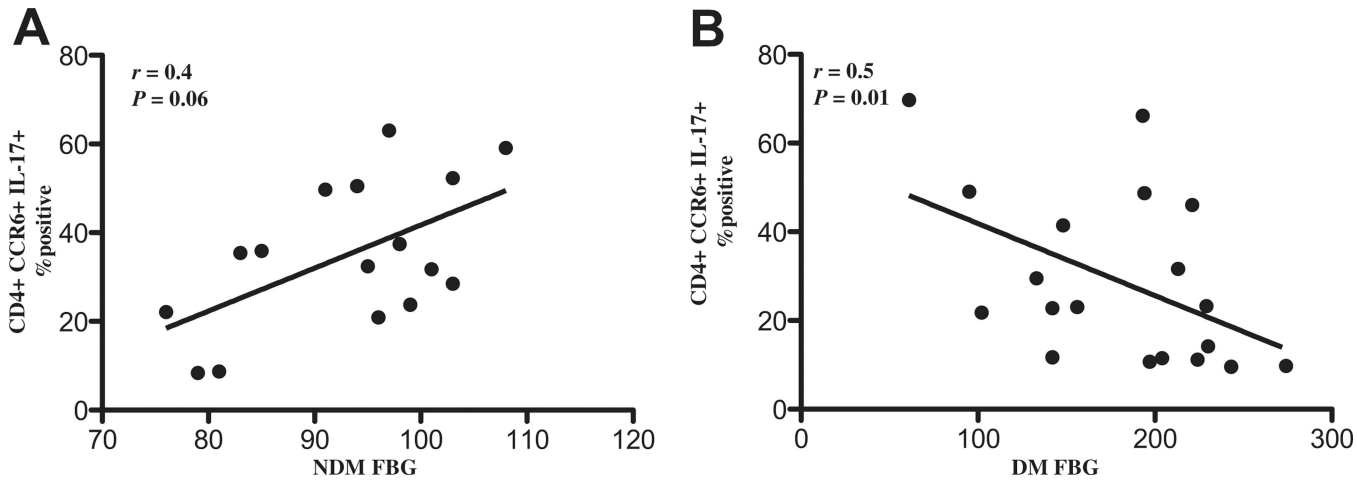

Fig 4.

(**A, B**) The relationship between fasting blood glucose (FBG) and percent positive CD4+, CCR6+, interleukin (IL)-17+, T-helper 17 (Th17) cells in patients without diabetes mellitus (NDM) (**A**) and with diabetes mellitus (DM) (**B**). No significant association was observed between FBG and percentage of positive Th17 cells in NDM patients. A strong inverse association was observed in DM patients between FBG and percentage of positive Th17 cells. Correlations were calculated using the Spearman nonparametric test.

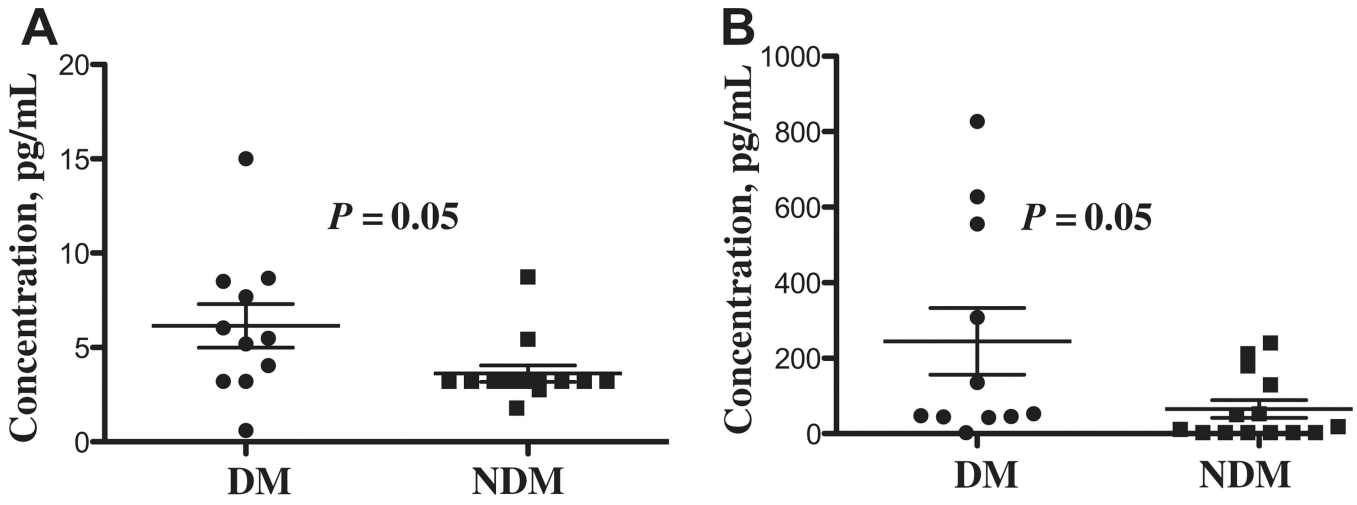

Fig 5.

Whole blood cell stimulatory response to capsule type 2 strain D39. (**A, B**) Significant increases in extracellular levels of interleukin (IL)-17 (**A**), and interferon γ (IFN-γ) (**B**) were observed after stimulation of whole blood with pneumococci. DM, diabetes mellitus; NDM, without diabetes mellitus.

Table I

Comparison of baseline participant characteristics between participants with and without diabetes

Variable	Diabetes (n = 20)	No diabetes (N = 16)	*P* value
Male gender, % (n)	6.3 (1)	45.0 (11)	0.02*
Age, y	57.0 (49.5–61.5)	56.0 (21.0–64.0)	0.79
BMI, kg/m^2	30.4 (29.0–33.3)	28.9 (23.0–33.9)	0.22
FBG, mmol/L	194.0 (162.0–224.0)	95.5 (84.0–100.0)	<0.001†
%A1c	8.4 (7.1–9.6)	5.4 (4.9–5.8)	<0.001†
HOMA-IR, %	5.3 (3.3–8.1)	3.6 (1.8–5.0)	0.07
CRP, mg/L	4.7 (3.2–7.1)	2.5 (1.9–9.2)	0.49
HDL, mg/dL	45.0 (41.0–58.0)	50.5 (44.0–60.0)	0.17
LDL, mg/dL	98.4 (78.0–140.0)	124.4 (99.2–148.4)	0.10
Triglycerides, mg/dL	159.0 (111.0–186.0)	107.0 (76.0–147.0)	0.04*
Systolic BP, mmHg	117.0 (107.5–129.0)	117.5 (104.0–127.5)	0.70
Diastolic BP, mmHg	68.5 (64.0–76.0)	68.0 (62.5–72.0)	0.73

Abbreviations: %*A1c*, percent glycated hemoglobin A1c; *BMI*, body mass index; *BP*, blood pressure; *CRP*, C-reactive protein; *FBG*, fasting blood glucose; *HDL*, high-density lipoprotein; *HOMA-IR*, homeostatic model assessment of insulin resistance; *LDL*, low-density lipoprotein.

Values presented as medians (interquartile range) for continuous variables and percentages for categorical variables. *P*-values represent difference in the medians.

*$P = 0.01$.

†$P \leq 0.001$.

Table II

Comparisons of percent positive CD4 cells using diabetes status, BMI, MFBG, and percent A1c

Comparison groups	CD4-positive cells	*P* value
Diabetes status		
Diabetes	57.7 (49.7–64.0)	0.001*
No diabetes	70.0 (64.4–83.1)	
BMI		
≥25 mmol/L	62.0 (53.5–69.1)	0.06
<25 mmol/L	70.7 (62.3–83.1)	
≥30 mmol/L	65.4 (60.3–71.9)	0.13
<30 mmol/L	58.6 (49.7–75.9)	
MFBG		
≥110 mmol/L	56.6 (48.7–64.0)	0.006*
<110 mmol/L	67.5 (61.3–78.9)	
%A1c		
≥6.5	58.7 (49.7–64.9)	0.02†
<6.5	67.5 (60.8–75.9)	

Abbreviations: %*A1c*, percent glycated hemoglobin A1c; *BMI*, body mass index; *MFBG*, mean fasting blood glucose; *Th17*, T-helper cell 17.

P values represent difference in the medians (interquartile ranges).

*$P = 0.001$.

†$P = 0.01$.

Table III

Comparisons of percent positive CD4 cells using diabetes status, BMI, MFBG, and %A1c

Comparison groups	Th17-positive cells, %	*P* value
Diabetes status		
Diabetes	23.1 (11.6–43.7)	0.31
No diabetes	33.9 (23.0–50.1)	
BMI		
≥25 mmol/L	26.7 (17.6–47.4)	0.99
<25 mmol/L	33.9 (18.6–45.6)	
≥30 mmol/L	36.6 (23.5–50.7)	0.01*
<30 mmol/L	21.4 (11.1–33.9)	
MFBG		
≥110 mmol/L	22.9 (11.4–36.5)	0.06
<110 mmol/L	35.7 (23.0–50.1)	
%A1c		
≥6.5	26.4 (11.7–41.4)	0.39
<6.5	32.1 (22.1–50.5)	
Metabolic syndrome†		
Metabolic syndrome (n = 11)	31.6 (22.8–49.0)	0.89
No metabolic syndrome	32.4 (20.9–48.7)	

Abbreviations: %*A1c*, percent glycated hemoglobin A1c; *BMI*, body mass index; *MFBG*, mean fasting blood glucose; *Th17*, T-helper cell 17.

P values represent difference in the medians (interquartile ranges).

*$P = 0.01$.

†Metabolic syndrome defined as BMI ≥30 kg/m^2 plus at least 3 of the following: fasting blood glucose ≥100 mmol/L or diagnosis of diabetes, serum triglycerides ≥150 mg/dL, high-density lipoprotein cholesterol, <40 mg/dL (males) or <50 mg/dL (females), or hypertension (>130 mmHg systolic or >85 mmHg diastolic blood pressure).

Table IV

Comparisons of GMFI of IL-17 expression in Th17 cells using diabetes status, obesity, MFBG, and %A1c

Comparison groups	CCR6+, Il-17+, and GMFI (95% CI)	*P* value
Diabetes status		
Diabetes	3338 (2918–3788)	0.05*
No diabetes	4290 (3429–5151)	
MFBG		
≥110 mmol/L	3201 (2879–3524)	0.02*
<110 mmol/L	4209 (3465–4953)	
%A1c		
≥6.5	3573 (3006–4139)	0.41
<6.5	3950 (3194–4705)	
Obese/not obese		
BMI ≥30 kg/m^2	3795 (3104–4487)	0.87
BMI <30 kg/m^2	3718 (3088–4349)	
Overweight/normal		
BMI ≥25 kg/m^2	3699 (3205–4193)	0.61
BMI <25 kg/m^2	3978 (2629–5326)	
Age		
≥60 y	4497 (3548–5446)	0.02*
<60 y	3293 (2918–3667)	

Abbreviations: %*A1c*, percent glycated hemoglobin A1c; *BMI*, body mass index; *CI*, confidence interval; *GMFI*, geometric mean fluorescence intensity; *IL*, interleukin; *MFBG*, mean fasting blood glucose; *Th17*, T-helper cell 17.

P values represent comparisons of geometric means using a *t* test with unequal variance (Satterthwaite).

* $P = 0.01$.

Table V

Comparisons of cytokine fold increase for obese vs nonobese participants and participants with and without diabetes after stimulation with pneumococcal laboratory strain D39 for 1–3 days

Cytokines	DM	No DM	*P* value	Obese	Nonobese	*P* value
IL-17, day 3 vs unstimulated day 3	2.2 (1.0–3.4)	1.0 (1.0–1.5)	0.05*	1.5 (1.0–2.2)	1.0 (1.0–2.7)	0.65
IFN-γ, day 3 vs unstimulated day 3	31.2 (7.7–103.8)	4.5 (1.0–21.7)	0.05*	8.9 (1.4–25.0)	19.4 (2.8–79.3)	0.30

Abbreviations: *DM*, diabetes mellitus; *IFN-γ*, interferon γ; *IL*, interleukin.

P values represent difference in the median (interquartile ranges) fold increases in cytokine production.

*$P = 0.01$.