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Monocytes from Patients with Type 1 Diabetes Spontaneously Secrete Proinflammatory Cytokines Inducing Th17 Cells[1]

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Autoimmune diseases including type 1 diabetes (T1D) are thought to have a Th1/Th17 bias. The underlying mechanisms driving the activation and differentiation of these proinflammatory T cells are unknown. We examined the monocytes isolated directly from the blood of T1D patients and found they spontaneously secreted the proinflammatory cytokines IL-1β and IL-6, which are known to induce and expand Th17 cells. Moreover, these in vivo-activated monocytes from T1D subjects induced more IL-17-secreting cells from memory T cells compared with monocytes from healthy control subjects. The induction of IL-17-secreting T cells by monocytes from T1D subjects was reduced in vitro with a combination of an IL-6-blocking Ab and IL-1R antagonist. In this study, we report a significant although modest increase in the frequency of IL-17-secreting cells in lymphocytes from long-term patients with T1D compared with healthy controls. These data suggest that the innate immune system in T1D may drive the adaptive immune system by expanding the Th17 population of effector T cells. *The Journal of Immunology,* **2009, 183: 4432–4439.**

A distinct and separate lineage of Th cells secreting the proinflammatory cytokine IL-17 has been recently described (1). The discovery of these Th17 cells has had a major impact on our understanding of immune processes not readily explained by the Th1/Th2 paradigm. Th17 cells are intimately involved in promotion of autoimmunity (2), in particular rheumatoid arthritis (3), experimental autoimmune encephalomyelitis (4), and multiple sclerosis (5). Moreover, there is preliminary evidence that IL-17 is expressed in the pancreas in the course of T1D in the murine model of T1D (6), the nonobese diabetic (NOD)[4] mouse, and that reducing the number of Th17 cells with induction of IFN-γ inhibited IL-17 production and restored normoglycemia at the prediabetic stage (7). Two recent articles found that transfer of islet-specific Th17 cells induced diabetes, but only after the cells converted to IFN-γ-producing cells (8, 9).

We and others recently demonstrated that although in humans TGF-β and IL-21 can differentiate naive CD4 cells into Th17 cells secreting IL-17, central memory CD4 cells are driven to secrete IL-17 with a combination of IL-1β and IL-6 (10–13). Monocytes stimulated with the TLR2 agonist peptidoglycan and to a lesser extent the TLR4 agonist LPS secrete IL-6 and IL-1β, but not IL-12 and IL-23, and can induce naive (13) and memory (14) T cells to secrete IL-17 and IFN-γ.

Circulating monocyte-derived cytokines are known to be elevated in the sera of diabetic subjects. There is an increase in serum TNF-α, IL-6, IL-1β, and IL-1α in diabetic subjects compared with control subjects at onset of clinical disease (15, 16), as well as in healthy first-degree relatives (17). In long-standing diabetic subjects, monocytes have been investigated in the context of atherosclerosis or metabolic control. There is an increased secretion of IL-6, IL-1β, and TNF-α from monocytes with stimulation in diabetic subjects compared with controls (18–21). Recently, it was shown that there are two monocyte gene expression clusters in T1D, one of which is defined by having increased proinflammatory cytokine expression, such as IL-6, IL-1β, and TNF-α (22).

In this study, we investigated the activation state of monocytes in patients with T1D, hypothesizing that in vivo activation of proinflammatory, circulating monocytes were driving the differentiation/expansion of CD4 cells into Th17/Th1 cells. We found a striking activation of a subset of CD16$^-$ monocytes isolated ex vivo from patients with T1D that both spontaneously secreted and expressed mRNA transcripts for IL-1β/IL-6. These in vivo-activated monocytes from T1D subjects induced IL-17-secreting cells from memory T cells compared with monocytes from healthy control subjects and this in vitro induction was inhibited by a combination of an IL-6-blocking Ab and IL-1R antagonist (IL-1Ra). These data suggest a mechanism by which an activated, proinflammatory innate immune system drives the expansion of Th17 cells in patients with T1D through spontaneous secretion of IL-6 and IL-1β.

Materials and Methods

Patients

Peripheral venous blood was obtained from 21 recent-onset (<1 year from disease onset) T1D subjects (mean age ± SD, 21.3 ± 9.0 years; mean disease duration ± SD, 2.6 ± 3.4 mo; 7 females and 14 males), 27 long-term (>1 year from disease onset) T1D subjects (mean age ± SD, 30.3 ± 9.3 years; mean disease duration ± SD, 181.4 ± 130.4 mo; 13 females and 14 males), 15 type 2 diabetic (T2D) subjects (mean age ± SD, 41.9 ± 9.2

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[4] Abbreviations used in this paper: NOD, nonobese diabetic; T1D, type 1 diabetes; T2D, type 2 diabetes; IL-1Ra, IL-1R antagonist.

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years; mean disease duration ± SD, 8.6 ± 7.0 years; 8 females and 7 males), or 42 healthy subjects (mean age ± SD, 31.8 ± 10.2 years; 27 females and 15 males) in compliance with institutional review board protocols. PBMCs were separated using density centrifugation on Ficoll-Hypaque (GE Healthcare). PBMCs were frozen at a concentration of 1–3 × 10^7/ml in 10% DMSO (Sigma-Aldrich)/90% FCS (Atlanta Biologicals). After thawing the PBMCs were washed in PBS.

Monocyte, dendritic cell, B cell, and T cell isolation

Monocytes were isolated by negative selection using magnetic beads (Monocyte Isolation Kit II; Miltenyi Biotec), with ~90% purity as defined by CD11b staining. The monocyte-depleted cells were FACS sorted into $CD3^+$, $CD19^+$, and $CD3^-CD19^-CD11c^+$ populations. The monocyte CD16 subpopulations were FACS sorted using CD14, CD16, and CD56; the CD56 Ab was used to exclude NK cells in the $CD14^{dim}$ $CD16^+$ population. For the coculture experiments, the monocytes were FACS sorted after negative isolation (FACSAria; BD Biosciences) based on CD11b expression. Naive and memory $CD4^+$ T cells were FACS sorted using the following markers: $CD4^+$, $CD25^-$, $CD62L^+$, $CD45RA^+$, and $CD4^+$, $CD25^-$, $CD62L^+$, $CD45RA^-$, respectively (all Abs are from BD Biosciences).

Real-time PCR

RNA from negatively isolated monocytes or FACS- sorted populations was purified using the absolutely RNA microprep kit (Stratagene). cDNA was made using a TaqMan kit with supplied random hexamers (Applied Biosystems). All primers and probes were obtained from Applied Biosystems and used according to standard methodologies.

ELISPOT

In brief, 2.5 × 10^5 PBMCs/well were plated in HL-1 medium supplemented with 2 mM L-glutamine, 5 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin, 0.1 mM each nonessential amino acids, 1 mM sodium pyruvate (all from Lonza), and 1% heat-inactivated human male AB serum (Omega Scientific) in 96-well round-bottom plates (Corning). For IL-17 ELISPOT, the plates were first coated with anti-CD3 (OKT3, 1 μg/ml) in PBS. After 18 h at 37ºC/5% CO_2, the cells were transferred to coated ELISPOT plates and left for an additional 16 h at 37ºC/5% CO_2. For the isolated monocyte and monocyte-depleted PBMC ELISPOT, the cells were separated using magnetic beads (Monocyte Isolation Kit II; Miltenyi Biotec) before being loaded on the ELISPOT plate. Ab capture and detection pairs are as follows: IL-6 (eBioscience), IL-1β (R&D Systems), and IL-17 (eBioscience).

Monocyte-T cell cocultures

Cells were cultured in complete HL-1 medium and 5% human serum. Memory T cells were cultured with monocytes (1:1) in the presence of plate-bound anti-CD3 Ab (OKT3, 1 μg/ml) for 5 days. For cytokine-blocking experiments with memory T cells cultured with monocytes, IL-1Ra (125 ng/ml), anti-IL-6 (10 μg/ml), and anti-TNF-α (10 μg/ml) (all from R&D Systems) were added to the initial incubation with anti-CD3. Then T cells were transferred to a new 96-well plate and incubated with rIL-2 (20 U/ml) (The reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: human rIL-2 from Dr. M. Gately, Hoffmann-La Roche) (23)) for an additional 7 days.

Intracellular staining

T cells from the coculture experiments were stimulated with PMA (50 ng/ml) and ionomycin (250 ng/ml) (Sigma Aldrich) for 5 h, and GolgiStop (BD Biosciences) was added for the final 3 h. Cells were fixed with 4% paraformaldehyde and permeabilized with reagents and protocols from BD Biosciences. T cells were then stained with allophycocyanin-IL-17 (eBioscience) and PE-IFN-γ (BD Biosciences). The data were acquired on a FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Statistical analysis

Significant differences were calculated with Prism 4.0 software (GraphPad) using an unpaired two-tailed *t* test, with the exception of the monocyte-T cell coculture experiments, which used a paired two-tailed *t* test.

Results

Ex vivo cytokine analysis of PBMCs from T1D subjects

IL-6 and IL-1β have both been implicated in the differentiation and expansion of Th17 cells. Therefore, we examined the number of circulating IL-6- and IL-1β-secreting cells ex vivo with no additional stimulus from T1D subjects as well as healthy controls and T2D subjects. ELISPOT assays demonstrated that there was a marked increase of spontaneous IL-6- and IL-1β-secreting cells in recent-onset T1D subjects (and to a lesser extent long-term T1D subjects) compared with healthy, age-matched controls and T2D subjects (Fig. 1 and supplemental Fig. 1*A*[5]). The PBMCs derived from T2D subjects showed no difference in cytokine secretion compared with those from healthy controls. There was no correlation between the number of cytokine-secreting cells and age of the T1D patients or the age of disease onset (data not shown).

Ex vivo cytokine analysis of circulating T1D monocytes

To determine which PBMC populations were secreting these proinflammatory cytokines, monocytes from recent-onset T1D and healthy control subjects were isolated by negative selection and analyzed by ELISPOT assays with no additional stimulus. The corresponding monocyte-depleted PBMCs were also examined. To avoid activation of monocytes with surface molecule ligation by Ab, monocytes were negatively isolated by magnetic bead exclusion of other cell types. CD3, CD7, CD16, CD19, CD56, CD123, and glycophorin A-positive cells were removed; this isolation method purifies the classical $CD14^+CD16^-$ monocyte population which accounts for ~90% of circulating monocytes. The majority of IL-6-and IL-1β-secreting cells were observed in the monocyte population (Fig. 2). The frequency of IL-6- and IL-1β-secreting monocytes varied greatly, as would be predicted from the data in Fig. 1. The frequency of IL-6-secreting monocytes from T1D subjects ranged from 0.3 to 10%, with a mean of 3%, indicating that only a subset of the circulating monocytes was in the activated state. Interestingly, we observed an increased number of IL-6-secreting cells in the T1D monocyte-depleted PBMCs compared with those from healthy controls, suggesting that there may be other cell types with increased cytokine secretion. To address this, monocytes were negatively isolated, and the remaining cells were FACS sorted into CD11c-, CD19-, and CD3-positive populations. Quantitative RT-PCR was used to identify the relative gene expression of *IL-6* and *IL-1β* (Fig. 3). The negatively isolated monocytes had the highest expression, but the $CD11c^+$ population also expressed significant amounts of *IL-6* and *IL-1β* compared with the $CD19^+$ and $CD3^+$ cells. The CD11c population contains the myeloid dendritic cells as well as the $CD16^+$ monocytes that are removed in the negative isolation and are thought to be proinflammatory, more mature, and have a higher T cell stimulatory capacity than the $CD16^-$ negatively isolated monocytes (24). The $CD16^+$ monocytes can be divided into two populations: $CD14^+CD16^+$ and $CD14^{dim}CD16^+$; both produce more TNF-α than the classical monocytes when stimulated, but the $CD14^{dim}CD16^+$ do not produce IL-10 while the $CD14^+CD16^+$ do produce IL-10 upon stimulation (25). To examine the different populations of monocytes ex vivo in T1D subjects, we FACS sorted the $CD14^+CD16^-$, $CD14^+CD16^+$, and $CD14^{dim}$ $CD16^+$ populations and analyzed their *IL-6* relative gene expression. Both the $CD14^+CD16^-$ and the $CD14^+CD16^+$ populations had increased *IL-6* gene expression, while the $CD14^{dim}CD16^+$ did not (Fig. 4).

[5] The online version of this article contains supplemental material.

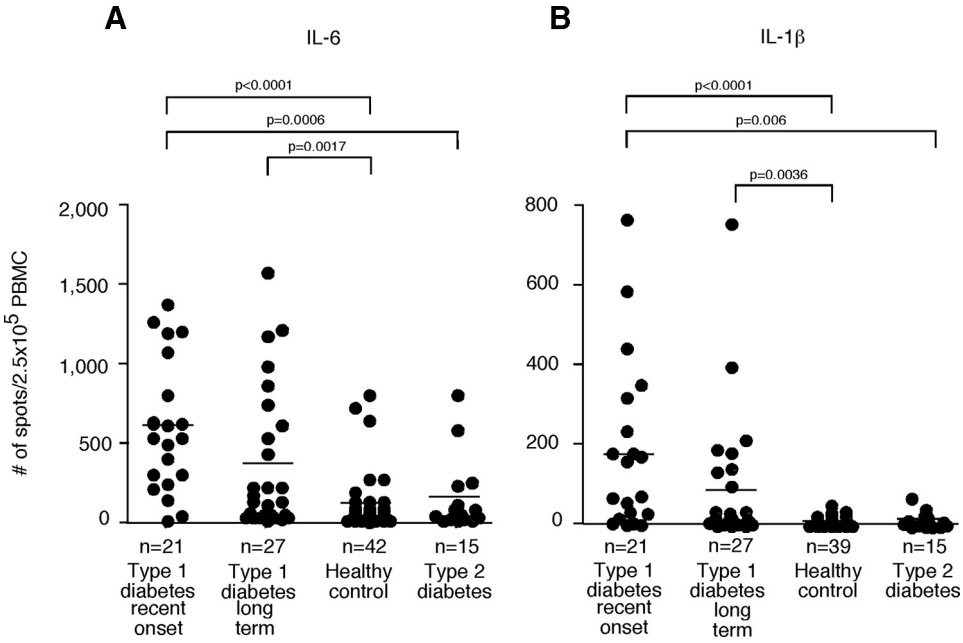

FIGURE 1. T1D subjects have a higher number of ex vivo IL-6- and IL-1β-secreting PBMCs compared with control subjects. Unstimulated PBMCs were incubated in HL-1 medium with 1% human serum for 18 h and then analyzed by ELISPOT. Each circle represents the number of positive PBMCs derived from a single subject. Two hundred fifty thousand PBMCs were added per well. Horizontal bars indicate the mean. Significant differences in cytokine-positive cells ($p < 0.05$) between groups are shown. *A*, The number of ex vivo IL-6-secreting cells from PBMCs from recent-onset T1D subjects was significantly greater than that from healthy control subjects or T2D subjects. Long-term T1D subjects also had a greater number of IL-6-secreting cells compared with T2D subjects. *B*, The number of ex vivo IL-1β-secreting cells from recent-onset and long-term T1D subjects was significantly greater than that from healthy controls. Recent-onset T1D subjects had a greater number of IL-1β-secreting cells compared with T2D subjects.

To directly examine the classical monocytes ex vivo for cytokine phenotype and avoid potential cell culture activation of this cell type, monocytes were isolated by negative selection and immediately deposited into RNA lysis buffer. Quantitative RT-PCR was used to evaluate mRNA levels of different monocyte-produced cytokines. Monocytes derived from recent-onset T1D subjects were found to have significantly increased gene expression of *IL-6*, *IL-1*β, and *TNF-*α compared with those from healthy subjects (Fig. 5, *A–C*). Increased production of TNF-α, IL-6, and IL-1β by circulating monocytes from T1D subjects is striking and suggests a systemic alteration in function in a subset of monocyte/macrophages. Moreover, the expression of *IL-6* and *IL-1*β mRNA from T1D-derived monocytes was strongly correlated, while expression of *IL-6* and *TNF-*α was not (Fig. 5, *D* and *E*). There were no significant differences of *IL-23* or *TGF*β expression seen between monocytes derived from T1D subjects or healthy controls (data not shown). The monocytes derived from T1D subjects also had increased *PDL-1* and *CD80* gene expression compared with those from healthy controls (Fig. 5, *F* and *G*); this is further evidence that the classical monocytes are in an activated state in T1D subjects.

Expansion and activation of memory Th17 cells by T1D monocytes

It has recently been demonstrated that IL-6 and IL-1β are involved in differentiation/expansion of Th17 cells (10–13). In this regard,

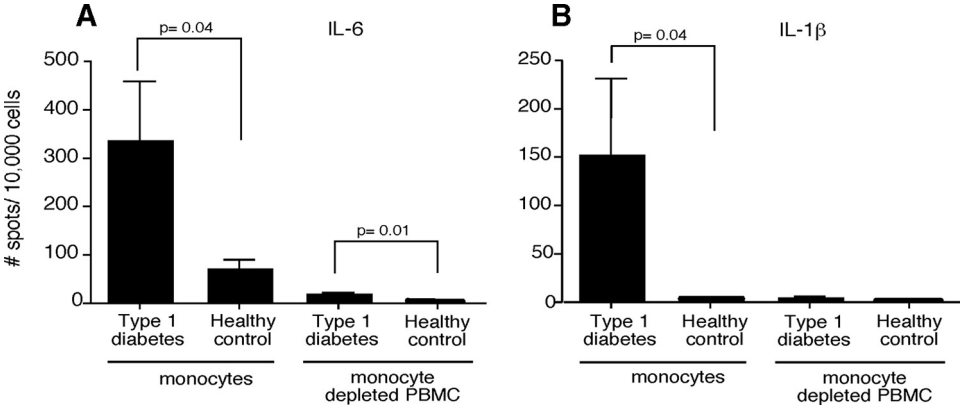

FIGURE 2. T1D subjects have a higher number of ex vivo IL-6- and IL-1β-secreting monocytes compared with control subjects. Monocytes from T1D and healthy control subjects were negatively selected and then both the monocytes and the monocyte-depleted PBMCs were analyzed by ELISPOT. Significant differences in cytokine-positive cells ($p < 0.05$) between groups are shown. *A*, The number of ex vivo IL-6-secreting cells from monocytes and monocyte-depleted PBMCs was significantly greater in the T1D subjects than that from healthy control subjects ($n = 7$). *B*, The number of ex vivo IL-1β-secreting cells from monocytes, but not monocyte-depleted PBMCs was significantly greater than that from healthy controls ($n = 8$).

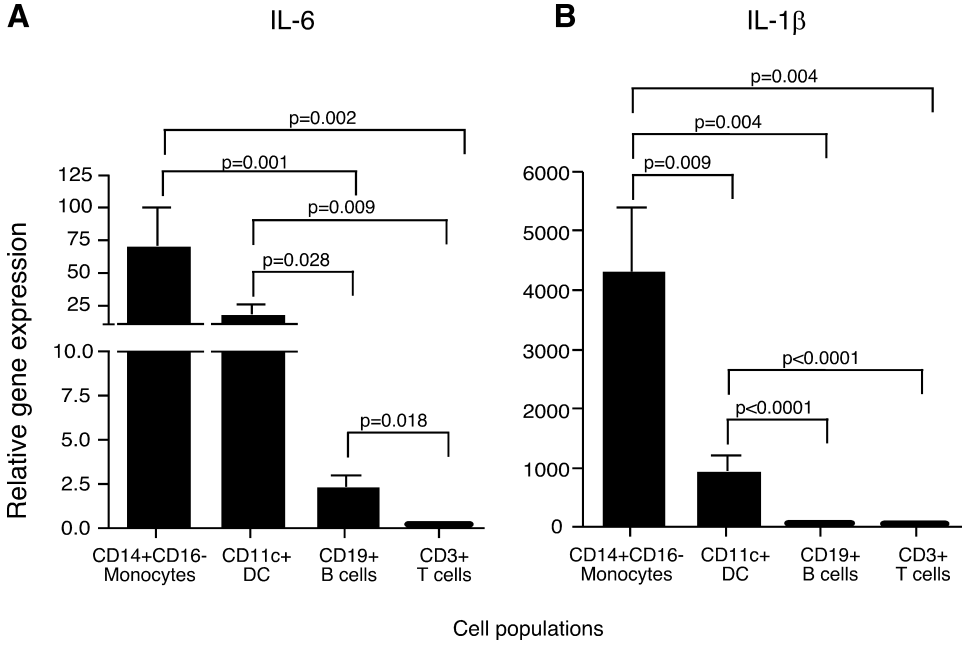

FIGURE 3. Negatively isolated monocytes and CD11c$^+$ cells from T1D subjects have high gene expression for *IL-6* and *IL-1β*. Gene expression of *IL-6* and *IL-1β* from negatively isolated monocytes and FACS- sorted CD11c$^+$, CD19$^+$, and CD3$^+$ cells from five T1D subjects were analyzed by quantitative RT-PCR. The CD16$^-$ monocytes were removed by negative isolation and the monocyte-depleted cells were FACS sorted into CD3$^+$, CD19$^+$, and CD3$^-$CD19$^-$CD11c$^+$ populations. Significant differences are shown. Relative gene expression of *IL-6* (*A*) and *IL-1β* (*B*) are increased in the classical monocyte and CD11c populations compared with the CD19 and CD3 populations. All gene expression is relative to β_2-*microglobulin*.

we previously observed that IL-1β with or without IL-6 induced the secretion of IL-17 from memory T cells. It has also been shown that monocytes, stimulated with peptidoglycan or LPS, secreted IL-1β and IL-6 and were able to induce IL-17-secreting cells (13, 14). Thus, a major consequence of T1D-derived monocyte IL-6/IL-1β secretion could be the induction of IL-17- secreting cells. To test this hypothesis, we examined whether unstimulated monocytes isolated ex vivo from patients with T1D induced naive or memory T cells from healthy control subjects to secrete IL-17 compared with monocytes isolated from control subjects. The CD4$^+$CD25$^-$CD62L$^+$CD45RA$^+$ naive and CD4$^+$CD25$^-$CD62L$^+$CD45RA$^-$ memory T cells from healthy controls were stimulated with anti-CD3 and classical monocytes from T1D or healthy control subjects for 5 days, and then further cultured in the presence of IL-2 for an additional 7 days. Although allogeneic responses result in cell proliferation and expansion, in this study the strong stimulus of immobilized anti-CD3 drove T cell proliferation (supplemental Fig. 2). Since the monocytes do not produce IL-21, a cytokine found to be critical to the differentiation of Th17 cells, we did not expect to see IL-17 secretion from the naive T cells and, as predicted, we saw few IL-17-secreting cells in the cocultures with the naive T cells (percent T cells expressing IL-17 cultured with monocytes from T1D: 0.89 ± 0.34% (mean ± SE); percent T cells expressing IL-17 cultured with monocytes from healthy control: 0.16 ± 0.06%; $n = 9$). We found that ex vivo, unstimulated T1D subject-derived monocytes preferentially induced IL-17 secretion from memory T cells compared with monocytes from healthy controls; interestingly, this IL-17-secreting population contains both IL-17$^+$IFN-γ^- and IL-17$^+$IFN-γ^+ populations as shown by intracellular staining (Table I). The IL-17$^+$IFN-γ^+ subset is known to express CXCR3, a Th1 chemokine receptor (26), which has been implicated in T1D (27) and has also been implicated in the experimental autoimmune encephalomyelitis model as the pathologic CD4 effector population (28). Few IL-17-secreting T cells were induced in the absence of monocytes from memory T cells under these conditions (supplemental Fig. 3).

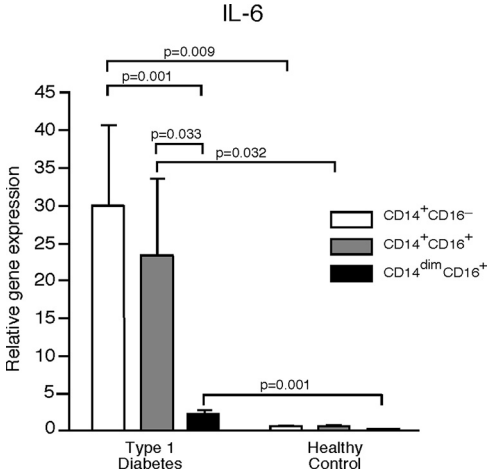

FIGURE 4. Unstimulated CD14$^+$CD16$^-$ and CD14$^+$CD16$^+$ monocytes from T1D subjects, but not CD14^{dim}CD16$^+$ monocytes have increased *IL-6* gene expression compared with healthy control subjects. Gene expression of *IL-6* from CD14$^+$CD16$^-$, CD14$^+$CD16$^+$, and CD14^{dim}CD16$^+$ FACS-sorted monocytes from five T1D subjects and five healthy control subjects were analyzed by quantitative RT-PCR. Significant differences are shown. All gene expression is relative to β_2-*microglobulin*.

To determine which of the proinflammatory cytokines were driving IL-17 secretion, blocking Abs against IL-6 or TNF-α and an IL-1Ra were added to cocultures of monocytes from T1D patients and memory T cells from healthy subjects. Cultures were analyzed by intracellular staining for IL-17- and IFN-γ-expressing T cells. Blocking IL-6 in combination with IL-1β or blocking IL-1β alone directly inhibited IL-17 secretion. The IL-1Ra reduced the number of IL-17-secreting cells by ~70%. Blocking IL-6 alone was not as effective as IL-1Ra, but did reduce by ~10% the number of IL-17-secreting cells; however, this was not statistically significant. Blocking TNF-α slightly increased the percentage of IL-17-secreting cells (Fig. 6).

Increased number of IL-17-secreting cells in T1D subjects

We directly measured the ex vivo frequency of IL-17-secreting CD4 cells. After 18 h of TCR cross-linking with anti-CD3, we found that the recent-onset T1D subjects do not have an increased frequency of IL-17 producers, but interestingly long-term T1D

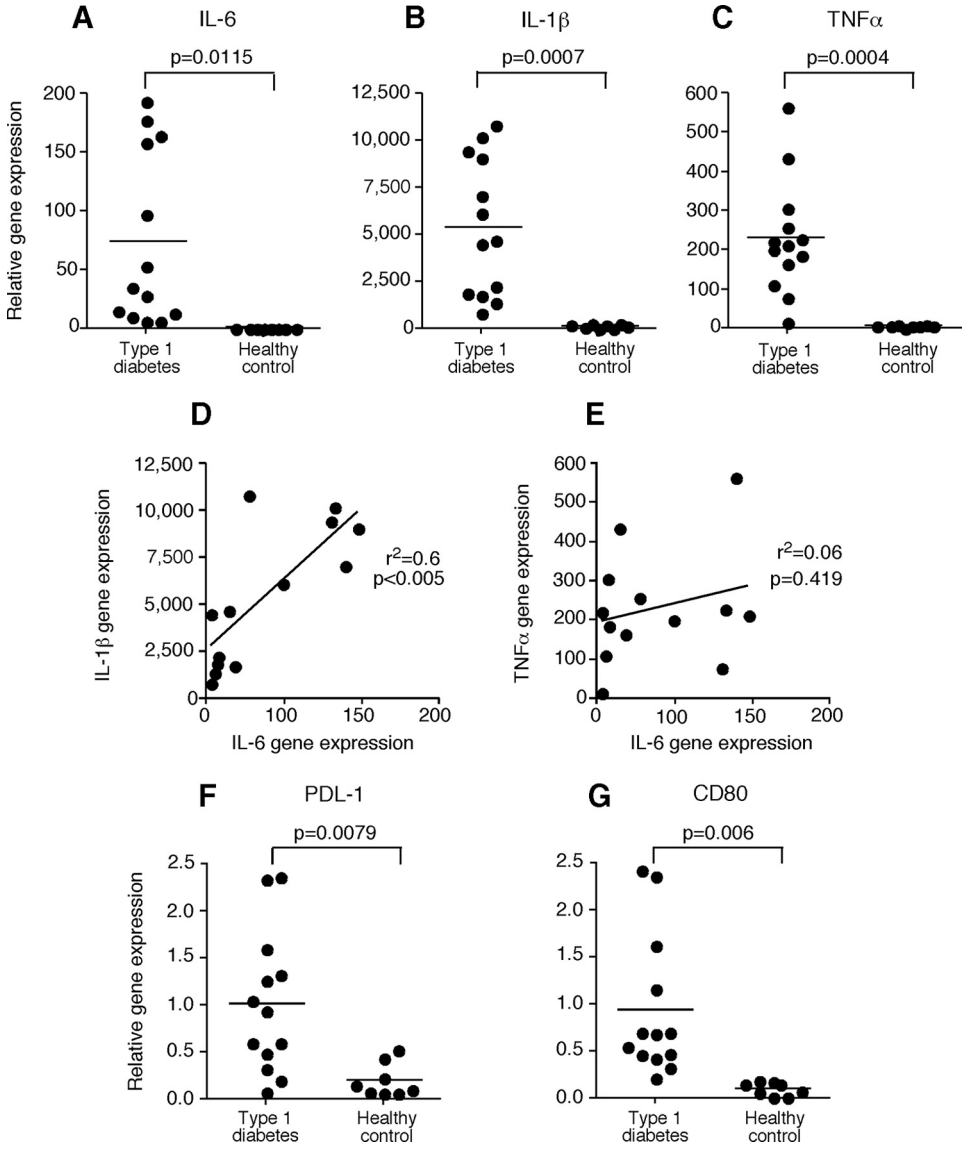

FIGURE 5. Ex vivo monocytes from T1D subjects have increased cytokine gene expression in comparison to healthy control subjects. Monocytes from recent onset (<1 year) T1D subjects were negatively selected, RNA was immediately isolated, and the relative gene expression was measured using quantitative RT-PCR. Relative *IL-6* (*A*), *IL-1β* (*B*), and *TNF-α* (*C*) gene expression for T1D and healthy control subjects is shown. All gene expression is relative to β_2-*microglobulin*. Each circle represents a subject; 13 recent-onset T1D subjects were compared with 8 healthy control subjects. Horizontal bars indicate the mean. Correlation of gene expression between *IL-6* and *IL-1β* (*D*) and *IL-6* and *TNF-α* (*E*) for monocytes from recent-onset T1D is shown. The monocytes derived from T1D subjects are in a more activated state as shown by increased expression of *PDL1* (*F*) and *CD80* (*G*) compared with those from healthy controls. Significant differences are shown.

subjects have a moderate increase of IL-17- secreting cells compared with healthy controls (Fig. 7). To confirm that the positive cells in the ELISPOT assay were derived from T cells, PBMCs were activated by PMA and ionomycin for 3 h and then stained for CD3 and IL-17 expression, followed by FACS analysis. The percentage of CD3$^+$IL-17$^+$ T cells corresponds to the number of positive spots determined by ELISPOT from total PBMCs (supplemental Fig. 4).

Table I. *Monocytes from T1D subjects induce a higher percentage of IL-17-positive cells from healthy control memory CD4$^+$ T cells than do monocytes from healthy control subjects*[a]

% Cytokine-Positive T Cells	Monocytes from T1D Subjects	Monocytes from Healthy Control Subjects
% IL-17$^+$*	24.16 ± 5.044	16.51 ± 3.750
% IL-17$^+$IFN-γ^+*	10.55 ± 2.072	6.690 ± 1.523
% IL-17$^+$/IFN-γ^-*	13.49 ± 3.095	9.686 ± 2.276
% IL-17/IFN-γ^+	44.11 ± 3.251	39.84 ± 3.751

[a] Healthy control CD4$^+$, CD25$^-$, CD62L$^+$, and CD45RA$^-$ T cells were cultured with monocytes from T1D or healthy control subjects for 5 days in the presence of plate-bound anti-CD3. T cells were then expanded for 7 days with the addition of IL-2. The cells were stimulated with PMA and ionomycin and analyzed by intracellular staining for IL-17/IFN-γ expression. Data are presented as mean ± SE for the number of cytokine-staining positive cells (n = 22).

*, $p < 0.01$.

Discussion

In this study, we examined a mechanism for the increased induction of Th17 cells observed in patients with autoimmune disease. Although there have been several reports implicating Th17 cells in the NOD model, this is the first report to correlate increased IL-17-secreting T cells with human T1D. We also observed a marked increase in the frequency of a subset of circulating IL-1β- and IL-6-secreting monocytes in recent-onset T1D patients compared with age-matched healthy controls or patients with T2D. These results were confirmed by direct PCR examination of mRNA where increases in proinflammatory cytokines and costimulatory molecules were observed. These activated, IL-1β- and IL-6-secreting monocytes from patients with T1D drove the in vitro induction of IL-17$^+$ CD4 cells that may be associated with the increase in the frequency of IL-17- secreting CD4 cells in patients with the disease.

There is previous evidence that circulating monocytes in patients with T1D can be induced to secrete proinflammatory cytokines. Although these previous studies demonstrate that monocytes

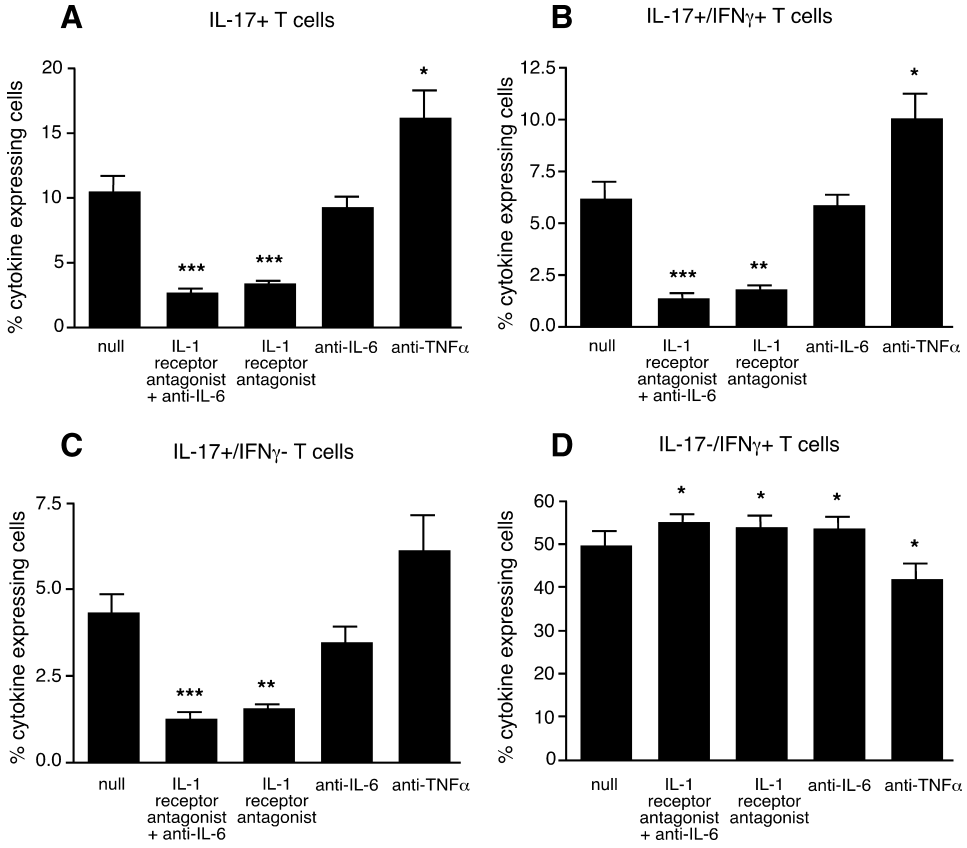

FIGURE 6. IL-1Ra reduced the number of IL-17-secreting memory T cells in monocyte-T cell cocultures. Healthy control memory T cells were cultured with monocytes from T1D subjects for 5 days in the presence of plate-bound anti-CD3. T cells were then expanded for 7 days with the addition of IL-2. The cells were stimulated with PMA and ionomycin and analyzed by intracellular staining for IL-17/IFN-γ expression. The number of (*A*) IL-17$^+$, (*B*) IL-17$^+$/IFN-γ$^+$, and (*C*) IL-17$^+$/IFN-γ-secreting cells, but not (*D*) IL-17$^-$/IFN-γ$^+$ ($n = 8$), could be reduced by IL-1Ra and anti-IL-6 or IL-1Ra alone, but were slightly increased by anti-TNF-α. ***, $p < 0.0005$; **, $p < 0.005$; and *, $p < 0.05$.

from patients with T1D can be induced to secrete a more inflammatory cytokine milieu through activation with LPS or IFN-γ, here we show that classical monocytes isolated by negative selection ex vivo spontaneously secrete proinflammatory cytokines that can drive the secretion of IL-17 from memory T cells. We demonstrated that not all of the monocytes are actively secreting cytokine; only a small subpopulation was actively secreting IL-6 and IL-1β. Interestingly, we found that both the CD14$^+$CD16$^-$ and the CD14$^+$CD16$^+$ populations had increased *IL-6* expression. It has been shown that CD14$^+$CD16$^-$ monocytes can convert to CD14$^+$CD16$^+$ monocytes upon stimulation (29). The relationship between these two monocyte populations in T1D requires further examination.

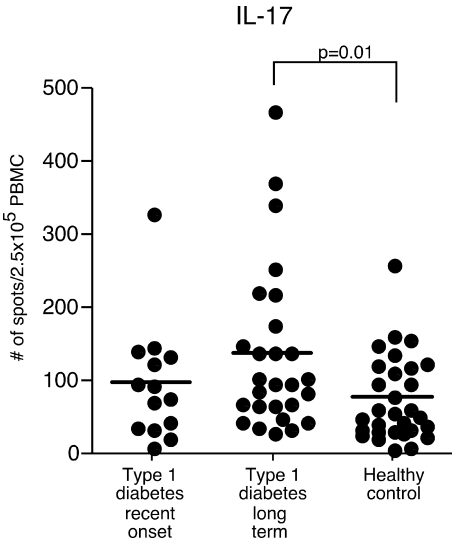

FIGURE 7. IL-17-secreting cells from anti-CD3-stimulated PBMCs from long-term T1D subjects was greater compared with those from healthy control subjects. Anti-CD3-stimulated PBMCs were incubated in HL-1 medium with 1% human serum for 18 h and then analyzed by ELISPOT. Each circle represents the number of positive PBMCs derived from a single subject. Two hundred fifty thousand PBMCs were added per well. Horizontal bars indicate the mean. Significant differences in cytokine-positive cells between groups are shown.

Other studies have compared monocytes and monocyte-derived cytokines from T1D and T2D subjects with differing results. Microarray analysis of PBMCs found that *IL-1β* was highly overexpressed in PBMCs derived from both T1D and T2D subjects (30). mRNA expression profiling has also been performed in monocytes from T2D patients compared with healthy controls and T1D patients as measured by quantitative real-time RT-PCR (20). Stimulated monocytes from T2D patients showed significantly higher expression levels of cytokines including *TNF-α*, *IL-6*, and *IL-1* compared with controls and T1D patients. In another investigation, TNF-α and IL-6 levels were evaluated in LPS-stimulated monocytes obtained from T1D and T2D patients, and although TNF-α secretion was elevated in monocytes derived from both T1D and T2D subjects, IL-6 was only elevated in T1D-derived monocytes compared with those from controls (19). A recent gene array study found an increase in proinflammatory cytokine secretion predominantly from adult-onset and latent autoimmune diabetes of the adult T1D subjects, but not from juvenile onset T1D subjects (22). A fundamental difference between that study and ours is the separation of patients into groups based on disease onset (22) or by length of disease duration (<1 year from disease onset or long-term T1D subjects, as described here). In the study presented here, recent-onset subjects were predominantly adult onset, whereas the long-term disease group (mean age of onset 15 years old with 15 years of disease) was more similar to the juvenile-onset group in

the referenced study (22). Larger studies will be needed to follow monocyte cytokine secretion as the disease progresses in each group of T1D subjects.

Previously, we and others have demonstrated that the combination of IL-6 and IL-1β resulted in secretion of both IL-17 and IFN-γ from memory T cells (10, 14). In this study, we show that monocytes from T1D subjects, which spontaneously secrete IL-6 and IL-1β, preferentially expanded memory T cells secreting IL-17, but did not expand the IFN-γ^+ memory T cell population compared with monocytes from healthy control subjects. The IL-17$^+$IFN-γ^+ subpopulation of inflammatory T cells is of particular interest because a similar population has been observed in the CNS of mice with experimental autoimmune encephalomyelitis (28). In the NOD model, it has been shown that Th17 cells are required to convert to IFN-γ-secreting cells for the initiation of diabetes (8, 9). The mechanism of induction of IL-17 secretion in T1D was partially through the proinflammatory cytokines IL-6 and IL-1β, as blocking their interaction with their receptor reduced the number of IL-17- secreting cells. These findings are of particular clinical interest as a phase I clinical trial in newly diagnosed T1D subjects using the IL-1 antagonist anakinra is in progress (clinicaltrials.gov/NCT00645840).

There has been increasing attention regarding the role of the innate immune system in driving the activation of CD4 cells into a pathologic effector state. Although there have been multiple reports on monocyte cell function after their ex vivo activation in human autoimmune diseases, we report here the presence of a strikingly activated innate immune system in patients with T1D. Moreover, we demonstrate that these monocytes from diabetic subjects, partly by secretion of IL-1β and IL-6, can drive memory CD4 T cells to secrete IL-17. Although it is possible that the hyperglycemic state of diabetic subjects could induce the secretion of these cytokines by monocytes, in these assays we do not observe the same amount of spontaneous cytokine secretion from PBMCs from patients with T2D, consistent with some previous reports examining cytokine secretion in these patients.

It has been demonstrated that islet-infiltrating macrophages and dendritic cells in patients with T1D secrete TNF-α and IL-1β (31), suggesting that activated monocytes may recruit to the islets in T1D. However, the origin of these monocytes still needs to be determined. A significant proportion of the immune system resides in the gastrointestinal system in homeostasis with gut flora, and the pancreatic lymph nodes are linked not only to the pancreas, but also to the gastrointestinal tract. Recently, it was shown that the innate immune system's interaction with intestinal microbes is important in NOD mice developing diabetes (32). Interestingly, many similar allelic variants are being observed between inflammatory bowel disease and human autoimmune diseases including T1D. Future investigations will focus on elucidating the source of these circulating, proinflammatory monocytes.

In conclusion, we demonstrated a mechanism of Th17 expansion in T1D; a subset of monocytes isolated directly from the circulation of recent-onset T1D patients express mRNA of proinflammatory proteins and spontaneously secrete IL-1β and IL-6 that induce potentially pathogenic IL-17/IFN-γ-secreting T cells. Blocking the monocyte-derived cytokines, IL-6 and IL-1β, but not TNF-α, resulted in a reduced number of IL-17-secreting cells, confirming their role in Th17 expansion. These observations may allow a simple measure of innate immune system activation in patients with autoimmune diseases. Finally, elucidation of the mechanism and site of monocyte activation in patients with T1D may provide critical insight into disease pathogenesis.

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Disclosures

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