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

Elizabeth M. Bradshaw,* Khadir Raddassi,* Wassim Elyaman,* Tihamer Orban,† Peter A. Gottlieb,‡ Sally C. Kent,* and David A. Hafler*1

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 Th 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 Th 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) 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 naïve 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 naïve (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 years; 8 females and 7 males), and 27 healthy control subjects (mean age ± SD 26.7 ± 5.0 years; 12 females and 15 males). Written informed consent was obtained from all subjects and the study was approved by the ethics committees at Brigham and Women’s Hospital and Harvard Medical School.

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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 \(\pm SD, 8.6 \pm 7.0\) years; 8 females and 7 males), or 42 healthy subjects (mean age \(\pm SD, 31.8 \pm 10.2\) 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 \(\times 10^7\) cells/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 from the CD14\(^+\)CD16\(^+\) populations. For the coculture experiments, the monocytes were FACs sorted after negative isolation (FACsAria; BD Biosciences) based on CD11b expression. Naïve 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 \(\times 10^7\) PBMCs/well were plated in HL-1 medium supplemented with 2 mM L-glutamine, 5 mM HEPES, 100 U/ml penicillin and 100 \(\mu\)g/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-CD1 (OKT3, 1 \(\mu\)g/ml in PBS. After 1 h at 37\(^\circ\)C/5% CO\(_2\), the cells were transferred to coated ELISPOT plates and left for an additional 16 h at 37\(^\circ\)C/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-1\(\beta\) (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 monocytoids (1:1) in the presence of plate-bound anti-CD3 Ab (OKT3, 1 \(\mu\)g/ml) for 5 days. For cytokine-blocking experiments with memory T cells cultured with monocytes, IL-1Ra (125 ng/ml), anti-IL-6 (10 \(\mu\)g/ml), and anti-TNF-\(\alpha\) (10 \(\mu\)g/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-\(\gamma\) (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-1\(\beta\) have both been implicated in the differentiation and expansion of Th17 cells. Therefore, we examined the number of circulating IL-6- and IL-1\(\beta\)-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-1\(\beta\)-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. 1A\(^\)\(^\_\)\(^\_\)\(^\_\)). 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 glycoporphuin A-positive cells were removed; this isolation method purifies the classic CD14\(^+\)CD16\(^+\) monocyte population which accounts for \(\sim 90\%\) of circulating monocytes. The majority of IL-6- and IL-1\(\beta\)-secreting cells were observed in the monocyte population (Fig. 2). The frequency of IL-6- and IL-1\(\beta\)-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-1\(\beta\) (Fig. 3). The negatively isolated monocytes had the highest expression, but the CD11c\(^-\) population also expressed significant amounts of IL-6 and IL-1\(\beta\) 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\(^+\)CD16\(^+\); both produce more TNF-\(\alpha\) than the classical monocytes when stimulated, but the CD14\(^+\)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\(^+\)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\(^+\)CD16\(^+\) did not (Fig. 4).

\(^3\) The online version of this article contains supplemental material.
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-1β, 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-1β 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 TGFβ 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 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 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.

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 controls (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).
PCR. The CD16 were analyzed by quantitative RT-expression is relative to monocytes from five T1D subjects and five healthy control subjects were analyzed by quantitative RT-PCR. The CD16 monocytes were removed by negative isolation and the monocyte-depleted cells were FAC 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 β₂-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⁺memory cells and IL-17⁺IFN-γ⁺memory cells 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).

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).

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 FAC 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 β₂-microglobulin.

FIGURE 4. Unstimulated CD14⁺CD16⁻ and CD14⁺CD16⁺ monocytes from T1D subjects, but not CD14⁺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⁺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 β₂-microglobulin.

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
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).

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-1-/IFN-α- 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-1-/IFN-α- 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

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

<table>
<thead>
<tr>
<th>Cytokine-Positive T Cells</th>
<th>Monocytes from T1D Subjects</th>
<th>Monocytes from Healthy Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>% IL-17+</td>
<td>24.16 ± 5.044</td>
<td>16.51 ± 3.750</td>
</tr>
<tr>
<td>% IL-17+/IFN-α+</td>
<td>10.55 ± 2.072</td>
<td>6.690 ± 1.523</td>
</tr>
<tr>
<td>% IL-17+/IFN-γ+</td>
<td>13.49 ± 3.095</td>
<td>9.680 ± 2.276</td>
</tr>
<tr>
<td>% IL-17+/IFN-γ−</td>
<td>44.11 ± 3.251</td>
<td>39.84 ± 3.751</td>
</tr>
</tbody>
</table>

*p < 0.01.
from patients with T1D can be induced to secrete a more inflam-
matory cytokine milieu through activation with LPS or IFN-γ (92).
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 demon-
strated that not all of the monocytes are actively secreting cyto-
kines; 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.

Other studies have compared monocytes and monocyte-derived
cytokines from T1D and T2D subjects with differing results. Mi-
croarray analysis of PBMCs found that IL-1β was highly overex-
pressed 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 pa-
tients as measured by quantitative real-time RT-PCR (20). Stim-
ulated monocytes from T2D patients showed significantly higher
expression levels of cytokines including TNF-α, IL-6, and IL-1β
compared with controls and T1D patients. In another investigation,
TNF-α and IL-6 levels were evaluated in LPS-stimulated mono-
cytes 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 predom-
antly 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 sep-
aration 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

**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 ad-
dition of IL-2. The cells were stimu-
lated 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.

**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.
and IL-17 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 subpopulation on monocyte cell function after their ex vivo activation in T1D 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.

The authors have no financial conflict of interest.

References


