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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,2* and David A. Hafler2,3*

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.


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; mean HbA1c ± SD, 6.9 ± 1.7); and 12 healthy control subjects (mean age ± SD, 27.6 ± 4.3 years; mean disease duration ± SD, 41.9 ± 9.2 years; mean HbA1c ± SD, 5.7 ± 0.6).
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$^7$/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 $\sim$90% purity as defined by CD11b staining. The monocyte-depleted cells were FACs sorted into CD3$^+$, CD19$^+$, and CD3$^+$CD19$^-$CD11c$^+$ populations. The monocyte CD16 subpopulations were FACs sorted using CD14, CD16, and CD56; the CD56$^+$ Ab was used to exclude NK cells for the CD14$^{a}$CD16$^+$ pop-

ulation. 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 $\times$ 10$^4$ 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 (Oxoid Scientific) in 96-well round-bottom plates (Corning). For IL-17 ELISPOT, the plates were first coated with anti-CD3 (OKT3, 1 $\mu$g/ml) in PBS. After 18 h at 37°C/5% CO$_2$, the cells were transferred to coated ELISPOT plates and left for an additional 16 h at 37°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 monocytes (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) 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$^\dagger$). 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 glycoporphin A-positive cells were removed; this isolation method purifies the classical 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$^{a}$CD16$^+$; both produce more TNF-$\alpha$ than the classical monocytes when stimulated, but the CD14$^{a}$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$^{a}$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$^{a}$CD16$^+$ did not (Fig. 4).

$^\dagger$ 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,
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-17 from memory T cells compared with monocytes from healthy controls; percent T cells expressing IL-17 cultured with monocytes from T1D subjects have high gene expression compared with healthy control subjects. Gene expression of IL-17 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).

**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\(^+/\)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\(^+/\)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.

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-(\gamma)(^+)*</td>
<td>10.55 ± 2.072</td>
<td>6.69 ± 1.523</td>
</tr>
<tr>
<td>% IL-17(^+/)IFN-(\gamma)(^-)*</td>
<td>13.49 ± 3.095</td>
<td>9.68 ± 2.276</td>
</tr>
<tr>
<td>% IL-17(^+)/IFN-(\gamma)(^+)(^-)*</td>
<td>44.11 ± 3.251</td>
<td>39.84 ± 3.751</td>
</tr>
</tbody>
</table>

*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-\(\gamma\) expression. Data are presented as mean ± SE for the number of cytokine-staining positive cells (n = 22).

\(p < 0.01\).

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-1\(\beta\) (B), and TNF-\(\alpha\) (C) gene expression for T1D and healthy control subjects is shown. All gene expression is relative to B2-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-1\(\beta\) (D) and IL-6 and TNF-\(\alpha\) (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.

4436 CD16\(^-\) MONOCYTES SPONTANEOUSLY SECRETE IL-6 AND IL-1\(\beta\) IN T1D

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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.

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 both 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.

Acknowledgments
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Disclosures
The authors have no financial conflict of interest.

References


Supplementary Figure 1. Representative ELISpot experiments for a T1D and healthy control subject. PBMCs were either (a) unstimulated (as described in Figure 1) or (b) stimulated with plate-bound anti-CD3 for 18 hours in complete HL-1 media with 1% human serum with or without an IL-1 receptor antagonist (as in Figure 7), 250,000 cells were added per well, and then (a) IL-6, IL-1β and (b) IL-17 positive cells were analyzed by ELISpot; null is with media alone. The number of positive spots per well is shown.
Supplementary Figure 2. The T cell proliferation is driven by the anti-CD3 stimulus and not the allogeneic response. Negatively-isolated naive T cells were cocultured with negatively-isolated monocytes from T1D or HC subjects in a one to one ratio, in the presence or absence of immobilized anti-CD3. Proliferation on day four was measured with thymidine incorporation. N=9.
Supplementary Figure 3. Memory T cells from healthy control subjects stimulated with anti-CD3 alone have a much lower frequency of IL-17 producing cells compared with memory T cells stimulated with monocytes derived from T1D subjects. Memory T cells were stimulated with plate-bound anti-CD3 with or without monocytes from T1D subjects in a 1:1 ratio for 5 days. 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/IFNγ expression. For each population of IL-17+ cells (+/− IFNγ expression), monocytes from T1D subjects induced more T cells to produce IL-17 than with T cells alone (A-C) except when T cells were IFNγ single positive (D). N=4.
Supplementary Figure 4. The IL-17 secreting cells detected by ELISpot are CD3+ T cells. PBMCs were stimulated with plate-bound anti-CD3 for 18 hours in complete HL-1 media with 1% human serum. Cell were then activated with PMA plus ionomycin for 3 hours in the presence of Golgistop® and stained with CD3 and IL-17. The IL-17 positive cells were in the CD3+ population indicating that they were T cells.