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IL-17 Immunity in Human Type 1 Diabetes

Jarno Honkanen,* Janne K. Nieminen,* Ru Gao,* † Kristiina Luopajarvi,* Harri M. Salo,* Jorma Ilonen,‡,§ Mikael Knip,¶‖,# Timo Otonkoski,¶‖,# and Outi Vaarala*†

Th17 immunity has been shown to regulate autoimmune diabetes in mice. IL-17 neutralization prevented development of diabetes when given postinitiation of insulitis but not earlier, suggesting interference with the effector phase of the disease. Islet-cell Ag-specific Th17 cells converted into IFN-γ-secreting Th1-like cells and caused diabetes in mice recipients. The role of IL-17 in human type 1 diabetes (T1D) is, however, not established. In this study, we show upregulation of Th17 immunity in peripheral blood T cells from children with T1D. This was characterized by increased IL-17 secretion and expression of IL-17, IL-22, and retinoic acid-related orphan receptor C isoform 2, but also FOXP3 transcripts upon T cell activation in vitro. Also, circulating memory CD4 cells from children with T1D showed the same pattern of IL-17, IL-22 and FOXP3 mRNA upregulation, indicating IL-17 pathway activation in vivo. IL-17–positive T cells appeared to be CD4+ cells expressing TCR-αβ and CCR6, and a subpopulation showed coproduction of IFN-γ. Given the Th17 immunity in T1D, we demonstrated that IL-17 had detrimental effects on human islet cells in vitro; it potentiated both inflammatory and proapoptotic responses. Our findings highlight the role of IL-17 immunity in the pathogenesis of human T1D and point to a potential therapeutic strategy. The Journal of Immunology, 2010, 185: 1959–1967.

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Abbreviations used in this paper: COX-2, cyclooxygenase-2; Ct, cycle threshold; F, female; HO, Hoehst 33342; M, male; NOS2A, NO synthase 2A; PI, propidium iodide; RORC2, retinoic acid-related orphan receptor C isoform 2; RT-qPCR, reverse transcription-quantitative PCR; SOD2, superoxide dismutase 2; T1D, type 1 diabetes.

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of IL-17 immunity in human T1D, which provides a new view on the pathogenesis of the disease and implies a novel potential therapeutic strategy in T1D based on the control of IL-17 immunity.

Materials and Methods

Patients and control subjects

We studied 24 children with T1D (13 boys, mean age 8.7 y, SD 4.4, median and mean duration of diabetes, 12.5 and 87 d) and 20 nondoabetic children (10 boys, mean age 8.2 y, SD 4.4) for the comparison of IL-17 immunity in peripheral blood between diabetic and healthy children. The characteristics of the patients and controls are given in Table I. Eight children (three boys) with recently diagnosed T1D and eight unrelated siblings (three boys) of diabetic children were included in the study of peripheral blood-derived monocyte activation (Table II). The parents gave informed consent and children 10 y of age or older their informed assent, and the studies were approved by the ethics committees of the participating hospitals.

T cell activation

PBMCs were separated from the fresh heparinized blood samples by Ficoll (GE Healthcare, Uppsala, Sweden) isogradient centrifugation. Cells were washed three times with PBS (Lonza, Verviers, Belgium) and resuspended in RPMI 1640 culture medium (Life Technologies, Paisley, U.K.) containing 2 mmol L-glutamine, 25 mmol/l HEPES, 25 mg/ml gentamycin (Sigma-Aldrich, St. Louis, MO), and 5% of heat-inactivated male AB serum (Innovative Research, Novi, MI). For T cell stimulation, PBMCs were cultured 2 × 10^5 cells/200 μl/well for 40 h as three replicates in 96-well round-bottom culturing plates (Nunc, Roskilde, Denmark) precoated with anti-CD3 Ab (50 μg/ml anti-CD3 at 5μg/ml in PBS) (BD Pharmingen, San Diego, CA) and in the presence of soluble anti-CD28 Ab (BD Pharmingen). We collected cells in Qiagen RLT lysis buffer (Qiagen, Hilden, Germany) with anti-CD3 Ab (50 μg/ml anti-CD3 at 5μg/ml in PBS) (BD Pharmingen, San Diego, CA) and in the presence of soluble anti-CD28 Ab (BD Pharmingen). We collected cells in Qiagen RLT lysis buffer (Qiagen, Hilden, Germany) with anti-CD3 Ab (50 μg/ml anti-CD3 at 5μg/ml in PBS) (BD Pharmingen, San Diego, CA) and in the presence of soluble anti-CD28 Ab (BD Pharmingen). We collected cells in Qiagen RLT lysis buffer (Qiagen, Hilden, Germany) with anti-CD3 Ab (50 μg/ml anti-CD3 at 5μg/ml in PBS) (BD Pharmingen, San Diego, CA) and in the presence of soluble anti-CD28 Ab (BD Pharmingen). We collected cells in Qiagen RLT lysis buffer (Qiagen, Hilden, Germany) with anti-CD3 Ab (50 μg/ml anti-CD3 at 5μg/ml in PBS) (BD Pharmingen, San Diego, CA) and in the presence of soluble anti-CD28 Ab (BD Pharmingen).

Isolation of memory T cells

Memory T cells were purified from the freshly isolated PBMCs by two-step magnetic separation. CD4+ T cells were purified using a CD4 Multisort Kit (Miltenyi Biotec, Bensheim, Germany), and then naive T cells positive for CD45RA isoform were depleted using CD45RA microbeads according to the manufacturer’s instructions (Miltenyi Biotec). CD45RA+ memory T cells were lysed in Qiagen RLT Buffer (Qiagen) for RT-qPCR analysis. More than 90% purity of the memory T cells was achieved.

Isolation of monocytes

Negative isolation of monocytes from frozen and thawed PBMCs was performed with a Monocyte Isolation Kit II and LS columns according to the manufacturer’s instructions (Miltenyi Biotec). Postisolation of monocytes, cells were then washed with 4°C PBS and lysed with RLT buffer (Qiagen) supplemented with 1% 2-ME. The lysate was stored at −80°C prior to the extraction of RNA. The purity of isolated CD14+ monocytes was assessed by flow cytometry (mean 86.1%, median 88.4%).

Flow cytometry assays

Golgistop (BD Biosciences, San Jose, CA) was added for at least the last 4 h of T cell activation of PBMCs, and then PBMCs were stained for surface Ags using anti-CD4–FITC (BD Biosciences), anti-CCR6–PE (BD Pharmingen), anti-CCR7–PE (BD Pharmingen), anti-TCRγδ–FITC (BD Biosciences), and anti-TCRβ–PE (BD Biosciences). After washing, cells were fixed and permeabilized using the Cytofix/Cytoperm Plus Fixation and Permeabilization Kit (BD Biosciences). Intracellular IFN-γ and IL-17 were stained with anti–IFN-γ–PE (BD Biosciences) and anti–IL-17–Alexa 647 (eBioscience, San Diego, CA). Appropriate isotype controls were used to determine specific binding for each fluorescent channel: IgG1–FITC (BD Biosciences), IgG1–PE (BD Biosciences), and IgG1–Alexa 647 (eBioscience). Plasma levels of IL-6 were measured with the CBA kit according to the instructions of the manufacturer (BD Biosciences).

Cytokine treatment of islet cells

Human islets were isolated as described earlier in the Central Laboratory of the Nordic Network for Clinical Islet Transplantation in Uppsala, Sweden (8). After Ficoll gradient purification, fractions rich in islets (66 ± 10%, n = 7) were collected and shipped on ice to Helsinki, Finland. All procedures were approved by institutional Ethics Committees in Sweden and Helsinki, Finland. Batches of islets were incubated in nonadherent 24-well plates for 24 h or 72 h in RPMI 1640 medium (Life Technologies) supplemented with antibiotics and 10% FCS (Lonza) and treated without (as negative control) or with one or more of following recombinant human cytokines: IL-17 (100 ng/ml; eBioscience), IL-1β (5 ng/ml; PeproTech, Rocky Hill, NJ), and IFN-γ (50 ng/ml; PeproTech). MIN6 cells, a mouse insulinoma cell line provided by Dr. Jun-ichi Miyazaki, University of Osaka, Osaka, Japan, were cultured in 12-well tissue-culture plates with or without glass coverslips in DMEM medium (4.5 g/l glucose; Lonza) supplemented with antibiotics, 2-ME (0.25 μmol; Life Technologies), and 15% FCS (Lonza). Cytokine treatment was the same as above for human islet cells.

Islet cell viability and apoptosis

The percentages of necrotic and apoptotic cells were determined by nuclear double staining with 2 μg/ml 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and 5 μg/ml propidium iodide (PI; Sigma-Aldrich). After the cytokine treatment, the cells were exposed to the dyes for 30 min at 37°C and then washed with PBS twice. The human islet cells were further dissociated with 0.05% trypsin and 0.02% EDTA and cyto centrifuged (700 rpm, 8 min) to the slides pre fixation, whereas MIN6 cells on coverslips were directly fixed with 4% paraformaldehyde for 20 min at room temperature. The staining was observed under a fluorescence microscope, and pictures were taken for later counting. HO freely passes all cell membranes and stains DNA blue, whereas PI only enters necrotic and late-phase apoptotic cells and stains DNA red. Thus, necrotic plus apoptotic cells could be identified by the white nuclear staining (HO plus PI) and the fragmented nuclei.

RT-qPCR

Total RNA from the cultured human PBMCs and human islet cells was isolated with the Qiagen RNeasy Mini kit (Qiagen). DNase I treatment option of the RNA isolation kit was applied to the protocol. From the purified human monocytes, total RNA was isolated using the Qia gen RNeasy Micro Kit (Qiagen). From the CD4+ memory T cells, total RNA was isolated by using the Sigma-Aldrich Mammalian Total RNA kit (Sigma-Aldrich). cDNA was synthesized according to recommendations by Applied Biosystems using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA).

A comparative ΔΔ Ct method was used for quantification of the relative mRNA copy number of the gene of interest (User Bulletin #4, Applied Biosystems). ΔΔ Ct values of each sample were compared with the ΔΔ Ct value of the in-house calibrator sample. The calibrator was RNA derived from PHA-stimulated PBMCs for T cell and monocyte studies or from human islets for islet cell studies.

The quantitative PCR TaqMan Gene Expression assays (Applied Biosystems) were used to amplify IL-17A (catalog number Hs00174383_m1), IL-17RA (catalog number Hs01064648_m1), IL-17RC (catalog number Hs.00262062_m1), RORC2 (catalog number Hs01076112_m1), IL-22 (catalog number Hs00220924_m1), IFN-γ (catalog number Hs00174143_m1), T-bet (catalog number Hs00203436_m1), FOXP3 (catalog number Hs00203958_m1), NO syn thase 2A (NOS2A, catalog number Hs00167248_m1), and cyclooxygenase-2 (COX-2; catalog number Hs00153133_m1)–specific transcripts. As an endogenous reference, gene ribosomal 18s was amplified using TaqMan Gene Expression assay (catalog number Hs00999990_s1) (Applied Biosystems).

Statistical analysis

Data from T cell activation experiments were compared between the two groups by Mann-Whitney U test, whereas the expression levels of the RNA of target genes in memory CD4+ cells were compared between the two groups with the two-sided Fisher’s exact test. The changes between untreated and cytokine treated human islets or MIN6 cells were compared with paired t tests. The Spearman test was used for correlation analyses (r). Data were analyzed with Prism 4 (GraphPad, San Diego, CA).

Results

Increased IL-17, RORC2, and IL-22 response upon T cell activation in children with T1D

To address the role of Th17 cells in human T1D, we activated peripheral blood T cells with anti-CD3 plus anti-CD28 stimulation. We observed enhanced IL-17 secretion and IL-17A transcript

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expression in children with T1D when compared with healthy children ($p = 0.018$ and $p = 0.012$, respectively) (Fig. 1A, 1B, Table I). Also, RORC2 mRNA, the signature transcription factor for Th17 cells, and the IL-22 cytokine-specific mRNA were expressed at higher levels in response to T cell activation in children with T1D than in healthy children ($p = 0.02$ and $p = 0.005$) (Fig. 1C, 1D). The levels of RORC2 and IL-17 transcripts induced upon T cell activation correlated with secreted IL-17 in children with T1D ($r_s = 0.599$, $p = 0.018$; and $r_s = 0.717$, $p = 0.003$). We found no difference in the IFN-γ response or in T-bet mRNA upregulation between diabetic and healthy children (Fig. 1E, 1F). The enhanced IL-17 immunity did not show any association with the age of the patients or the duration of the disease, and the number of patients studied was too small to determine the associations with the HLA genotype (Supplemental Table I).

**Enhanced IL-17, RORC2, and IL-22 mRNA expression in circulating memory CD4 cells in T1D**

Having shown that anti-CD3 plus anti-CD28 activation of T cells induced an IL-17 response in children with T1D, but not in healthy children, we examined in an independent series of nine children with T1D whether IL-17 upregulation could be observed in vivo (i.e., in circulating memory CD4 cells). IL-17A and IL-22 mRNA was expressed in the population of freshly purified peripheral blood CD45RO+CD4+ memory cells from children with T1D (Fig. 2A, 2B), whereas minimal or undetectable expression levels were seen in healthy children (six out of eight versus zero out of eight; $p = 0.007$ for IL-17; and five out of eight versus zero out of seven; $p = 0.026$ for IL-22). In naive CD45RA−/CD45RO+ cells, no expression of IL-17A mRNA could be observed in diabetic or healthy children. The expression levels of RORC2 mRNA were not detectable in most CD45RO−/CD4+ cells. No differences in the expression of IFN-γ in CD45RO+CD4+ cells were seen between diabetic and healthy children (Fig. 2C). Thus, the upregulation of markers characterizing Th17 cells occurred in diabetic children in vivo without accompanied aberrancies mirrored in Th1-related markers. FACS analysis showed that T cell stimulation resulted in an increase of IL-17–positive cells among CD4+ cells, whereas IL-17CD4+ cells were rare in children with T1D (Fig. 3A). As shown in Fig. 3B, >90% of IL-17CD4+ T lymphocytes expressed CCR6 (median 95.2%, range 81–99.4%, $n = 7$ T1D children), an established Th17 cell-associated chemokine receptor, which is involved in mucosal immunity. IL-17–positive CD3+ cells were TCR-αβ-expressing cells (Fig. 3D; $n = 8$ T1D children). The characteristics of IL-17–positive CD4 cells in terms of CCR6 and TCR was similar in healthy children (Supplemental Fig. 1).

**Coproducers of IFN-γ/IL-17 cells do exist in children with T1D**

Because coproducers of IL-17 and IFN-γ have been observed in autoimmune diseases, we examined the relation of IL-17 and IFN-γ response in T1D. Despite comparable IFN-γ and T-bet upregulation in children with T1D and healthy children, the IFN-γ response, both at the mRNA and protein level, correlated with IL-17A secretion induced by T cell activation in children with T1D ($r_s = 0.652$, $p = 0.006$; and $r_s = 0.735$, $p = 0.002$). A subpopulation of IL-17–positive CD4+ cells coexpressed also IFN-γ in 4 out of 11 diabetic children tested, the proportion of double-positive cells varying from 15–35% of IL-17–positive CD4 cells (Fig. 3C). Accordingly, it seems that some CD4+IL-17+ cells are IFN-γ coproducers also in T1D.

**Upregulation of FOXP3 transcripts upon T cell activation and in memory T cells in T1D**

Because plasticity between Th17 cells and FOXP3-positive regulatory T cells exists, we determined the expression of FOXP3 mRNA, the major regulator of regulatory T cell differentiation. Increased levels of FOXP3 transcripts were observed in response to anti-CD3 and anti-CD28 stimulation in children with T1D.

![Image](https://www.jimmunol.org/)

**FIGURE 1.** Pronounced IL-17 pathway upon T cell activation in human T1D. ELISA of IL-17 levels in supernatant samples (A) and real-time PCR expression levels of IL-17 (B), RORC2 (C), IL-22 (D), IFN-γ (E), T-bet (F), and FOXP3 (G) mRNA transcripts in anti-CD3 plus anti-CD28–activated PBMCs in 15 children with T1D and 12 nondiabetic children. Levels of target gene mRNA transcripts are normalized to rRNA (18S) and in-house calibrator (mitogen-stimulated PBMC-derived RNA). Values of $p$ were calculated with the Mann-Whitney $U$ test.
The FOXP3 expression induced upon T cell activation correlated with both RORC2 mRNA expression levels and IL-17 secretion in children with T1D ($r_s = 0.846, p < 0.0001$; and $r_s = 0.706, p = 0.003$). Also, high expression levels of FOXP3 in CD45RO+CD4+ cells were seen more often in children with T1D than in healthy children (five out of nine versus zero out of nine; $p = 0.009$) (Fig. 2D). The mRNA levels of FOXP3 and IL-17 in memory T cells correlated strongly in diabetic children ($r_s = 0.89, p < 0.0001$). Thus, upregulation of the RORC2/IL-17 pathway was associated with enhanced FOXP3 transcription in T1D.

No evidence of enhanced activation of IL-6 and IL-1β in monocytes from children with T1D

Simultaneous expression of FOXP3 and RORC2 has been related to the counteracting role of IL-6 in FOXP3-mediated inhibition of the IL-17 response. When we compared plasma IL-6 levels between children with T1D and healthy children, we did not, however, find elevated systemic IL-6 levels among children with T1D (data not shown). We next analyzed the expression of IL-1β and IL-6 mRNA in monocytes separated from thawed PBMCs of eight children with T1D and eight unrelated auto-Ab–negative siblings of diabetic children matched for age, sex, and HLA genotype. We did not confirm the upregulation of IL-1β and IL-6 transcripts in the resting monocytes from patients with T1D (Fig. 4, Table II) as reported by others (6). However, the patients with T1D also included adults in the study by Bradshaw and coauthors (7), whereas we studied children. In addition, the controls in our study were healthy siblings who all actually carried increased HLA-conferred susceptibility to T1D.

IL-17 potentiated inflammatory and apoptotic response in human islet cells

Our findings of IL-17 immunity in T1D raise the question of the effects of IL-17 on β cells. We examined the effects of IL-17...
on human islet cells in vitro, as such or in combination with other inflammatory mediators, namely IL-1β and IFN-γ, which contribute to human β cell death. Remarkable expression levels of IL-17RA and IL-17RC mRNA transcripts were detected in human islets (mean 0.06-fold and mean 3.8-fold for IL-17RA and 0.14-fold and 17.34-fold for IL-17RC in relation to the expression level of β2-microglobulin and BCL-2 transcripts, respectively, in human islet cells; n = 6). Furthermore, IL-17 increased transcription of SOD2, and in synergy with IL-1β and IFN-γ transcription of the inducible isoform of NOS2A and COX-2 (Fig. 5A–C), which are involved in the inflammatory response in islet cells. We also analyzed the potential apoptotic effect of IL-17 and observed that IL-17 inhibited the mRNA expression of the antiapoptotic gene BCL-2 (Fig. 5D) and enhanced the proapoptotic effect of IL-1β+IFN-γ in mouse insulinoma cells and primary human islet cells (Fig. 6).

Discussion
We demonstrated overwhelming IL-17 response upon T cell activation and upregulation of IL-17A transcripts in the circulating memory CD4 cells in about half of the children with T1D. Most of the diabetic children studied were newly diagnosed, but IL-17

FIGURE 2. Activation of IL-17 immunity in circulating memory cells in T1D. Real-time PCR expression levels of IL-17 (A), IL-22 (B), IFN-γ (C), and FOXP3 (D) mRNA transcripts in memory CD4+CD45RO+ cells in children with T1D and nondiabetic children. Levels of target gene mRNA transcripts are normalized to rRNA (18S) and in-house calibrator (mitogen-stimulated PBMC-derived RNA). Values of p were calculated with the Fischer’s exact test.

FIGURE 3. Phenotype analysis of IL-17–positive cells in T1D. A, Percentage of CD4+ and IL-17–positive cells in lymphocyte gate in unstimulated and anti-CD3 plus anti-CD28–activated PBMCs from a representative child with T1D. B, A representative dot plot of IL-17–expressing cells among CD4+CCR6+ and CD4+CCR6− cells from anti-CD3 plus anti-CD28–stimulated PBMCs from a child with a newly diagnosed T1D. C, Percentage IL-17+ and IFN-γ+ cells in CD4-gated lymphocytes in unstimulated and anti-CD3 plus anti-CD28–activated PBMCs from a representative child with T1D. D, A representative dot plot of anti-CD3 plus anti-CD28–stimulated PBMCs from a child with newly diagnosed T1D showing that CD3+ T cells expressing IL-17 are TCR-αβ+. 

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FIGURE 4. No difference in the levels of IL-6 and IL-1β mRNA transcripts in monocytes from children with T1D and controls. IL-6 and IL-1β mRNA expression in monocytes isolated from frozen and thawed PBMCs of eight children with recent T1D and eight auto-Ab-negative siblings of diabetic children. Levels of target gene mRNA transcripts are normalized to rRNA (18S) and calibrator.

immunity was seen as well in the children with longer duration of the disease. Upregulation of the IL-17–inducing transcription factor RORC2 and the cytokine IL-22 associated with IL-17 immunity was seen in both activated T cells and memory CD4 cell population from diabetic children. These findings indicate that activation of the IL-17/IL-22 pathway is a hallmark of human T1D, at least in pediatric diabetes. The number of circulating Th17 cells was low because the signal for IL-17A mRNA from memory CD4 cells was weak although detectable in diabetic children.

The IL-17–producing T cells in children with T1D expressed CCR6, an established chemokine receptor for Th17 cells (9–11). CCR6-expressing T lymphocytes show specific recruitment to the mucosal surfaces. Indeed, Th17 immunity is considered a crucial element in the mucosal immune protection against bacterial and fungal pathogens (6). Its activation is also seen in autoimmune conditions and inflammatory bowel disease (6, 12). In autoimmune diabetes, the lymphocytes in the inflamed islets show mucosal homing properties as demonstrated both in animal models and human T1D (13–15). Also, the aberrancies of the gut immune system, such as subclinical intestinal inflammation (16, 17) and increased gut permeability (18, 19), have been associated with human T1D. Accordingly, the upregulation of IL-17 immunity may be a marker of altered mucosal immune response in T1D. IL-17–producing T cells in mice have been described to express TCRγδ (20, 21), which is typically found in the intraepithelial T cells, whereas only a minor population of circulating T cells have TCRγδ. Peripheral blood IL-17–producing T cells from diabetic children showed, however, expression of the conventional TCRαβ, which may be of mucosal origin, too.

The induction of naive and memory human T cells to IL-17–secreting cells seems to be promoted by different sets of cytokines. IL-6 and IL-1β induce RORC2 and IL-17 activation in memory T cells, whereas TGF-β and IL-21 in combination induce differentiation of Th17 cells from naive T cells (22). Bradshaw and coauthors (7) reported recently that monocytes from patients with T1D showed spontaneous activation of IL-6 and IL-1β, and the monocytes isolated from diabetic patients were able to induce IL-17 deviation in allogeneic memory T cells in vitro. We could not, however, demonstrate increased systemic IL-6 activation in plasma samples from diabetic children or spontaneous activation of IL-6 or IL-1β transcripts in monocytes from diabetic children. It should be noted that we compared the diabetic children to the unrelated siblings who all carried the HLA risk genotype associated with the disease.

In addition to the upregulation of IL-17 immunity in T1D, we observed increased expression of FOXP3 transcripts in both activated T cells and memory CD4 cells, which suggests simultaneous upregulation of regulatory mechanisms and the IL-17 pathway in T1D, but not necessarily in the same cell population. Accumulating evidence shows that human regulatory T cells show plasticity and may differentiate IL-17–producing cells (23–27). In the presence

Table II. Levels of IL-6 and IL-1β transcripts and the characteristics of patients and control children

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<th>Diagnosis</th>
<th>Age (y)</th>
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6HLA genotype indicates the presence of HLA-DR-DQ haplotypes associated with T1D risk. DR3-DQ2 = (DR3)*DQA1*05-DQB1*02, DR4-DQ8 = DRB1*0401/4- (DQA1*03)-DQB1*0302. All other haplotypes marked by x.

A-6IL-6 and IL-1β mRNA levels are normalized to an endogenous control (18S) and to an in-house calibrator (PHA-stimulated PBMCs). The purity of the enriched monocyte fraction is expressed as the percentage of CD14+ monocytes of the total CD45+ leukocytes, as determined by flow cytometry.

7Controls are unrelated auto-Ab-negative (negative for islet cell Abs, insulin auto-Abs, glutamic acid decarboxylase Abs, and IA-2 Abs) siblings of patients with T1D.

F, female; M, male.
of IL-6, the inhibition of RORC2 by FOXP3 is abrogated, and IL-17 production is seen in regulatory T cells still expressing FOXP3 mRNA (23–25). CCR6-expressing regulatory T cells possess suppressive function, although they produce IL-17 upon activation (26). IL-2 and IL-15 have been shown to induce the differentiation of human regulatory T cells into IL-17–producing cells, and this is further enhanced in particular when exogenous IL-1β, IL-23, or IL-21 is present (27). We demonstrated the presence of IL-17–producing cells in anti-CD3 plus anti-CD28–activated T cells. This treatment also results in FOXP3 upregulation (28). Thus, FOXP3 staining would not have revealed whether the IL-17–producing cells were originally FOXP3–expressing regulatory T cells in the current study design. In unstimulated peripheral blood T cells, the IL-17–producing T cells were too rare to be analyzed by flow cytometry. The small volume and availability of fresh blood samples from children with T1D further suggests that some of the IL-17–secreting T cells associated with T1D are not functionally committed memory T cells. IL-17–secreting T cells are able to convert into Th1 cells when TGF-β is not present (32).

The tissue destruction associated with Th17 immunity has been suggested to be dependent on the coproduction of IFN-γ (4, 5). IFN-γ secretion by Th17 cells may result in additive β cell damage due to the synergistic effects mediated by IFN-γ and IL-17 on human islet cells, as we demonstrated in this paper. The expression level of IL-17RA and IL-17RC was remarkable in human islets, and IL-17 potentiated the inflammatory and apoptotic response in human islet cells in vitro. The upregulation of

**FIGURE 6.** IL-17 increases proapoptotic responses in human islet cells and mouse β cells. A, The proportion of necrotic and apoptotic cells in pancreatic islet cell preparation treated with IL-17 alone, IL-1β and IFN-γ, or IL-17 together with IL-1β and IFN-γ. B, The proportion of necrotic and apoptotic cells in a mouse insulinoma cell line, MIN6, treated with IL-17 alone, IL-1β and IFN-γ, or IL-17 together with IL-1β and IFN-γ. Data represent mean ± SD of three experiments. The proportion of necrotic and apoptotic cells was assessed by nuclear double staining with HO and PI. Examples of necrotic plus apoptotic cells identified by the white nuclear staining (as a result of the combined blue HO and red PI signals) and the fragmented nuclei (arrows indicating positive cells) in human islet cell preparation (C) and MIN6 cells (D) when treated with IL-17 together with IL-1β and IFN-γ (lower panels) and untreated control (upper panels). Original magnification ×20.
inducible NOS and SOD2 results in increased production of free radicals that interfere with β cell function and cause β cell damage (33, 34). The induction of inducible NOS by IL-17 has been earlier shown in a mouse insulinoma cell line, confirming the effect of IL-17 on β cells (34). Furthermore, IL-17 together with IFN-γ and IL-1β potentiated the upregulation of COX-2, the expression of which is associated with β cell dysfunction (35, 36). Downregulation of the antiapoptotic gene BCL-2 (37) was seen as well as increased apoptosis of human islet cells and mouse insulinoma cells when treated with IL-17 together with other inflammatory cytokines, such as IL-1β and IFN-γ. Accordingly, the synergistic effect of IFN-γ and IL-17 in the induction of inflammation and apoptosis in the human islet cells indeed suggests that the coproducers of IL-17 and IFN-γ might be of clinical importance, as implicated in animal models of autoimmune diabetes (3, 5). Interestingly, IL-17 secretion has been reported in glutamic acid decarboxylase-specific T cell clones (38), which provides a link to the pathogenesis of TID. Recent evidence from experimental animal models suggests that so-called natural Th17 cells are generated in the thymus, and they share a developmental relationship with FOXP3+ regulatory cells, showing self-reactivity and specific recruitment to mucosal surfaces (39). These natural Th17 cells also secrete IL-22 and express CCR6. In inflammatory conditions, these kinds of self-reactive natural Th17 cells could contribute to the proinflammatory response and promote autoimmune and self-destruction. We could not explore in this study the Ag specificity of the IL-17–producing T cells in TID, but our finding of Th17 cells in TID is especially interesting in light of these recent observations because the alleles of the insulin gene finding of Th17 cells in T1D is especially interesting in light of Immunologic activity in the intestinal mucosa of pediatric patients with type 1 diabetes. Diabetes 52: 2287–2295.


20. Rader, S. T., E. F. Machold, F. M. Strober, and D. A. Hafler. 2008. IL-21 and TGF-beta are potentiated the upregulation of COX-2, the cell damage. We further demonstrate detrimental effects of IL-17 on human islet cells, providing a link between Th17 immunity and β cell damage.

Disclosures

The authors have no financial conflicts of interest.


Supplementary figure. Phenotype analysis of IL-17 positive cells in a non-diabetic child. (a) Percentage of CD4 and IL-17 positive cells in lymphocyte gate in unstimulated and anti-CD3 plus anti-CD28 activated peripheral blood mononuclear cells. (b) A dot plot of IL-17 expressing cells among CD4+ CCR6+ and CD4+CCR6- cells from anti-CD3 plus anti-CD28 stimulated peripheral blood mononuclear cells. (c) Percentage IL-17 and IFN-γ positive cells in CD4-gated lymphocytes in unstimulated and anti-CD3 plus anti-CD28 activated peripheral blood mononuclear cells. (d) A dot plot of anti-CD3 plus anti-CD28 stimulated peripheral blood mononuclear cells showing that CD3+ T cells expressing IL-17 are TCR αβ positive.
Supplementary table. The correlation of IL-17 related immunity with the patient characteristics.

Correlation of disease duration (days) and gene expression (mRNA) and IL-17 secretion (IL-17prot) in anti-CD3+anti-CD28 stimulated PBMC from patients with type 1 diabetes

<table>
<thead>
<tr>
<th>mRNA</th>
<th>IL-17 mRNA</th>
<th>RORC2 mRNA</th>
<th>IL-22 mRNA</th>
<th>FOXP3 mRNA</th>
<th>IFNG mRNA</th>
<th>IL-17prot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman r</td>
<td>0.072</td>
<td>-0.25</td>
<td>-0.07</td>
<td>-0.06</td>
<td>-0.24</td>
<td>-0.07</td>
</tr>
<tr>
<td>p-value</td>
<td>0.8</td>
<td>0.36</td>
<td>0.82</td>
<td>0.83</td>
<td>0.4</td>
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Correlation of age (years) and gene expression (mRNA) and IL-17 secretion (IL-17prot) in anti CD3+anti-CD28 stimulated PBMC from patients with type 1 diabetes

<table>
<thead>
<tr>
<th>mRNA</th>
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<th>IL-22 mRNA</th>
<th>FOXP3 mRNA</th>
<th>IFNG mRNA</th>
<th>IL-17prot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman r</td>
<td>0.46</td>
<td>0.26</td>
<td>0.37</td>
<td>0.2</td>
<td>0.44</td>
<td>0.21</td>
</tr>
<tr>
<td>p-value</td>
<td>0.09</td>
<td>0.35</td>
<td>0.19</td>
<td>0.47</td>
<td>0.1</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Correlation of disease duration (days) and gene expression in CD4+CD45RO+ memory T cells from patients with type 1 diabetes

<table>
<thead>
<tr>
<th>mRNA</th>
<th>IL-17 mRNA</th>
<th>RORC2 mRNA</th>
<th>IL-22 mRNA</th>
<th>FOXP3 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman r</td>
<td>0.36</td>
<td>0.58</td>
<td>0.31</td>
<td>0.15</td>
</tr>
<tr>
<td>p-value</td>
<td>0.39</td>
<td>0.11</td>
<td>0.46</td>
<td>0.7</td>
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</tbody>
</table>

Correlation of age (years) and gene expression in CD4+CD45RO+ memory T cells from patients with type 1 diabetes

<table>
<thead>
<tr>
<th>mRNA</th>
<th>IL-17 mRNA</th>
<th>RORC2 mRNA</th>
<th>IL-22 mRNA</th>
<th>FOXP3 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman r</td>
<td>0.12</td>
<td>0.32</td>
<td>0.07</td>
<td>-0.27</td>
</tr>
<tr>
<td>p-value</td>
<td>0.79</td>
<td>0.41</td>
<td>0.88</td>
<td>0.49</td>
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</table>

The median levels of gene expression (mRNA) and cytokine secretion (P) in anti-CD3+anti-CD28 stimulated PBMC from the patients positive for HLA DR3-DQ2 or DR4-DQ8 haplotype. The genotyping result was not available in two patients and one patient had both HLA DR3-DQ2 and DR4-DQ8.

<table>
<thead>
<tr>
<th></th>
<th>DR3-DQ2 positive, n=3</th>
<th>DR4-DQ8 positive, n=10</th>
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</thead>
<tbody>
<tr>
<td>FOXP3</td>
<td>median: 56.2, range: 34.4 - 103.2</td>
<td>median: 43.3, range: 9.3 - 103.2</td>
</tr>
<tr>
<td>IL-17</td>
<td>median: 441.5, range: 26.1 - 3952</td>
<td>median: 27.9, range: 0.0 - 2098</td>
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<tr>
<td>RORC2</td>
<td>median: 93.9, range: 23.9 - 146.9</td>
<td>median: 82.3, range: 3.5 - 770.2</td>
</tr>
<tr>
<td>IL-22</td>
<td>median: 6.5, range: 3.7 - 144.3</td>
<td>median: 3.5, range: 0.2 - 27.4</td>
</tr>
<tr>
<td>IFNGP</td>
<td>median: 6943, range: 1959 - 13128</td>
<td>median: 4368, range: 0.0 - 10048</td>
</tr>
<tr>
<td>IL-17P</td>
<td>median: 172.8, range: 99.3 - 805.7</td>
<td>median: 111.6, range: 0.0 - 593.2</td>
</tr>
</tbody>
</table>

The median levels of gene expression (mRNA) in CD4+CD45RO+ memory T-cells from the patients positive for HLA DR3-DQ2 or DR4-DQ8 haplotype. The genotyping result was not available in one patient and one patient had no HLA DR3-DQ2 or DR4-DQ8. All patients positive for HLA DR3-DQ2 were also positive for HLA DR4-DQ8.
<table>
<thead>
<tr>
<th></th>
<th>DR3-DQ2</th>
<th></th>
<th>DR4-DQ8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median</td>
<td>range</td>
<td>median</td>
</tr>
<tr>
<td>FOXP3</td>
<td>2.0</td>
<td>1.1 - 2.0</td>
<td>9.3</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.44</td>
<td>0.0 - 0.72</td>
<td>0.75</td>
</tr>
<tr>
<td>RORC2</td>
<td>0.95</td>
<td>0.0 - 2.2</td>
<td>2</td>
</tr>
<tr>
<td>IL-22</td>
<td>0</td>
<td>0.0 - 184.1</td>
<td>40.3</td>
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