Peripheral and Islet Interleukin-17 Pathway Activation Characterizes Human Autoimmune Diabetes and Promotes Cytokine-Mediated β-Cell Death

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OBJECTIVE—CD4 T-cells secreting interleukin (IL)-17 are implicated in several human autoimmune diseases, but their role in type 1 diabetes has not been defined. To address the relevance of such cells, we examined IL-17 secretion in response to β-cell autoantigens, IL-17A gene expression in islets, and the potential functional consequences of IL-17 release for β-cells.

RESEARCH DESIGN AND METHODS—Peripheral blood CD4 T-cell responses to β-cell autoantigens (proinsulin, insulinoma-associated protein, and GAD65 peptides) were measured by IL-17 enzyme-linked immunospot assay in patients with new-onset type 1 diabetes (n = 50). mRNA expression of IL-17A and IFNG pathway genes was studied by qRT-PCR using islets obtained from subjects who died 5 days and 10 years after diagnosis of disease, respectively, and from matched control subjects. IL-17 effects on the function of human islets, rat β-cells, and the rat insulinoma cell line INS-1E were examined.

RESULTS—A total of 27 patients (54%) showed IL-17 reactivity to one or more β-cell peptides versus 5 of 30 (10%) control subjects (P = 0.0001). In a single case examined close to diagnosis, islet expression of IL17A, RORC, and IL22 was detected. It is noteworthy that we show that IL-17 mediates significant and reproducible enhancement of IL-1β/interferon (IFN)-γ-induced and tumor necrosis factor (TNF)-α/IFN-γ-induced apoptosis in human islets, rat β-cells, and INS-1E cells, in association with significant upregulation of β-cell IL17RA expression via activation of the transcription factors STAT1 and nuclear factor (NF)κB.

CONCLUSIONS—Circulating IL-17+ β-cell–specific autoreactive CD4 T-cells are a feature of type 1 diabetes diagnosis. We disclose a novel pathway to β-cell death involving IL-17 and STAT1 and NFκB, rendering this cytokine a novel disease biomarker and potential therapeutic target.

In organ-specific autoimmune diseases such as type 1 diabetes, key pathological checkpoints are priming and differentiation of T-cells specific for β-cell autoantigens, migration of these autoreactive cells to the islets of Langerhans, and β-cell–selective death (1). The recent identification of the importance of CD4 T-cells that secrete interleukin (IL)-17 in various rodent disease models (2,3) has focused attention on how T helper 17 (Th17) effector cells might participate in critical disease pathways in humans. In multiple sclerosis, for example, IL-17 is expressed at high levels by circulating T-cells, and IL17 gene expression is elevated in plaques in the affected brain (4,5). It is noteworthy that a clear link to immunopathogenesis has been made through the demonstration that Th17 cells disrupt blood-brain barrier tight junctions, enabling transmigration of immune-competent cells into the brain parenchyma and the establishment of local inflammation (6). Likewise, the presence of circulating and synovial tissue-infiltrating Th17 cells is well documented in rheumatoid arthritis (7,8), and IL-17 stimulates osteoclast-mediated bone resorption, a key pathological feature of the disease (7).

In contrast, the impact of IL-17 on the key checkpoints of human type 1 diabetes development has not been sufficiently addressed to enable a pathogenic role to be assigned to it. After polyclonal stimulation of peripheral blood cells, patients with type 1 diabetes upregulate IL-17 mRNA (9) and demonstrate a higher proportion of IL-17–secreting CD4 T-cells (10), but the lack of β-cell specificity of these responses limits their disease relevance. To address this important knowledge gap, we examined IL-17 effector responses to β-cell autoantigens in patients with newly developed type 1 diabetes. Here we report that IL-17–secreting CD4 T-cells primed to recognize β-cell autoantigens are a major feature of disease development and that an IL-17 signature is present in islets of Langerhans obtained from a patient who died very close to disease onset. It is important to note that IL-17R upregulation by the proinflammatory cytokines IL-1β and interferon (IFN)-γ renders human β-cells highly susceptible to death by IL-17/IL-1β/IFN-γ–induced apoptosis. These studies provide a strong rationale for early interference in the IL-17 pathway as a therapeutic strategy for type 1 diabetes.

RESEARCH DESIGN AND METHODS

Fresh heparinized blood samples were obtained from 50 patients with newly diagnosed type 1 diabetes (age range 18–43 years) (duration of type 1 diabetes ≤20 weeks) and 30 healthy control subjects (age range 20–48 years; summarized in Table 1 and detailed in Supplementary Table 1). These studies were...
carried out with the approval of the Local Research Ethics Committee, and informed consent was obtained from all participants.

**Detection of β-cell-specific IL-17-secreting CD4+ T-cells.** Peptides based on sequences of naturally processed and HLA-DRB4 presented IIA (708-738, 752-775, and 853-872), prosolin (C19-A3), and GAD65 epitopes (335-352 and 554-575), as well as overlapping regions of the insulin B (1-29, 6-25, and 11-30) and A chain (1-21), were synthesized by Fmoc chemistry and purified by high-performance liquid chromatography (Thermo Hybaid, Germany), a peptide of the CS protein of *Plasmodium falciparum* (363-377) that was used as a negative control. Pediacel, a penta-vaccine consisting of purified diphtheria toxoid, purified tetanus toxoid, acellular pertussis vaccine, inactivated poliovirus, and *Haemophilus influenzae* type b polysaccharide, was obtained from Sanofi Pasteur (Berkshire, U.K.) and used at 1 μL/mL as a positive control.

Detection of IFN-γ production by CD4+ T-cells in response to peptide stimulation was carried out using an enzyme-linked immunospot (ELISPOT) assay that has significant discriminative ability for type 1 diabetes in blinded proficiency testing, as described previously (11,12). The ELISPOT platform was adapted to a direct format to detect IL-17-secreting CD4+ T-cells using plates precoated with monoclonal anti-IL-17 capture antibody 72-h incubation and biotinylated anti-IFN-γ detector antibody (R&D Systems, Abingdon, U.K.). Data are expressed as the mean number of spots per triplicate and compared with the mean spot number in the presence of diluent alone (stimulation index).

**Statistical analysis.** Frequency data between patient and control groups were compared with the two-sided Fisher exact test. Data for the islet and insulinoma experiments are presented as mean ± SEM. Comparisons were performed by ANOVA followed by a Student t test with Bonferroni correction. A P value <0.05 was considered statistically significant.

**RESULTS**

**Detection of circulating β-cell-specific CD4+ T-cells secreting IL-17.** We examined peripheral blood responses of 50 patients with new-onset type 1 diabetes and 30 age-, HLA-, and sex-matched healthy nondiabetic control subjects against a well-defined panel of β-cell autoantigenic peptides using a sensitive ELISPOT to detect IL-17 secretion. IL-17 responses were observed in 27 of 50 patients (54%) compared with 3 of 30 (10%) healthy control subjects (P = 0.0001) (Fig. 1A). Among patients, 21 of 50 (42%) had responses against two or more β-cell autoantigenic peptides compared with 2 of 30 (8%) healthy control subjects (P = 0.0007; median responses 1 and 0 peptides, respectively).

In keeping with our previous findings, IFN-γ production against the same peptide panel was also observed and was
significantly more frequent in patients than in control subjects (31 of 50 [62%] compared with 4 of 30 [13%] \(P < 0.0001\); Fig. 1B). IL-17 responses were attributable to CD4 T-cells, since reactivity is lost after immunomagnetic depletion of these cells (data not shown).

Five patients were retested for IL-17 responsiveness a median of 12 months later (range 9–14 months) against the entire peptide panel. In three patients, the number of peptides provoking an IL-17 response declined. In each of these, the responses detected in the second sample had the same peptide specificities as in the original sample. In two patients, the number of peptides (and specific peptides targeted) remained the same (Fig. 1C).

The pattern of IL-17 reactivity against β-cell autoantigenic peptides did not differ markedly from the IFN-γ response (Fig. 1D), suggesting complementarities of the priming and maintenance stimuli for these two types of responses; however, the number of IL-17+ autoreactive and recall antigen-specific responder cells was typically fewer than for IFN-γ (Fig. 1E). All but one patient had received insulin therapy before testing, and it is conceivable that the responses we observed against overlapping peptides of insulin were induced as a result. However, IL-17 responses to overlapping peptides of insulin (A1–21, B1–20, B6–25, and B11–30) were no more frequent overall than responses to proinsulin, IA-2, and GAD65 epitopes, and the single patient tested before insulin therapy commenced showed IL-17 responses to insulin peptides A1–21 and B11–30 as well as GAD 554–575, IA-2 752–775, and IA-2 853–72.

Studies on at-risk subjects will be required to provide conclusive evidence that insulin treatment-naïve subjects make IL-17 responses against this autoantigen.

In 12 of the patients (24%), both IL-17 and IFN-γ responses were observed for the same β-cell peptide, although no single peptide emerged as dominant. These assays were unable to discriminate dual-secreting cells from populations of single-secretors.

There was no significant relationship between age of onset of type 1 diabetes, duration of disease, the presence of islet cell autoantibodies or HLA genotype, and the

**FIG. 1.** β-Cell autoantigen-specific IL-17 reactivity. A: IL-17 responses to β-cell autoantigens in patients with type 1 diabetes (■; \(n = 50\)) are significantly more prevalent than in healthy control subjects (□; \(n = 30\)), whereas the prevalence of reactivity to recall antigens is similar. B: For comparison, IFN-γ responses to β-cell autoantigens in patients with type 1 diabetes are also significantly more prevalent than in healthy control subjects, and the prevalence of reactivity to recall antigens is similar (**\(P < 0.0001\))). Representative examples of IL-17 ELISPOT responses in type 1 diabetes are shown. C: Graph shows number of peptides provoking IL-17 production in five patients tested in the original sampling near diagnosis and again a median of 12 months (range 9–14) later. There is a nonsignificant trend for the number of peptides testing positive to decline over time (paired \(t\) test; \(P = 0.09\)). D: Frequency of responses to individual β-cell autoantigenic peptides among type 1 diabetic patients as measured by production of IL-17 (■) and IFN-γ (□). E: Photomicrographs of wells in which cells have been cultured with negative control (peptide diluent), β-cell autoantigen, and recall stimulus (Pediacel vaccine) for IL-17 (upper row) and IFN-γ (lower row). Spots indicate responder antigen-specific CD4 T-cells. It is noteworthy that the frequency of IFN-γ responder cells for recall antigens is severalfold higher than for IL-17+ cells.
detection of an IL-17 response. The frequency of HLA-DR3/DR4 heterozygosity was 13 of 46 (28%) patients compared with 9 of 28 (32%) control subjects (P = 0.79). There was no relationship between possession of either HLA-DR4 or HLA-DR3 and the presence of an IL-17 response to β-cell peptides. Among patients, the frequency of IL-17 responses was similar in DR3/DR4 heterozygous and nonheterozygous individuals (6/13 [54%] vs. 17/33 [53%], respectively). IL-17 responses were observed more frequently in males (19/32, 59%) than females (8/18, 44%) but this was not significant (P = 0.38). Patients and control subjects did not differ in the frequency of response to recall antigens (present in the pentavalent vaccine Pediacel) or an irrelevant peptide of Plasmodium falciparum (circumsporozoite 363–377).

Collectively, these data indicate that, at diagnosis, type 1 diabetes is characterized by the presence of circulating β-cell autoantigen-specific CD4 T-cells secreting the proinflammatory cytokine IL-17.

**Detection of IL-17A gene expression in the pancreas in type 1 diabetes.** To examine whether the diabetes-related IL-17 signature that we detected in peripheral blood near diagnosis is relevant to the process of inflammation that leads to β-cell damage, we obtained purified islets of Langerhans from two patients, one of whom died within 5 days of diagnosis (designated T1D1) and one after 10 years of disease (T1D2) as well as from three nondiabetic control subjects (13). As previously reported (13), the islets in the diseased organs showed infiltration by mononuclear cells (insulitis), a characteristic pathological feature of type 1 diabetes. Notably, CD4 T-cells were detected in a higher proportion of islets when studied near diagnosis (69 vs. 28% islets positive in T1D1 and T1D2, respectively) and were present in greater number (approximately threefold higher), compared with longstanding disease. Quantitative RT-PCR analysis of these samples showed a relative sixfold higher expression of IL17A over the control samples in the islets obtained close to diagnosis, whereas IL17A was not detectable in the longstanding patient (Fig. 2A). A similar pattern was seen for IFNG expression, which showed a relative 32-fold higher level of transcripts in T1D1 but was barely detectable in T1D2 (Fig. 2B). We next examined whether the transcription factors associated with TH17 and TH1 differentiation, RORC and TBX21, respectively, were detectable. Transcripts for both TH17- and TH1-associated transcription factors were detected at elevated levels in T1D1 islets, and TBX21 was also detected in T1D2 islets (Fig. 2C and D). Further support for the presence of cells with a TH17 differentiation pathway in islets near to diagnosis is demonstrated by the detection of IL22 in T1D1 but not T1D2 (Fig. 2E). Quantitative RT-PCR analysis of pancreas blocks from the same samples was less informative, presumably as a result of dilutional effects (islets represent <1% of pancreatic cells). IL17A and IFNG transcripts were elevated in T1D1 but not T1D2 pancreas, whereas those for the transcription factors were similar to control subjects (data not shown).

**Effects of IL-17 on β-cells.** The finding that IL-17–secreting CD4 T-cells specific for β-cell autoantigens are present in the circulation and that IL17A transcripts are elevated in the pancreatic islets near to diagnosis of type 1 diabetes prompted us to examine the effects of IL-17 on human β-cells. It is well established that human β-cells are highly sensitive to the action of the proinflammatory cytokines IL-1β and TNF-α, which mediate a proapoptotic effect in combination with IFN-γ (22,26). Whereas IL-17A alone had no proapoptotic effect on cultured human islets, its addition significantly exacerbated the apoptosis induced by the combination of IL-1β and IFN-γ or TNF-α and IFN-γ (Fig. 3A–F). This step was associated with significantly increased release of nitrite, suggesting increased NO production by human islets exposed to these cytokine combinations. With the addition of the inhibitor of nitric oxide

**FIG. 2.** Real-time quantitative PCR analysis of expression of IL17 (A), IFNG (B), IL22 (C), RORC (D), and TBX21 (E) in islets of Langerhans obtained from two patients with type 1 diabetes (T1D1, who died within 5 days of diagnosis, and T1D2, who died 10 years after diagnosis). Mean (SEM) values (technical replicates) are also shown for three control subjects (organ donors). Levels of target gene mRNA transcripts are normalized to the housekeeping gene HPRT and compared with an in-house calibrator sample as described.
synthase (NOS), L-NMA substantially decreased nitrite accumulation in the medium, indicating diminished NO production by the cytokine-treated islets (Fig. 3G). L-NMA, however, did not prevent cytokine-induced islet cell apoptosis (Fig. 3B), in keeping with our previous observation that cytokine-induced human \( \beta \)-cell death is not mediated by NO formation (18,27). Furthermore, transcripts for IL17RA, but not IL17RC, were significantly upregulated by IL-1\( \beta \) and IFN-\( \gamma \) treatment of human islets (Fig. 3H–I), indicating that conditioning of \( \beta \)-cells by these cytokines renders them susceptible to the apoptosis-promoting actions of IL-17A via specific cytokine-receptor induction.

We next examined the transcriptional pathways involved in IL-1\( \beta \)/IFN-\( \gamma \)-mediated IL-17RA induction using either siRNA targeting of STAT1 or a superrepressor of NF-\( \kappa \)B activity. We first confirmed that the IL-1\( \beta \)/IFN-\( \gamma \) cytokine combination induces IL-17RA in the rat insulin-producing INS-1E cells and in primary rat \( \beta \)-cells, in association with a proapoptotic signal that is significantly enhanced by addition of IL-17A (Supplementary Figs. 1A–C).

**FIG. 3.** Mechanisms through which IL-17 promotes cytokine-induced \( \beta \)-cell apoptosis. A: Human islets were treated with IL-1\( \beta \) (50 units/mL), TNF-\( \alpha \), and IFN-\( \gamma \) (both at 1,000 units/mL) in the presence of 20 ng/mL rIL-17A for 48 h. Apoptosis was evaluated using H&E staining. Results are means \pm SEM of six to nine independent experiments (**\( P < 0.001 \) vs. untreated cells; §§§\( P < 0.001 \) vs. IL-1\( \beta \) + IFN-\( \gamma \)-treated cells; §§§§\( P < 0.001 \) vs. TNF-\( \alpha \) and IFN-\( \gamma \)-treated cells (ANOVA with Bonferroni correction). B: Human islets were treated with IL-1\( \beta \) (50 units/mL) and IFN-\( \gamma \) (1,000 units/mL) in the presence of 20 ng/mL rIL-17A for 48 h with or without the addition of L-NMA. Apoptosis was evaluated using H&E staining. Results are means \pm SEM of three independent experiments (**\( P < 0.001 \) vs. untreated cells; §§\( P < 0.01 \) vs. IL-1\( \beta \) + IFN-\( \gamma \)-treated cells; §§§\( P < 0.05 \) vs. IL-1\( \beta \) + IFN-\( \gamma \) + L-NMA–treated cells (ANOVA with Bonferroni correction). Panels show representative images of cell death in whole human islets without cytokine (C), islets cultured with IL-17A (20 ng/mL) (D), islets cultured with IL-1\( \beta \) (50 units/mL) + IFN-\( \gamma \) (1,000 units/mL) (E), and islets stimulated with IL-1\( \beta \) (50 units/mL) + IFN-\( \gamma \) (1,000 units/mL) + IL-17A (20 ng/mL) (F). G: Human islets were treated with cytokines as described above for 48 h in the presence of NO-suppressing L-NMA, and supernatants were assayed for nitrite content. Nitrite is significantly raised in the presence of IL-1\( \beta \) (50 units/mL) + IFN-\( \gamma \) (1,000 units/mL) and further increased when supplemented with 20 ng/mL rIL-17A. In the presence of L-NMA, nitrite is significantly decreased. Results are mean \pm SEM of three independent experiments (**\( P < 0.05 \) vs. untreated cells; §\( P < 0.05 \) vs. IL-1\( \beta \) + IFN-\( \gamma \) + IL-17A–treated cells; ANOVA with Bonferroni correction). H: The combination of cytokines IL-1\( \beta \) and IFN-\( \gamma \) upregulate IL-17RA mRNA expression in human islets after 24 h. Human islets were treated with IL-1\( \beta \) (50 units/mL) and IFN-\( \gamma \) (1,000 units/mL) for 24 h; IL-17RA mRNA expression was assayed by RT-PCR and normalized for the housekeeping gene \( \beta \)-actin (ACTB); *\( P < 0.05 \) vs. untreated (Student t test). Results are means \pm SEM of six independent experiments. Results are expressed as fold variation compared with untreated control. I: In contrast, under the same conditions as in H, IL-1\( \beta \) and IFN-\( \gamma \) do not increase IL-17RC mRNA expression in human islets after 24 h. Results are means \pm SEM of six independent experiments. Results are expressed as fold variation as compared with untreated control. (A high-quality color representation of this figure is available in the online issue.)"
and 2). INS-1E cells treated with siRNA targeting STAT1 showed significantly reduced IL17RA induction after treatment with the IL-1β/IFN-γ combination (Fig. 4), as did primary rat β-cells under equivalent conditions (Supplementary Fig. 2). Similarly, INS-1E cells infected with an adenovirus encoding a superrepressor IκBα to inhibit NF-κB activity showed significantly reduced IL17RA induction compared with control infected cells (Fig. 4). These data indicate that the induction of IL-17RA expression in β-cells depends on both STAT1 and NF-κB transcriptional pathways.

**DISCUSSION**

Despite the intense interest provoked by the discovery of CD4 effector T-cells that secrete the signature cytokine IL-17, to date, there have been few examples in which the antigen specificity of such cells has been explored and none in the context of human autoimmune disease. Here, we report that peripheral blood CD4 T-cells from patients with type 1 diabetes secrete IL-17 in response to the TH1 cytokines IFN-γ and TNF-α, and demonstrate for the first time that this is a process mediated via NF-κB and STAT1, respectively. As a result, β-cells are rendered highly susceptible to the destructive actions of IL-17, which we have shown is a feature of islet inflammation and a product of CD4 T-cell autoreactivity.

INS-1E cells were infected with an adenovirus encoding a superrepressor IκBα (10 units/mL) + IFN-γ (100 units/mL) for 24 h. IL-17RA mRNA expression was assayed by RT-PCR and normalized for the housekeeping gene GAPDH. Cytokine treatment significantly upregulates IL17RA in INS-1E cells (39). IL-17A is a novel finding, since hitherto, the signaling cascades leading to IL-17RA expression were not known. The requirement for IL-17RA upregulation on β-cells for IL-17 to mediate damage lends further support to the concept that β-cells are not passive bystanders in their own destruction. Rather, the β-cell participates actively in its own demise via a complex cytokine–transcription factor network involving IFN-γ–STAT1–IL17R and IL-1β–NF-κB–IL17R, whereby signaling by early inflammatory mediators secreted by T17 cells and macrophages renders β-cells susceptible to subsequent IL-17–mediated apoptosis (Fig. 5).

Our results suggest that responses by IL-17–producing cells essentially parallel those of T17 cells near diagnosis. There is much still to learn about the β-cell–specific CD4 T-cell response phenotype. It is technically challenging to examine whether the cells identified secrete both IL-17 and IFN-γ, and it remains to be established whether other types of CD4 T-cell polarization exist in the disease, the times course of the responses, and whether there is a general a tendency to generate autoreactive effector T-cells. The presence of circulating β-cell–specific CD4 T-cells in patients with type 1 diabetes, along with additional evidence that patients have an abnormal expansion of IL-17–secreting CD4 T-cells after polyclonal stimulation of peripheral blood cells (9; data not shown), highlights a further aspect of the possible contribution of these cells to type 1 diabetes immunopathology. These data suggest
that at the time of diagnosis of type 1 diabetes, polarization toward the IL-17 pathway is prominent. However, this was not evident in the IL-17 memory response to recall antigens, which we found to be normal in frequency and amplitude when tested at diagnosis (and which would have been primed some years before via vaccination). This result suggests that there is active promotion of IL-17 pathway differentiation for CD4 T-cells during the peri-diagnosis period, which may be important for two reasons. First, TH17 differentiation is known to be influenced by at least one ligand-receptor system (for aryl hydrocarbons) that is responsive to environmental stimuli, and there is ample evidence for a role for nongenetic factors in type 1 diabetes (34). Second, because IL-17-secreting cells are known to be more resistant to regulation by nTregs, our finding could explain the consistent observation that effector cells from type 1 diabetic patients are less "regulatable" (35). Taken together with the susceptibility of the human β-cell to IL-17, these studies offer clear rationale for contemplating intervention in the differentiation and effector pathways of Th17 cells, with several candidate biologics now available (36,37).

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