# 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 3 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 NF-κB, rendering this cytokine a novel disease biomarker and potential therapeutic target. *Diabetes* 60:2112–2119, 2011

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 (T$_H$17) effector cells might participate in critical disease pathways in humans. In multiple sclerosis, for example, IL-17 is expressed at high levels by circulating T-cells, and *IL17* gene expression is elevated in plaques in the affected brain (4,5). It is noteworthy that a clear link to immunopathogenesis has been made through the demonstration that T$_H$17 cells disrupt blood-brain barrier tight junctions, enabling transmigration of immune-competent cells into the brain parenchyma and the establishment of local inflammation (6). Likewise, the presence of circulating and synovial tissue-infiltrating T$_H$17 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-17 receptor (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

TABLE 1
Summary of clinical characteristics of patients with type 1 diabetes and healthy nondiabetic control subjects

| | Males (%) | Females (%) | HLA-DRB1* 0401 or 0301 (%) | Anti-GAD autoantibodies (% positive) | Anti–IA-2 autoantibodies (% positive) |
|---|---|---|---|---|---|
| Patients | 64 | 36 | 88 | 75 | 54 |
| Healthy control subjects | 30 | 70 | 71 | 0 | 0 |

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-DR4 presented IA2 (709–736, 752–775, and 853–872), proinsulin (C19-A3), and GAD65 epitopes (335–352 and 554–575), as well as overlapping regions of the insulin B (1–20, 6–25, and 11–30) and A chain (1–21), were synthesized by 9-fluorenylmethyl carbamate chemistry and purified by high-performance liquid chromatography (Thermo Hybaid, Ulm, Germany), a peptide of the CS protein of *Plasmodium falciparum* (363–377) 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–IL-17 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). A response is considered positive when the stimulation index is ≥3 using cutoffs determined using receiver-operator characteristic plots as previously described. Interassay coefficient of variation for the spot number in the IL-17 ELISPOT assay was 8.6% when evaluated by measuring spot number elicited when fresh peripheral blood mononuclear cells from the same donor were obtained on five separate occasions over a 3-month period and cultured with a low dose of Pediacel (0.2 μL/mL) to mimic the small number of spots observed in autoreactive responses.

**Analysis of gene expression in pancreas tissue.** Islet and pancreas samples were obtained from two patients who died at different stages of type 1 diabetes (5 days and 10 years after diagnosis) as described (13). RNA from cryostat sections obtained from pancreatic blocks or from fresh frozen purified islets was extracted with the RNeasy micro kit (QIAGEN, Hilden, Germany). A total of 1 μg RNA (total pancreas) or 200 ng RNA (purified islets) was reverse-transcribed using Oligo-dT (0.5 μg/μL; GeneLink, Hawthorne, NY) and Moloney Murine Leukemia Virus reverse-transcriptase (Promega, Madison, WI). Quantitative PCR assays were performed on a LightCycler 480 (Roche, Mannheim, Germany) using TaqMan universal assay conditions and using the following TaqMan Assays on Demand: *IL17A* (Hs00174383_m1), *IL22* (Hs01574154_m1), *IFNG* (Hs00989291_m1), *RORC* (Hs01076112_m1), *TBX21* (Hs00894392_m1), and *HPRT* (Hs99999909_m1) as housekeeping genes. Relative values were determined by normalizing the expression for each gene of interest to the housekeeping gene (*HPRT*) and to a calibrator sample as described in the $2^{-\Delta\Delta Ct}$ method (14). The calibrator sample was a pool of all of the samples included in the study (pancreases and islets). For *IL22*, a different calibrator sample was used because it was not detectable using the standard calibrator. This second calibrator was a pool of samples from type 1 diabetic patients (pancreata and islets).

**IL-17 effects on insulin-producing INS-1E cells and primary rat and human β-cells.** Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were housed and used according to the guidelines of the Belgian Regulations for Animal Care. Islets were isolated by collagenase digestion and hand-picked under a stereomicroscope. β-Cells were purified by autofluorescence-activated cell sorting (FACSAria; BD Bioscience, San Jose, CA) (15,16). The preparations contained 94 ± 2% β-cells (n = 5). β-Cells were cultured for 2 days in Ham's F-10 medium containing 10 mmol/L glucose, 2 mmol/L glutaMAX, 50 μmol/L 3-isobutyl-1-methylxanthine, 5% FBS, 0.5% charcoal-absorbed BSA (Boehringer, Indianapolis, IN), 50 units/mL penicillin, and 50 μg/mL streptomycin (16). During cytokine exposure, cells were cultured in the same medium but without serum (17). We have previously shown (18) that human islet exposure to proinflammatory cytokines induces apoptosis in the presence or absence of serum. The rat insulin-producing INS-1E cell line (a gift from Dr. C. Wollheim, Centre Medical Universitaire, Geneva, Switzerland) was cultured as previously described (19).

Human islets were obtained from nine donors (two donors at the Diabetes Research Unit at King's College London and seven isolated in the Department of Endocrinology and Metabolism, Metabolic Unit, University of Pisa, as previously described [20]); of note, similar effects of IL-17 were observed in islets isolated in London and Pisa and sent to Brussels [data not shown]), where they were cultured in complete medium as described above. β-Cell proportion was evaluated by insulin staining in each individual preparation and represented 48–72% of the dispersed islets (mean [± SEM] β-cell purity 62 ± 4.7%); these dispersed islets or whole islets were exposed to cytokines for 24 and 48 h, as indicated in the figures.

The following siRNAs were used in this study: BLOCK-iT Stealth Select siRNA siSTAT1: 5′-CCCUAGAAGACUUACAAGAUGAAUA-3′ (Invitrogen, Paisley, U.K.); Allstars Negative Control siRNA (siCtrl) was used for control-transfected conditions (Qiagen, Venlo, The Netherlands; sequence not provided). The transfection of siRNAs in INS-1E cells and primary fluorescence–activated cell-sorted rat β-cells was done as previously described, with an efficiency of transfection >90% (20).

Nuclear factor (NF)-κB inhibition was carried out using an adenovirus encoding a super-repressor IκBα that blocks NF-κB activation as previously described (21).

The following cytokine concentrations were used, based on previous dose-response experiments by our group (19,22,23): recombinant human IL-1β (specific activity [SA] $1.8 \times 10^7$ units/mg; a gift from C.W. Reinolds, National Cancer Institute, Bethesda, MD) at 10 or 50 units/mL as indicated; recombinant rat IFN-γ (SA $2 \times 10^7$ units/mg; R&D Systems) at 100 units/mL; recombinant human IFN-γ (R&D Systems) at 1,000 units/mL; recombinant human tumor necrosis factor (TNF)-α (R&D Systems) at 1,000 units/mL; and recombinant rat IL-17A (Biovision, Mountain View, CA) and recombinant human IL-17A (R&D Systems) at 20 ng/mL. When cells were treated with cytokines, culture supernatants were collected for nitrite determination (nitrite is a stable product of nitric oxide [NO] oxidation) at $OD^{540nm}$ using the Griess method. In some experiments, the inducible nitric oxide synthase inhibitor $N^G$-methyl-L-arginine (L-NMA) (Sigma, St. Louis, MO) was used at a concentration of 5 mmol/L to inhibit NO production.

The percentage of viable, apoptotic, and necrotic cells was determined after a 15-min incubation with the DNA-binding dyes propidium iodide (PI, 5 μg/mL; Sigma-Aldrich) and Hoechst dye 33342 (HO, 5 μg/mL; Sigma-Aldrich) (16). A minimum of 500 cells were counted in each experimental condition. Viability was evaluated by two independent observers, one of them being unaware of sample identity. The agreement between findings obtained by the two observers was >90%. Results are expressed as percent apoptosis, calculated as number of (apoptotic cells/total number of cells) × 100.

Poly(A)+ mRNA was isolated from INS-1E cells, rat primary β-cells, or human islets using the Dynabeads mRNA DIRECT kit (Invitrogen) and was reverse-transcribed and amplified by PCR as previously described (16,24), using SYBR Green and compared with a standard curve (25). Expression values were corrected for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). We have previously shown that mRNA expression of these genes is not affected by the present experimental conditions (20). The primers used in this study are listed in Supplementary Table 2.

**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-,
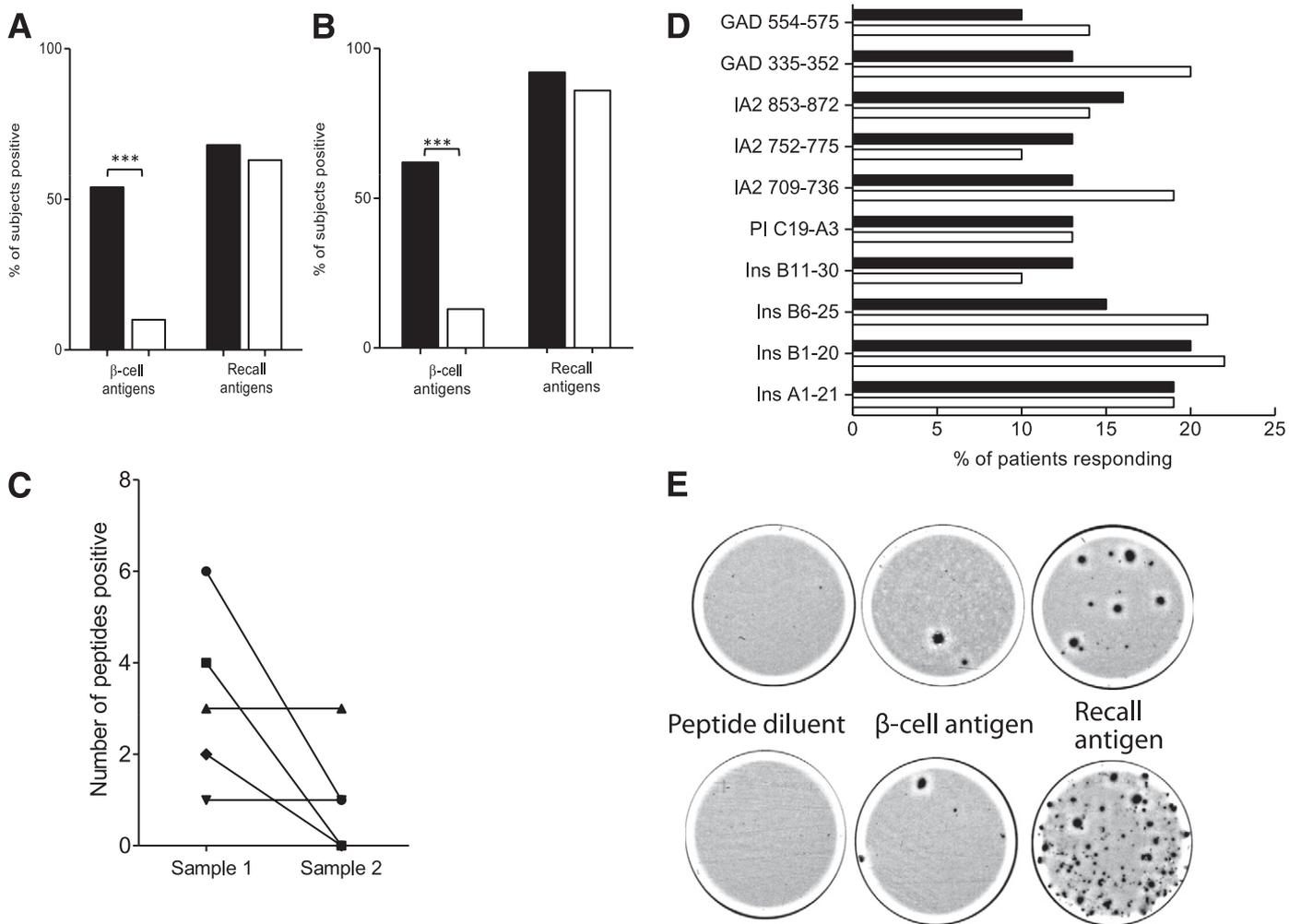
FIG. 1. β-Cell autoantigen-specific IL-17 reactivity. *A*: IL-17 responses to β-cell autoantigens in patients with type 1 diabetes (■; *n* = 50) are significantly more prevalent than in healthy control subjects (□; *n* = 30), whereas the prevalence of reactivity to recall antigens is similar. *B*: For 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). *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 non-significant 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.

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. 1*A*). 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. 1*B*]). 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. 1*C*).

The pattern of IL-17 reactivity against β-cell autoantigenic peptides did not differ markedly from the IFN-γ response (Fig. 1*D*), 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. 1*E*). 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–naive 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 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. 2*A*). 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. 2*B*). We next examined whether the transcription factors associated with T$_H$17 and T$_H$1 differentiation, *RORC* and *TBX21*, respectively, were detectable. Transcripts for both T$_H$17- and T$_H$1-associated transcription factors were detected at elevated levels in T1D1 islets, and *TBX21* was also detected in T1D2 islets (Fig. 2*C* and *D*). Further support for the presence of cells with a T$_H$17 differentiation pathway in islets near to diagnosis is demonstrated by the detection of *IL22* in T1D1 but not T1D2 (Fig. 2*E*). 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
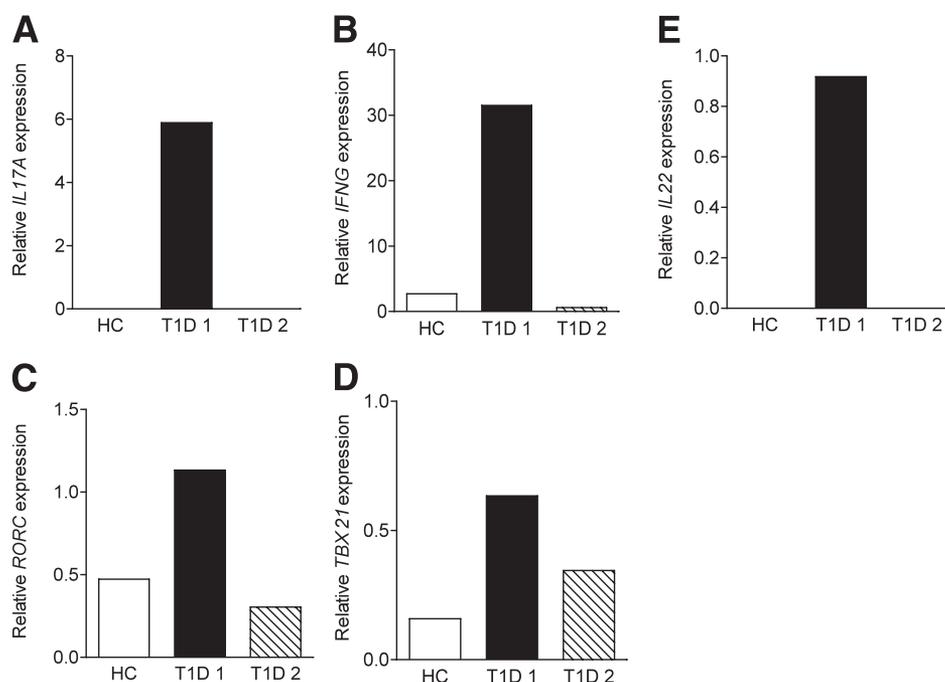


FIG. 2. Real-time quantitative PCR analysis of expression of *IL17* (*A*), *IFNG* (*B*), *RORC* (*C*), *TBX21* (*D*), and *IL22* (*E*) in islets of Langerhans obtained from two patients with type 1 diabetes (T1D1, who died within 5 days of diagnosis, and T1D2, who died 10 years after diagnosis). Mean (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. HC, healthy controls.
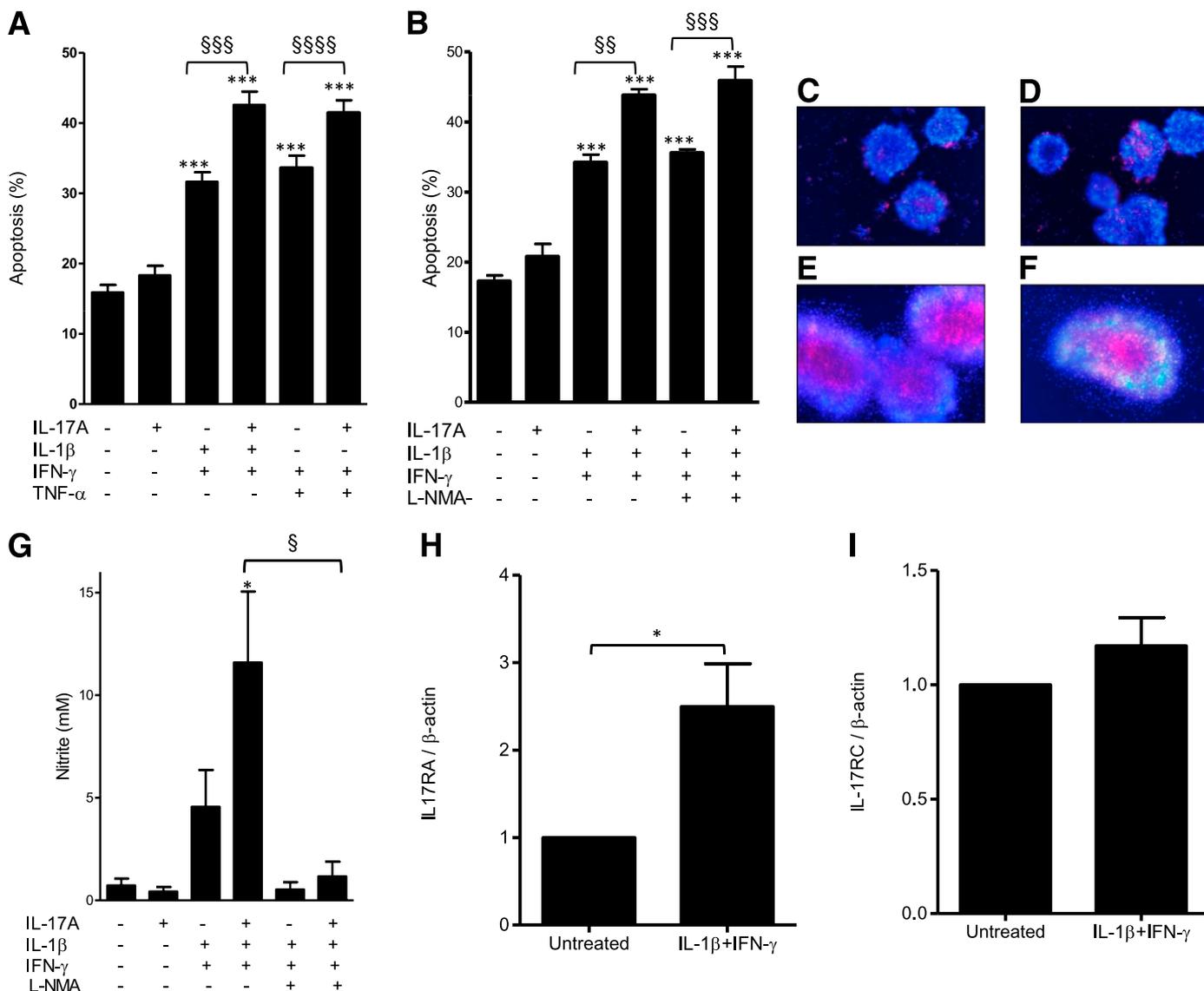
FIG. 3. Mechanisms through which IL-17 promotes cytokine-induced β-cell apoptosis. *A*: Human islets were treated with IL-1β (50 units/mL), TNF-α, and IFN-γ (both at 1,000 units/mL) in the presence of 20 ng/mL rIL-17A for 48 h. Apoptosis was evaluated using HO/PI staining. Results are means ± SEM of six to nine independent experiments (\*\*\**P* < 0.001 vs. untreated cells; §§§*P* < 0.001 vs. IL-1β + IFN-γ–treated cells; §§§§*P* < 0.001 vs. TNF-α and IFN-γ–treated cells) (ANOVA with Bonferroni correction). *B*: Human islets were treated with IL-1β (50 units/mL) and IFN-γ (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 HO/PI staining. Results are means ± SEM of three independent experiments (\*\*\**P* < 0.001 vs. untreated cells; §§*P* < 0.01 vs. IL-1β + IFN-γ–treated cells; §§§*P* < 0.05 vs. IL-1β + IFN-γ + L-NMA–treated cells) (ANOVA with Bonferroni correction). *C–F*: 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-1β (50 units/mL) + IFN-γ (1,000 units/mL) (*E*), and islets stimulated with IL-1β (50 units/mL) + IFN-γ (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 L-NMA, and supernatants were assayed for nitrite content. Nitrite is significantly raised in the presence of IL-1β (50 units/mL) + IFN-γ (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 means ± SEM of three independent experiments (\**P* < 0.05 vs. untreated cells; §*P* < 0.05 vs. IL-1β + IFN-γ + IL-17A–treated cells) (ANOVA with Bonferroni correction). *H*: The combination of cytokines IL-1β and IFN-γ upregulate IL-17RA mRNA expression in human islets after 24 h. Human islets were treated with IL-1β (50 units/mL) and IFN-γ (1,000 units/mL) for 24 h; IL-17RA mRNA expression was assayed by RT-PCR and normalized for the housekeeping gene β-actin (*ACTB*); \**P* < 0.05 vs. untreated (Student *t* test). Results are means ± 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-1β and IFN-γ do not increase IL-17RC mRNA expression in human islets after 24 h. Results are means ± 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.)

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. 3*A–F*). This step was associated with significantly increased release of nitrite, suggesting increased

NO production by human islets exposed to these cytokine combinations. The addition of the inhibitor of nitric oxide synthase (NOS), L-NMA, substantially decreased nitrite accumulation in the medium, indicating diminished NO production by the cytokine-treated islets (Fig. 3*G*). L-NMA, however, did not prevent cytokine-induced islet cell apoptosis (Fig. 3*B*), in keeping with our previous observation that cytokine-induced human β-cell death is not mediated by NO formation (18,27). Furthermore, transcripts for

*IL17RA*, but not *IL17RC*, were significantly upregulated by IL-1β and IFN-γ treatment of human islets (Fig. 3*H–I*), indicating that conditioning of β-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-1β/IFN-γ–mediated IL-17RA induction using either siRNA targeting of STAT1 or a super-repressor of NF-κB activity. We first confirmed that the IL-1β/IFN-γ cytokine combination induces IL-17RA in the rat insulin-producing INS-1E cells and in primary rat β-cells, in association with a proapoptotic signal that is significantly enhanced by addition of IL-17A (Supplementary Figs. 1*A–C* 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 super-repressor 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



FIG. 4. IL-17RA expressed in rat insulin-producing INS-1E cells is upregulated by cytokines via STAT1 and NF-κB activation. INS-1E cells were transfected with 30 nmol/L of either a control siRNA (□) or a siRNA targeting STAT1 (■). After 1 day of recovery, cells were left untreated or were treated with IL-1β (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 IL-17RA (**$P < 0.01$ vs. untreated cells; Student $t$ test). siRNA targeting of STAT1 significantly reduces IL-17RA induction (§$P < 0.05$ vs. control siRNA). Results are means ± SEM of five independent experiments. In another series of experiments, INS-1E cells were infected with an adenovirus encoding a super-repressor IκBα to inhibit NF-κB activity (■) or with adenovirus encoding luciferase as control (□). Cells were left untreated or treated with IL-1β (10 units/mL) + IFN-γ (100 units/mL) for 24 h. Cytokine treatment significantly upregulates IL-17RA (***$P < 0.001$ and *$P < 0.05$ vs. relevant untreated cells). Adenovirus-mediated inhibition of NF-κB significantly reduces IL-17RA induction. §$P < 0.05$ vs. control. Results are means ± SEM of four independent experiments.

from patients with type 1 diabetes secrete IL-17 in response to β-cell autoantigens. We show that this cytokine is actively produced in inflamed islets close to the onset of disease and provide evidence for the existence of a multistep pathway for β-cell destruction in which IL-17 has a key role.

Until the current study, the evidence for a role for IL-17 in autoimmune diabetes was largely based on animal studies, and the data are ambiguous. In the nonobese diabetic (NOD) mouse model, it is reported that blockade of IL-17 is protective (28), whereas other studies suggest that disease actually results from the destructive actions of $T_H1$ cells that have undergone conversion from $T_H17$ cells (29,30). Our results in human type 1 diabetes indicate a direct role for IL-17–secreting cells in pathways that lead to β-cell damage. Moreover, we provide supportive evidence to indicate that the IL-17 secretion that we detected in islets of Langerhans obtained near diagnosis can be attributed to bona fide $T_H17$ cells. We show the presence of the $T_H17$ transcription factor *RORC* in inflamed islets, as well as *IL22*, a cytokine typically secreted by $T_H17$ cells. However, these results represent a case report, and future studies on similar cases will be required to verify these preliminary findings. It is probable that there are multiple pathways through which β-cells die during the development of type 1 diabetes. It has been known from some years that β-cells are exquisitely susceptible to the proapoptotic actions of the combination of IL-1β and IFN-γ or TNF-α and IFN-γ (22), and the present and recent findings (9) highlight a prominent exacerbating role for IL-17 in β-cell destruction. Interestingly, this effect is independent of increased NO production. Our results show that IL-1β and IFN-γ condition human islets to express IL-17RA 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. STAT1 is a key mediator of biological responses to IFN-γ in both β-cells (31) and the immune system (32), but this is the first description of STAT1 affecting IL-17R expression. Furthermore, recent data examining genes in type 1 diabetes pancreata report the increased expression of *STAT1* in the pancreas (13), indicating that the pathway for upregulating IL-17RA expression on β-cells is active in diseased tissue. IL-1β exerts its effects on β-cells to a large extent via the NF-κB pathway (21,33), and in our studies, blockade of NF-κB resulted in a decrease in *IL17RA* expression in relevant cell lines. This STAT1- and NF-κB–dependent upregulation of *IL17RA* is a novel finding, since hitherto, the signaling cascades leading to IL-17RA expression were not known. Synergy between cytokines such as IL-1β, IFN-γ, and TNF-α in the induction of apoptosis of human β-cells has been well documented (26), but the involvement of IL-17 is a new finding. 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–IL-17R and IL-1β–NF-κB–IL-17R, whereby signaling by early inflammatory mediators secreted by $T_H1$ 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 $T_H1$ cells near diagnosis.

**FIG. 5. Schematic representation of possible synergistic effects of cytokines in mediating β-cell apoptosis. We propose that β-cell death could have two phases. In the first, $T_H1$ cells and macrophages in the local milieu secrete IFN-γ and IL-1β, respectively, which bind to specific receptors leading to the activation of STAT1 and NF-κB transcription factors. Both STAT1 and NF-κB lead to the expression of IL-17R through an as yet unknown mechanism. In the second phase, the inflammatory milieu includes $T_H17$ cells, which secrete IL-17A. Triggering through IL17RA/RC exacerbates apoptosis initiated by IL-1β and IFN-γ, leading to increased β-cell death.**

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 we 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 time course of the responses, and whether there is a generalized 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 peridiagnosis period, which may be important for two reasons. First, $T_H17$ 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 naturally occurring T regulatory cells, 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 $T_H17$ cells, with several candidate biologics now available (36,37).

performed experiments. C.M.D. and J.P. recruited and characterized patients.

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