IL-17 Silencing Does Not Protect Nonobese Diabetic Mice from Autoimmune Diabetes


*J Immunol* published online 23 November 2011
http://www.jimmunol.org/content/early/2011/11/23/jimmunol.1101215

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2011/11/23/jimmunol.1101215.DC1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-17 Silencing Does Not Protect Nonobese Diabetic Mice from Autoimmune Diabetes

Julie Joseph,* Stefan Bittner,† Fabian M. P. Kaiser,* Heinz Wiendl,† and Stephan Kissler*

The long-held view that many autoimmune disorders are primarily driven by a Th1 response has been challenged by the discovery of Th17 cells. Since the identification of this distinct T cell subset, Th17 cells have been implicated in the pathogenesis of several autoimmune diseases, including multiple sclerosis and rheumatoid arthritis. Type 1 diabetes has also long been considered a Th1-dependent disease. In light of the emerging role for Th17 cells in autoimmunity, several recent studies investigated the potential of this subset to initiate autoimmune diabetes. However, direct evidence supporting the involvement of Th17 cells in actual pathogenesis, particularly during spontaneous onset, is lacking. In this study, we sought to directly address the role of IL-17, the cytokine by which Th17 cells are primarily characterized, in the pathogenesis of autoimmune diabetes. We used lentiviral transgenesis to generate NOD mice in which IL-17 is silenced by RNA interference. The loss of IL-17 had no effect on the frequency of spontaneous or cyclophosphamide-induced diabetes. In contrast, IL-17 silencing in transgenic NOD mice was sufficient to reduce the severity of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis, consistent with reports that IL-17 deficiency is protective in this experimental model of multiple sclerosis. We concluded that IL-17 is dispensable, at least in large part, in the pathogenesis of autoimmune diabetes. The Journal of Immunology, 2012, 188: 000–000.

The pathogenesis of many autoimmune diseases, including type 1 diabetes (T1D), has long been thought to be orchestrated by Th1 cells. The dichotomy of Th cell subsets introduced by Mosmann et al. (1) led to the view that Th1, but not Th2, cells were responsible for the organ-specific autoimmunity underlying diseases such as multiple sclerosis (MS) and T1D. Inconsistencies in the role of IFN-γ during autoimmune pathogenesis were among the first indications that such a model may not be accurate (2–5). However, it was the discovery that IL-23, and not IL-12, is indispensable for experimental autoimmune encephalomyelitis (EAE) (6, 7), a model for MS, that marked the beginning of a new understanding of autoimmune pathogenesis. Subsequent to this seminal finding, the link was made between the role of IL-23 in autoimmunity and its requirement for the full differentiation and expansion of Th17 cells, a subset characterized primarily by the production of IL-17A (IL-17) (8, 9). Since then, Th17 cells have been implicated in the pathogenesis of several autoimmune diseases, including MS and rheumatoid arthritis. For example, IL-17 deficiency alone confers protection, albeit incomplete, from both EAE and collagen-induced arthritis (10, 11). These findings led to the hypothesis that Th17 cells may also contribute to the pathogenesis of T1D.

Recently, several groups investigated the potential involvement of Th17 cells in diabetogenesis. A report by Zaghouani and colleagues (12) suggested that tolerance induction, and the ensuing diabetes prevention, in the NOD mouse model by a glutamic acid decarboxylase peptide-Ig fusion construct was indirectly mediated by the inhibition of IL-17 production. Subsequently, it was further reported that anti–IL-17 Ab treatment of 10-wk-old NOD mice reduced diabetes frequency (13). However, two independent studies also demonstrated that although in vitro-generated Th17 cells could induce diabetes in NOD.SCID recipients, they did so by differentiating into Th1-like cells before causing β cell destruction (14, 15). In this transfer model, diabetes could be prevented by anti–IFN-γ, but not anti–IL-17, Ab. The evidence available to date indirectly suggests a role for Th17 cells in diabetes pathogenesis but is not fully conclusive. Therefore, we sought to directly assess the involvement of IL-17 in autoimmune diabetes in the NOD mouse model. An IL-17 knockout (KO) allele was generated in the 129/Sv background (11). Breeding this allele into the NOD background, in addition to being a lengthy process, entails the risk that genetic regions flanking the KO allele may themselves introduce gene variations and affect disease independently of IL-17. Earlier work indeed demonstrated that backcrossing a KO allele into the NOD background can lead to misinterpretation (16). To circumvent this issue, we generated an IL-17 knockout (KD) NOD line by directly introducing an appropriate short hairpin RNA (shRNA) construct into NOD zygotes by lentiviral transgenesis. We found that IL-17 silencing did not prevent autoimmune diabetes, whether spontaneous or cyclophosphamide induced (17), in transgenic NOD mice. However,
gene KD was effective, as evidenced by the fact that IL-17 KD mice were partially protected from EAE following challenge with myelin oligodendrocyte glycoprotein (MOG)-derived peptide. Our findings demonstrated that although IL-17 silencing in the NOD background reduces EAE severity, consistent with published findings (10), it does not alter diabetes susceptibility. In conclusion, our results suggested that IL-17, and consequently Th17 effector function, may be dispensable in the pathogenesis of autoimmune diabetes.

Materials and Methods

Mice

NOD/MrkTac mice were maintained under specific pathogen-free conditions at the University of Würzburg. Transgenic mice were generated, as described previously (18). Briefly, lentivirus containing an shRNA that targets the sequence CCAAGGACTTCCTCCAGAA in the \textit{Il17a} gene was microinjected into the perivitelline space of NOD zygotes. Injected embryos were reimplanted into pseudopregnant CD-1 mice. For diabetes testing, glycosuria was measured weekly (for spontaneous disease) or every 2 d (following cyclophosphamide treatment) using Diastix (Bayer). Mice with two consecutive readings $>250$ mg/dl were considered diabetic.

Luciferase assay

The \textit{Il17a} cDNA (GenBank: BC119303) was cloned into the dual-luciferase reporter plasmid psiCheck2 (Promega). HEK293 cells were transfected with 100 ng psiCheck2 plasmid together with 300-ng lentiviral vector containing shRNA sequences against \textit{Il17a}, and luminescence of cell lysates was measured after 48 h to assess reporter silencing.

EAE induction

Mice were immunized with 200 $\mu$g MOG$_{35-55}$ peptide (Biotrend) emulsified in CFA and injected with 400 ng pertussis toxin (Alexis) on the day of immunization and 2 d later. EAE was scored as follows: 1, limp tail tip; 2, limp tail; 3, moderate hind limb weakness; 4, complete hind limb weakness; 5, mild paraparesis; 6, paraparesis; 7, heavy paraparesis or paraplegy; 8, tetraparesis; 9, quadriplegia or premoribund; and 10, death.

FIGURE 1. Generation of IL-17 KD NOD mice. \textit{A}, Schematic representation of the pLBUG vector used for transgenesis, which contains a U6 promoter driving shRNA expression and a ubiquitin promoter driving GFP expression. \textit{B}, Validation of \textit{Il17a} shRNA-silencing efficiency by dual-luciferase assay. \textit{Renilla} luciferase activity (variable) was normalized to firefly luciferase activity (constant) to adjust for transfection efficiency. \textit{C}, Validation of \textit{Il17a} shRNA-silencing specificity. The luciferase reporters for \textit{Il17a} (left panel) and \textit{Ptpn22} (ctrl, right panel) were used in conjunction with empty vector and vectors containing shRNA against \textit{Il17a} (#176) or \textit{Ptpn22} (ctrl). Data in \textit{B} and \textit{C} are representative of two similar experiments. Mean and SEM of triplicate values are shown. \textit{D}, Representative GFP expression measured by flow cytometry in blood samples from a transgenic (black line) and wt (shaded graph) mouse.

FIGURE 2. T cells from IL-17 KD NOD mice secrete greatly reduced levels of IL-17. \textit{A}, Naive CD4$^+$CD62L$^{hi}$ T cells were differentiated using the indicated cytokine combinations, and IL-17 was measured following restimulation of differentiated cells. \textit{B}, Naive CD4$^+$CD62L$^{hi}$ T cells were differentiated under Th1- or Th17-polarizing conditions. IL-17 and IFN-$\gamma$ were measured following restimulation of differentiated cells. Results are representative of three similar experiments. $p < 0.0001$, difference in IL-17 secretion between wt and IL-17 KD cells under Th17-polarizing conditions. $p = 0.002$, difference in IL-17 secretion between wt and IL-17 KD cells under Th1-polarizing conditions.
FIGURE 3. IL-17 gene silencing is specific and does not affect overall Th17 cell differentiation. Naive T cells from IL-17 KD and wt NOD mice were differentiated into Th17 cells. Following re-stimulation, IL-17, IL-17F, and IL-21 were measured at the protein (A) and mRNA (B) level by ELISA and qPCR, respectively. Expression of RORγt mRNA was also measured in B. The data show the combined results from two identical experiments with cells from a total of five mice of each genotype tested individually. For IL-17, p = 0.047 in A and p = 0.0003 in B. Differences for IL-17F, IL-21, and RORγt were not statistically significant.

Cell transfer
Splenocytes from wild-type (wt) or IL-17 KD mice were magnetically depleted of CD26L+ and CD25+ using anti-CD26L and anti-CD25 Abs (Becton Dickinson), followed by sheep anti-rat Ab-coated magnetic Dynabeads (Invitrogen). NOD.SCID mice were injected with 10^6 (Becton-Dickinson), followed by sheep anti-rat Ab-coated magnetic CD28 Ab (1 μg/ml) in the presence of cytokines and were restimulated after 5 d with phorbol-myristate-acetate and ionomycin for 5 h prior to measurement. The following polarizing reagents were used: Th1–IL-2 (20 ng/ml), IL-12 (10 ng/ml), anti–IL-4 (1 μg/ml), Th17–TGF-β (2 ng/ml), IL-23 (15 ng/ml), IL-21 (10 ng/ml), and anti–IL-4 and anti–IFN-γ (Fig. 3, both 1 μg/ml). Alternatively, splenocytes from MOG55-65+ immunized mice were stimulated with MOG55-65 peptide (10 μg/ml) or CD3/CD28-coated beads (Invitrogen). Cytokine levels were measured in the supernatants of stimulated cells using ELISA kits (Bioscience and R&D Systems), following the manufacturer’s instructions.

Quantitative PCR
RNA was extracted using TRIzol reagent (Invitrogen), and cDNA was synthesized with a Transcriptor First Strand cDNA Synthesis kit (Roche), according to the manufacturer’s instructions. Quantitative PCR (qPCR) was performed with primer–probe pairs from the Universal Probe Library (UPL) and a Faststart Universal Probe Master mix (Roche) on an ABI 7900HT system (Applied Biosciences). The following forward and reverse primers, respectively, were used: GAPDH, 5′-AGCTGTGATCAACGCCTGAAG-3′ and 5′-TTTGATGTTAGTGGGGTCTCG-3′ UPL probe #5; IL-17, 5′-GTGGAGGGTCCAAGTCAATTCTC-3′ and 5′-GAAGGATATCCTATCGGTTCTCTAT-3′ UPL probe #4; IL-21, 5′-TCAGCTCCACAAAGTGAAGG-3′ and 5′-GCTTTCTGGAAACGGCAGGAAA-3′ UPL probe #10; IFN-γ, 5′-ATCTGGAGGACTTGCCGCAA-3′ and 5′-TTCAGAGCTTCAAAGGTGCTAGGA-3′ UPL probe #21; and RORγt, 5′-GCCAGAATGCCCATGAA-3′ and 5′-GACATTCCGGCAAGGTTG-3′ UPL probe #21.

Relative mRNA levels were calculated as follows: % mRNA = 100 × (2^(-ΔCT of group) − 2^(-ΔCT of wt group)). The data are shown (mean and SEM, n = 3) and IL-17 KD (% mRNA = 90 ± 9, n = 5) mice. The cycle difference between IL-17 and RORγt measurements is shown (mean and SEM, n = 4).

Statistics
Kaplan–Meier survival curves were compared using the log-rank test. EAE severity was compared using the Wilcoxon signed-rank test. ELISA and qPCR data were compared using the unpaired t test.

Results
Generation of IL-17 KD NOD mice
To investigate the involvement of Th17 cells in T1D, we generated NOD mice in which IL-17 is constitutively silenced by RNA interference. Several shRNA sequences targeting the Il17a gene were cloned into a lentiviral vector also encoding the GFP (Fig. 1A). The efficiency of gene KD was validated in a luciferase reporter assay. The Il17a cDNA was cloned into the 3′-untranslated region of the Renilla luciferase, and silencing efficiency was evaluated by luminescence measurement following cotransfection of the Il17a reporter construct with different shRNA-containing lentiviral

FIGURE 4. IL-17 is effectively silenced in islets and pancreatic lymph nodes (pLN) from IL-17 KD mice. A, IL-17 and RORγt mRNA was measured in purified islets from wt (n = 3) and IL-17 KD (n = 5) mice. The cycle difference between IL-17 and RORγt measurements is shown (mean and SEM, left panel) as a measure of relative IL-17 mRNA levels normalized to RORγt expression (right panel). B, IL-17 mRNA was measured in pLN from wt (n = 9) and IL-17 KD (n = 4) mice. The mean and SEM of ΔCT values (IL-17 relative to GAPDH measurements, left panel) and the relative cytokine mRNA level in pLN (right panel) are shown.
vectors (Fig. 1B). Among the sequences tested, shRNA#176 was reproducibly the most potent. To further control for specificity in this assay, shRNA#176 and an shRNA against a distinct target gene (*Ptpn22*) were both tested against reporter constructs for *Il17a* and *Ptpn22* (Fig. 1C). The vector containing shRNA#176 potently and specifically silenced only the reporter for *Il17a*. Therefore, we chose this construct to produce high-titer lentivirus that was subsequently injected into NOD zygotes for the generation of IL-17 KD mice. We identified one transgenic founder line in which virtually all hematopoietic cells expressed the GFP reporter gene, as measured by flow cytometry of blood samples (Fig. 1D). The expression of the lentiviral transgene was found to be uniformly high in all immune cell lineages (Supplemental Fig. 1).

Validation of IL-17 gene silencing in transgenic mice

To verify gene KD in transgenic animals, naïve CD4+CD62Lhi cells from IL-17 KD or control wt NOD mice were differentiated into Th17 cells in vitro, and IL-17 secretion was measured following their restimulation. Cells from IL-17 KD mice produced vastly reduced amounts of IL-17 compared with wt Th17 cells (Fig. 2). In contrast, IFN-γ production following Th1 polarization was unaffected by the transgene (Fig. 2B). Th17 cells are also characterized by their secretion of IL-17F and IL-21 (8, 20), as well as by their expression of the transcription factor RORγt (21). We further examined the specificity of gene silencing by quantifying the expression of these additional molecules both at the mRNA and at the protein level (Fig. 3). Again, Th17 cells from IL-17 KD mice secreted lower levels of IL-17 (74% inhibition, Fig. 3A), but neither IL-17F nor IL-21 secretion was significantly affected by the transgene. In parallel, we measured mRNA levels for these cytokines and for RORγt in the same cells (Fig. 3B). qPCR results confirmed significant gene silencing for IL-17 (average of 1.87-cycle increase, equivalent to 73% inhibition) in transgenic cells. None of the other mRNAs measured differed significantly in their expression between wt and IL-17 KD cells. These results confirmed that the KD of IL-17 is both effective and specific and that it does, in itself, disrupt Th17 differentiation of transgenic cells, which express RORγt, IL-17F, and IL-21 in amounts comparable to wt cells.

We next sought to measure IL-17 levels ex vivo, more specifically in the most disease-relevant tissue (i.e., pancreatic islets of Langerhans). Because we found cytokine expression in purified islets to be highly variable between mice, we measured both IL-17 and RORγt expression in islets from wt and IL-17 KD mice and compared IL-17 expression relative to that of RORγt to account for overall variations in infiltration by Th17 cells (Fig. 4A). IL-17 mRNA was markedly lower in islets from IL-17 KD mice compared with wt NOD islets (average 1.7-cycle increase, equivalent to a 70% reduction), confirming that gene silencing measured in vitro faithfully reflected IL-17 KD in vivo. Measurements of IL-17 mRNA in pancreatic lymph nodes from wt and IL-17 KD mice provided similar results (Fig. 4B).

**FIGURE 5.** Loss of IL-17 does not affect autoimmune diabetes in the NOD model. A, Comparison of spontaneous diabetes frequency of wt (*n* = 63) and IL-17 KD (*n* = 47) female NOD mice (*p* = 0.76). B, Comparison of cyclophosphamide-induced (200 mg/kg) diabetes frequency of wt (*n* = 23) and IL-17 KD (*n* = 18) female NOD mice (*p* = 0.24). Data in B show the combined results of two similar experiments. C, Comparison of the diabetes frequency between groups of NOD.Scid mice injected with splenocytes from wt (*n* = 9) or IL-17 KD (*n* = 9) mice depleted of CD62Llo and CD25+ T cells (*p* = 0.23).

**FIGURE 6.** IL-17 KD NOD mice are partially protected from MOG35–55-induced EAE. A, Mean EAE score of wt (*n* = 6) and IL-17 KD (*n* = 6) female NOD mice (*p* = 0.0014). B, Cumulative disease burden (individual scores were integrated for each mouse, group averages are shown with SEM; *p* < 0.0001). IL-17 (C and D) and IFN-γ (C) secretion was measured following restimulation of splenocytes from diseased mice using MOG35–55 peptide (C) or Ab-coated beads (D; *p* < 0.0001).
Loss of IL-17 does not affect the diabetes susceptibility of NOD mice

Having generated NOD mice in which IL-17 expression is silenced, albeit not completely absent, we assessed whether the loss of IL-17 had an effect on diabetes susceptibility. Our hypothesis was that, given the demonstrated role for IL-17 in the pathogenesis of EAE and collagen-induced arthritis (10, 11), IL-17 silencing may similarly reduce diabetes frequency in transgenic NOD mice. However, both the frequency and the onset kinetics of disease in IL-17 KD mice were indistinguishable from those of the wt NOD cohort (Fig. 5A). We further tested the susceptibility of IL-17 KD NOD mice to cyclophosphamide-induced diabetes, a treatment that accelerates onset and results in synchronous diabetes within 2–3 wk (17). Again, wt and IL-17 KD mice did not differ in their disease susceptibility (Fig. 5B). Finally, we tested the capacity of splenocytes depleted of naïve CD62L hi T cells and CD25+ regulatory T cells to induce diabetes following transfer into immunodeficient NOD SCID recipients. CD62L hi/CD25+–depleted cells isolated from IL-17 KD mice transferred disease susceptibility similarly to wt cells (Fig. 5C), confirming the diabetogenicity of effector T cells from IL-17 KD animals.

IL-17 KD NOD mice are partially protected from EAE

Although we had found efficient IL-17 gene silencing both in vitro, following the generation of Th17 cells from transgenic animals, and ex vivo in transgenic islets, it was possible that IL-17 KD in vivo was quantitatively insufficient to hinder the onset of autoimmunity. Previous work by Iwakura and colleagues (10) demonstrated that IL-17–deficient mice were partially protected from EAE. As a validation of the silencing efficiency of IL-17 at the systemic level, we compared the susceptibility of IL-17 KD and wt NOD mice to EAE. We previously found that mice in our NOD colony developed a relapsing form of EAE after challenge with MOG35–55 peptide, with the relapse increasing in severity compared with the first acute phase of paralysis (18). IL-17 KD mice displayed significant protection from disease during the first phase of disease (Fig. 6A). The overall cumulative disease burden was also significantly reduced (Fig. 6B), indicating that IL-17 silencing in transgenic NOD mice was sufficient to affect disease in a manner consistent with previous findings using IL-17 KO animals (10). Restimulation of T cells from diseased mice in vitro confirmed the impaired capacity of transgenic cells to produce IL-17. Following restimulation of MOG35–55–reactive T cells with the disease-inducing peptide, IL-17 was undetectable in cultures from IL-17 KD mice, whereas cells from wt animals produced significant amounts of this cytokine (Fig. 6C). In contrast, cells from both groups of mice produced comparable amounts of IFN-γ ex vivo in response to MOG35–55 peptide restimulation. A non–Ag-specific stimulation using Ab-coated beads showed that T lymphocytes from IL-17 KD mice were capable of producing IL-17 when maximally stimulated but that IL-17 levels were significantly lower than in wt cells (Fig. 6D), confirming results obtained with in vitro-differentiated Th17 cells.

Discussion

To investigate the role of Th17 cells in T1D within the NOD model, we used RNA interference to target IL-17, one of the main effector cytokines produced by this T cell subset. We found that IL-17 KD was sufficient to confer partial protection from EAE, consistent with published data obtained with gene-deficient mice (10). Our results demonstrated that gene silencing in transgenic NOD mice was effective and quantitatively sufficient to impair Th17-dependent autoimmunity, yet IL-17 KD mice were not protected from diabetes. We concluded that IL-17 is not essential to the autoimmune pathogenesis of diabetes in the NOD model. To our knowledge, our results provide the first direct evidence that IL-17 may not be required for autoimmune diabetes. Consequently, our study suggests that Th17 cells are not a critical component of the autoimmune response that causes T1D, at least not owing to the production of their characteristic cytokine IL-17. Earlier work suggested a role for IL-17 in immune-mediated diabetes. Zaghouani and colleagues (12) found that protection from diabetes induced by a glutamic acid decarboxylase peptide-Ig fusion construct involved the modulation of IL-17 levels. Shapiro and colleagues (13) further reported that IL-17 Ab treatment had a protective effect in the NOD model, albeit only when administered to 10-wk-old mice and not in younger mice. These results could be reconciled with ours in a model in which Th17 cells are facultative to the diabetogenic immune response and in which IL-17 may only become indispensable later in disease if the initial response was skewed toward a Th17 profile. Arguing against such an interpretation is the fact that Th17 cells were found to be diabetogenic when transferred into immunodeficient NOD SCID recipients, but only after converting into Th1-like cells (14, 15), and that anti–IL-17 Ab was not protective in this model. Together, previous studies are inconclusive as to the role of Th17 cells in the autoimmune response that causes T1D. Our experiments with IL-17 KD NOD mice now indicate that IL-17 is not critical to the pathogenesis of autoimmune diabetes. Notably, IL-21 signaling was shown to be indispensable for T1D in the NOD model (22–24). This cytokine is produced by Th17 cells and was also proposed to reinforce the differentiation of this T cell subset (25). Furthermore, the pathogenicity of Th17 cells in EAE was very recently shown to critically depend on their secretion of GM-CSF (26, 27). Therefore, it is conceivable that Th17 cells may yet be involved in diabetes through effector mechanisms distinct from IL-17 secretion, and further work is warranted to test whether the complete absence of the Th17 subset would indeed be protective. However, our results illustrated that a characteristic Th17 response involving IL-17 secretion is not necessarily central to all T cell–driven autoimmune disorders, particularly not autoimmune diabetes. This finding adds to the notion that the role of Th17 cells in autoimmunity may be overstated and that caution should be exercised in the evaluation of data pertaining to this T cell subset in human patients (28–31).

Acknowledgments

We thank Katharina Herrmann for technical assistance and Nicole Hain for microinjection, colony management, and diabetes testing.

Disclosures

The authors have no financial conflicts of interest.

References

IL-17 IS DISPENSABLE IN AUTOIMMUNE DIABETES