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IL-21 Promotes CD8⁺ CTL Activity via the Transcription Factor T-bet

Andrew P. R. Sutherland,*†‡ Nicole Joller,*† Monia Michaud, ‡ Sue M. Liu,*†‡§ Vijay K. Kuchroo,*† and Michael J. Grusby‡

CD8⁺ T cells are fundamental for immune-mediated clearance of viral infections and contribute to immune pathology in autoimmune diseases such as type 1 diabetes. To execute these functions, CD8⁺ T cells must differentiate into CTLs, a process that is precisely regulated by a variety of cytokines, costimulatory molecules, and transcription factors. IL-21 is an IL-2 family cytokine and a growth factor for multiple lymphocyte effector lineages, including cytotoxic CD8⁺ T cells. Recent studies demonstrate that loss of IL-21 signaling results in reduced viral clearance in models of lymphocytic choriomeningitis virus infection, and also protection from type 1 diabetes in the NOD model. This is most likely the result of impaired CD8⁺ CTL function in the absence of IL-21 signaling. Currently, the mechanisms by which IL-21 promotes CTL differentiation in CD8⁺ T cells remain unclear, particularly the identity of the relevant transcription factor(s). We show that IL-21 promotes CTL function in vitro and killing of pancreatic islets in vivo via the use of transgenic mice expressing IL-21 in pancreatic β cells. We demonstrate that IL-21 induces the expression of the transcription factor T-bet in CD8⁺ T cells, predominantly via STAT1, and that T-bet is required for the induction of cytolytic molecules, including perforin and granzyme B in response to IL-21. Finally, we show that IL-21–induced CTL function is T-bet dependent, as T-bet deficiency results in defective IL-21–dependent cytotoxicity in CD8⁺ T cells in vitro and in vivo. Thus, IL-21 drives CD8⁺ CTL differentiation via the actions of the transcription factor T-bet. The Journal of Immunology, 2013, 190: 000–000.
IL-21–INDUCED CTL DIFFERENTIATION IS T-bet DEPENDENT

Materials and Methods

Mice

All mice were housed in microisolation cages under specific pathogen-free conditions at the Harvard Institutes of Medicine, and all animal studies were performed according to institutional and National Institutes of Health guidelines for animal use and care. In accordance with these guidelines, blood glucose levels were monitored weekly using a handheld Ascensia Contour glucometer ( Bayer). Diabetic incidence was calculated in terms of the percentage of diabetic mice per group. IL-21R–deficient mice were generated as described (27). STAT4-deficient mice were generated as described (31). C57BL/6, OT-I (C57BL/6-Tg[ThraThrb]1100Mjb/J), RIP-OVA−/−(C57BL/6-Tg[Ins2-OVA]307Wehi/Wehi), RIP-mOVA (C57BL6-Tg[Ins2-1T2R5/OVA]296Wehi/Wehi), and IFN-γ–deficient (B6.129Stg(Ighm129129Igh129129)) mice were purchased from The Jackson Laboratory (Bar Harbor, MA). The 129S6/SvEvTac, Stat1−/−deficient (B6.129S6Stat1tm1Rds/J) mice were purchased from The Jackson Laboratory and used for experiments. C57BL/6 J, OT-I (B6.129S7-Tga1Sro/J), RIP-OVA low (C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/Wehi), and Stat1−/−deficient mice generated in our laboratory were used for experiments. N12) were purchased from Taconic. T-bet–deficient mice (B6.129S6-Thrb2tm1(Pell)J) were provided by B. Lichtman (Brigham and Women’s Hospital).

Flow cytometry

Single-cell suspensions were prepared from spleen and peripheral lymph nodes by mechanical disruption. Cells were filtered through a 70-μm cell strainer (BD Biosciences, San Jose, CA), and then subjected to erythrocyte lysis using ACK buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA [pH 7.2–7.4]). CD8+ T cells were isolated using magnetic separation (Miltenyi Biotec) and cultured at 37˚C in 10% CO₂ in DMEM supplemented with 1% heat-inactivated FCS, sodium pyruvate, t-glutamine, penicillin/ streptomycin, nonessential amino acids, arginine/asparagine, folic acid, vitamins, and 2-ME. CD8+ T cells were activated at 1 × 10⁶ cells/well in 24-well plates previously coated with anti-CD3 (clone 145-2C11) and anti-CD28 (clone PV-1) Abs at 2 μg/ml IL-12, IL-21, and IL-27 were used at 25 ng/ml and purchased from R&D Systems. Lymphocytes were isolated from pancreatic tissue by fine dissection with razor blades, followed by collagenase digestion in HBSS for 20 min at 37˚C.

RNA extraction, cDNA synthesis, and quantitative RT-PCR

Total RNA was isolated with QiaGen RNeasy Plus Minikit. A quantity amounting to 1 μg total RNA was used to prepare cDNA using the iScript cDNA synthesis kit (Bio-Rad). Quantitative RT-PCR was performed using TaqMan probes and the 7500 Fast Real-Time PCR System (Applied Biosystems). All samples were normalized to β-actin internal control.

CFSE labeling and cell transfers

CD8+ T cells were resuspended at 2 × 10⁶/ml in 0.1% BSA in PBS. CFSE (Invitrogen) was added to a final concentration of 10 μM, and cells were incubated at 37˚C for 15 min. Cells were washed once with 10% FCS in PBS, twice with 0.1% BSA in PBS, and then resuspended in PBS for transfer to recipient animals. A total of 5 × 10⁶ OT-1 cells was transferred to IL-21Tg × RIP-OVA−/− double-transgenic or control mice for CFSE proliferation and diabetes incidence experiments. A total of 1 × 10⁶ activated OT-1 cells was transferred to RIP-mOVA mice for diabetes incidence studies.

Flow cytometry

Cells were harvested and resuspended in PBS containing 0.5% BSA and 0.01% sodium azide (FACS buffer). A total of 1 × 10⁶ cells was used per stain, with 1 μg/ml test mAb, or control mAb. Primary mAbs were washed for 25 min at 4˚C, after which cells were washed twice with FACS buffer. Secondary staining was performed after resuspension in 100 μl containing appropriate secondary staining reagents. After incubating for 15 min at 4˚C, cells were washed twice in FACS buffer and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences). Intracellular staining was performed using BD Cytofix/Cytoperm kit, FITC-, PE-, PerCP-, and allophycocyanin-conjugated mAbs to CD3, CD4, CD8, B220, NK1.1, and T-bet were obtained from BioLegend (San Diego, CA) and used according to the manufacturer’s instructions. mAbs to pSTAT1, pSTAT3, pSTAT4, and pSTAT5 were obtained from BD Biosciences.

Antiphospho-STAT staining

Cells were harvested and fixed in 4% paraformaldehyde for 12 min at 37˚C, rinsed with PBS, and fixed in 90% methanol solution on ice for 30 min. Cells were washed twice with Perm buffer (eBiosciences) and then blocked in Perm buffer containing 2% FBS and Fc block (1:100) for 10 min at room temperature. Ab staining was performed in Perm buffer for 45 min at room temperature, and cells were then washed three times with Perm Buffer and resuspended in FACS buffer for flow cytometry.

In vitro CTL assays

OVA-expressing (EG7) and a nontransfected control cell line (EL4) were seeded at 1 × 10⁶ cells/well into 96-well plates. CTLs were added at a range of concentrations and incubated with target cells overnight, after which cells were stained with PE-conjugated anti-CD8 mAb and 7-ami-noactinomycin D (7-AAD) in FACS buffer and dead cells identified by 7-AAD positivity.

Statistical analysis

Statistical significance was determined for in vivo studies using one-way ANOVA analysis, log rank test, and Bonferroni’s multiple comparison posttest using t test. Statistical significance for other studies was determined using Student t test with relation to comparison data. Statistical significance values are indicated as follows: *p < 0.05, **p < 0.01, and ***p < 0.005.

Results

IL-21 has previously been shown to promote the differentiation of CD8+ CTLs in vitro and in vivo (20). To confirm these data, we isolated CD8+ T cells from wild-type mice and performed in vitro activations with anti-CD3/CD28 mAbs in the presence of IL-21 or control cytokotikes IL-27 and IL-12 (both of which induce CTL differentiation) for 72 h. Cells were harvested and mRNA levels were measured by quantitative RT-PCR. IL-21 induced the expression of cytotoxic molecules, including perforin (Fig. 1A), granzyme B (Fig. 1B), and granzyme A (Fig. 1C). To determine whether IL-21 stimulation also resulted in increased CTL function, CD8+ T cells were isolated from OT-I mice and activated in vitro with anti-CD3/CD28 mAbs for 5 d in the presence of IL-21 or control cytokotike IL-27 and IL-12. Cells were harvested, and in vitro CTL activity was quantified against an OVA-expressing cell line, which demonstrated that IL-21 increased the cytolytic activity of the cultured CD8+ T cells (Fig. 1D).

We have previously reported that transgenic mice expressing IL-21 in pancreatic β-cells develop spontaneous TID (27); however, the cellular mechanisms responsible for this phenotype have not been defined. Given that IL-21 can promote the development of CTL function, we hypothesized that CD8+ T cells could play an important role in the TID in this model. To address this issue, IL-21 transgenic mice were crossed to the RAG2-deficient background to determine whether T or B cells were required to initiate autoimmunity in IL-21 transgenic mice. RAG2-deficient mice were completely protected from the development of TID in IL-21 transgenic mice, clearly demonstrating the dependence on adaptive immunity (Fig. 1E). The pancreatic infiltrates from prediabetic IL-21 transgenic mice were analyzed to identify cells that may be involved in the induction of diabetes. Most notably, we observed an increased abundance of CD8+ T cells and B cells in the pancreas of transgenic mice compared with littermate controls (Fig. 1F), with smaller increases in CD4+ T cells and NK cells also observed. CD8+ T cells were isolated from spleen and lymph nodes, and the expression of perforin, granzyme A, granzyme B, and IFN-γ was quantified by RT-PCR analysis. This revealed an increased expression of these effector molecules in IL-21 transgenic mice, suggesting increased CTL differentiation (Fig. 1G). To test whether diabetogenic CTL activation and differentiation were enhanced by overexpression of IL-21, IL-21 transgenic mice were crossed to RIP-OVA−/−3 mice to generate double-transgenic mice (IL-21Tg × RIP-OVA−/−3) and relevant controls. OVA-specific TCR transgenic OT-1 T cells were purified and labeled with CFSE, and 5 × 10⁶ unactivated cells were transferred i.p. into double-transgenic recipient mice and controls. Mice were sacri-
10 d later, lymphoid organs were isolated, and division of transferred cells was quantified by CFSE dye dilution (Fig. 1H). This demonstrated activation and proliferation of OT-I cells in double-transgenic mice, and, as expected, no proliferation was observed in single-transgenic controls. In addition, transfer of OT-I cells into double- but not single-transgenic recipient mice resulted in the onset of T1D from 10 d posttransfer (Fig. 1I and data not shown). In contrast, IL-21R–deficient OT-I cells were not able to induce T1D upon transfer to double-transgenic recipient mice (Fig. 1I), demonstrating that CD8+ T cells require direct stimulation by IL-21 to induce T1D in this system. Together, these data confirm previous findings that IL-21 can promote the expression of cytolytic molecules and CTL differentiation in CD8+ T cells and demonstrate the IL-21 transgenic mice have enhanced CTL responses, resulting in destruction of pancreatic islets and onset of T1D.

IL-21 is therefore a potent stimulator of CTL differentiation; however, the underlying transcriptional events mediating this process remain unclear. The transcription factors T-bet and eomesodermin are central regulators of CD8+ CTL differentiation, which coordinate the expression of important effector molecules, including perforin, granzyme B, and IFN-γ, and are required for optimal CTL function (7, 12, 19). We therefore hypothesized that IL-21 may induce CTL function in CD8+ T cells via induction of T-bet and/or eomesodermin. To test this hypothesis, wild-type CD8+ T cells were activated in vitro with anti-CD3/CD28 alone or in the presence of IL-21 (and IL-27 or IL-12 as positive controls), and the expression of T-bet and eomesodermin was analyzed over a 72-h time course. These experiments did not reveal any significant regulation of T-bet by IL-21 during this time period (Fig. 2A); in contrast, IL-21 significantly suppressed the expression of eomesodermin (Fig. 2B). To determine whether IL-21 was exerting positive effects at an earlier time point, we repeated the previous experiments over a 24-h time course. These experiments revealed that IL-21 induced a transient 70% increase in T-bet mRNA compared with controls at 4 h poststimulation (Fig. 2C);
suppression of eomesodermin by IL-21 was again observed during this time course (Fig. 2D). We then analyzed T-bet protein expression by flow cytometry, which demonstrated that IL-21 induced a 30% increase in T-bet protein expression at 8 h (Fig. 2E). CD8+ T cells produce high levels of IFN-γ in response to TCR activation and costimulation and, because IFN-γ is a potent driver of T-bet expression (32), we reasoned that the true capacity for IL-21–dependent induction of T-bet may be obscured in our in vitro culture system by excessive IFN-γ production. To test this hypothesis, we measured T-bet expression in response to IL-21 stimulation in IFN-γ knockout mice. We observed a 6-fold increase in T-bet mRNA at 4 h (Fig. 2F), indicating that the magnitude of IL-21–dependent T-bet induction was drastically increased in the context of IFN-γ deficiency. Analysis of T-bet protein expression by flow cytometry demonstrated that the magnitude of T-bet protein induction was enhanced in the absence of IFN-γ, with a 2-fold increase in T-bet protein observed at 8 h (Fig. 2G, 2H). Similar results were observed when wild-type CD8+ T cells were cultured in the presence of IL-21 and IFN-γ-blocking Abs (data not shown). Thus, IL-21 can rapidly induce T-bet expression during the activation of CD8+ T cells, and T-bet induction is even more pronounced in the absence of IFN-γ.

IL-21 mediates many of its effects via the function of STAT family transcription factors (20); thus, we next determined which STAT molecules were required for induction of T-bet expression by IL-21. CD8+ T cells were activated in vitro in the presence of IL-21, IL-27 as a positive control, or no cytokine (control). Cells were harvested at the indicated time points, and levels of STAT phosphorylation were quantified by intracellular staining. These experiments demonstrated that IL-21 induced the phosphorylation of STAT1, 3, and 5, as previously described (20), but unexpectedly also STAT4 (Fig. 3A). The specificity of pSTAT4 detection in this assay system was confirmed by the complete lack of signal observed in STAT4 knockout mice (Supplemental Fig. 1A). Because both STAT1 and STAT4 are able to induce the expression of T-bet (7, 32), we next determined whether STAT1 and/or STAT4 were required for IL-21–dependent induction of T-bet. CD8+ T cells from STAT1-deficient mice and wild-type controls were activated in vitro in the presence of IL-21 or no cytokine (control) and T-bet mRNA and protein levels quantified at the indicated time points. STAT1 knockout CD8+ T cells exhibited a 50% decrease in IL-21–induced T-bet mRNA at 4 h (Fig. 3B) and a corresponding 35% reduction in T-bet protein level at 8 h poststimulation (Fig. 3C). Similarly, CD8+ T cells from STAT4-deficient mice and wild-type controls were activated in vitro in the presence of IL-21 or no cytokine (control). These data showed a 20% decrease in IL-21–induced T-bet mRNA at 4 h (Fig. 3D) and a corresponding 20% reduction in T-bet protein level at 8 h poststimulation in STAT4 knockout CD8+ T cells (Fig. 3E), neither of which reached statistical significance. However, IL-21–induced IFN-γ production was STAT4 dependent over the same time course, indicating that IL-21 can induce functional effects via activation of STAT4 in this system (Supplemental Fig. 1B). Together, these data indicate that IL-21 induction of T-bet is predominantly STAT1 dependent.

IL-21 induces the expression of cytotoxic molecules such as perforin and granzyme B (20), which are targets of T-bet and are
upregulated during CTL differentiation. Thus, we tested whether the IL-21–induced expression of T-bet target genes, such as perforin and granzyme B, was in fact dependent on T-bet. We stimulated CD8⁺ T cells isolated from T-bet–deficient mice and wild-type controls with IL-21 for 72 h and assayed gene induction by RT-PCR. This demonstrated that IL-21–induced expression of Hlx-1 (Fig. 4A), IL-12Rβ2 (Fig. 4B), perforin (Fig. 4C), and granzyme B (Fig. 4D) was dependent on T-bet (7, 32, 34). Interestingly, similar levels of granzyme A expression were observed in T-bet knockout mice and wild-type controls after IL-21 stimulation (Fig. 4E), indicating that IL-21 also regulates T-bet–independent transcriptional circuits that are required for optimal cytolytic molecule expression.

We then tested whether IL-21–induced CTL function required T-bet. To this end, OT-I mice were crossed to the T-bet–deficient background, activated in vitro with anti-CD3/CD28 for 5 d in the presence of IL-21, IL-27, or IL-12 (both of which induce T-bet–dependent CTL differentiation). Cells were harvested, and in vitro CTL activity was quantified against an OVA-expressing cell line. IL-21 enhanced in vitro CTL activity in wild-type OT-I, but this effect was completely ablated in the absence of T-bet (Fig. 5A). In keeping with previous studies, CTL activity induced by IL-27 and IL-12 was also dependent on T-bet (Supplemental Fig. 2A). Quantification of perforin, granzyme B, and granzyme A expression after 5 d of culture showed that upregulation of perforin and granzyme B by IL-21 was T-bet dependent (Supplemental Fig. 2B–D), as observed at earlier time points (Fig. 4). To test whether T-bet was required for IL-21–induced cytotoxicity in vivo, the cultured wild-type and T-bet–deficient OT-I cells were transferred to RIP-mOVA recipient mice, and blood glucose levels were monitored to determine diabetes onset. IL-21–stimulated OT-I cells were able to induce diabetes after transfer into RIP-mOVA recipient mice (Fig. 5B); however, in contrast, IL-21–stimulated T-bet–deficient OT-I cells were unable to induce diabetes (Fig. 5B). Wild-type and T-bet–deficient OT-I cells stimulated with IL-27 or IL-12 demonstrated a similar pattern of T-bet dependency. To corroborate this finding, we used a second model, in which unactivated T-bet–deficient OT-I cells or wild-type OT-I controls were transferred to IL-21Tg RIP-OVAlow double-transgenic recipient mice. In contrast to OT-I controls, recipients of T-bet–deficient OT-I cells were almost completely protected from the onset of T1D (Fig. 5C). As this model is completely dependent on IL-21R signaling in transferred CD8⁺ T cells (as shown in Fig. 1I), these data indicate that T-bet is required for IL-21–induced CTL activity in vivo. Thus, our data demonstrate that IL-21–induced expression of cytotoxic molecules and in vitro and in vivo CTL function is dependent on the transcription factor T-bet.

**Discussion**

Common γ-chain cytokines can regulate both effector and memory differentiation in CD8⁺ T cells. Previous studies demonstrate that IL-2 is a differentiation factor for effector CD8⁺ T cells (19), whereas IL-7 and IL-15 play critical roles in the generation and
IL-21 promotes the development of both effector and memory characteristics in CD8+ T cells, inducing CTL differentiation in some experimental systems (20, 36, 37), while promoting the acquisition of a memory phenotype in others (38–40). Our present studies further support the notion that IL-21 is a differentiation factor for effector CD8+ T cells. We demonstrate that IL-21 promotes the development of CD8+ CTLs both in vitro and in vivo by inducing the transcription factor T-bet, which is critical for the development of effector CD8+ T cells (7). Thus, IL-21 can be grouped with a panel of other cytokines, such as IL-27 and IL-12, which induce effector function in CD8+ T cells via T-bet. Whereas these cytokines all induce T-bet expression, there are qualitative differences between their functions. We suggest that these differences could be ascribed in part to their differing kinetics of T-bet induction. IL-21 induces a more rapid and transient induction of T-bet compared with that observed with IL-27, and particularly IL-12, which does not show significant induction until at least 48 h after activation. This rapid and transient induction of T-bet by IL-21 results in the upregulation of T-bet–dependent genes required for CTL differentiation, such as perforin and granzyme B; however, the lack of sustained T-bet expression may be an important prerequisite for the subsequent development of a memory phenotype in response to IL-21 in some experimental systems (38–40). Previous studies demonstrate that CD8+ T cells lacking eomesodermin have impaired memory CD8+ T cell development, indicating that eomesodermin is important for the differentiation of memory CD8+ T cells (18). However, our experiments, and those of other groups, indicate that IL-21 is a potent inhibitor of eomesodermin (39, 41), thus suggesting that IL-21 would prevent memory CD8+ T cell development. It should be noted that these studies were performed at relatively early time points (3–5 d postactivation), and it is possible that IL-21 suppression of eomesodermin is subsequently relieved, which could allow memory CD8+ T cell development to proceed in response to IL-21 stimulation.

FIGURE 4. IL-21 induces T-bet–dependent gene expression. CD8+ T cells were isolated from spleen and lymph nodes of C57BL/6 and T-bet–deficient mice via magnetic separation and activated in the presence of IL-21 or no cytokine (control), and quantitative RT-PCR was performed for (A) Hlx-1, (B) IL-12Rβ2, (C) perforin, (D) granzyme B, and (E) granzyme A (n = 2 for all groups). *p < 0.05.

FIGURE 5. IL-21–induced CD8+ CTL activity is T-bet dependent. CD8+ T cells were isolated from OT-I and T-bet–deficient OT-I mice and activated for 5 d in the presence of IL-21, IL-27, IL-12, or no cytokine (control). (A) CTLs were incubated overnight with OVA-expressing cell line (EG7), and cell death was measured by 7-AAD incorporation. A representative experiment is shown. (B) A total of 1 × 10^6 cells was transferred i.p. into RIP-mOVA recipients, and blood glucose levels were monitored daily (n = 5 for IL-21 from two independent experiments, n = 2 for all other groups). (C) Unactivated CD8+ T cells were isolated from OT-I and T-bet–deficient OT-I mice via magnetic separation; 5 × 10^6 cells were transferred i.p. into IL-21TG × RIP-OVA^low double-transgenic recipients; and blood glucose levels were monitored daily (OT-I, n = 5; T-bet–deficient OT-I, n = 9 from two independent experiments). *p < 0.05, **p < 0.01.
expression in CD8+ T cells. Our experiments demonstrate that IL-21 can induce STAT4-dependent IFN-γ expression, indicating that the activation of STAT4 by IL-21 has functional effects. In contrast, STAT4 was not required for the optimal induction of T-bet expression in response to IL-21. This is in keeping with previous studies demonstrating that IL-21–induced CTL function in CD8+ T cells is STAT4 independent (37), and, along with experiments performed in STAT1-deficient mice, indicates that STAT1 is the primary inducer of T-bet in response to IL-21. The exact mechanisms of STAT4 activation and the relative importance of STAT4–mediated gene induction in response to IL-21 remain to be determined.

Recent studies indicate that IL-21R–deficient mice fail to clear chronic viral infections (21–23) and that IL-21R NOD mice are protected from T1D (24–28), most likely via impaired CD8+ CTL function and reduced expression of T-bet (44). Our data suggest that this generalized impairment of the IL-21 signaling may result from impaired induction of T-bet and reduced expression of T-bet–dependent transcriptional programs that promote CTL function. In keeping with this hypothesis, T-bet–deficient mice have attenuated CD8+ T cell responses and are protected from the development of T1D in a model of virally induced T1D and on the NOD background (16, 45). IL-21 is located in the Idd3 disease-susceptibility locus, and protection conferred by this congenic interval is correlated with reduced expression of IL-21 (24). We would hypothesize that this may cause Nod.Idd3 mice to have impairments in CTL responses due to reduced IL-21–dependent expression of T-bet, and that this may account in part for the protection from diabetes that is observed in Nod.Idd3 mice.

In conclusion, we demonstrate that IL-21 promotes CTL function in vitro and in vivo, as overexpression of IL-21 in pancreatic β cells leads to CD8+ CTL differentiation and T1D. We show that IL-21 induces the expression of the transcription factor T-bet in CD8+ T cells and the expression of cytolytic molecules, and the functional differentiation of CTLs in response to IL-21 is T-bet dependent. These data illuminate a mechanistic basis for the helper effects of this cytokine during the development of effector CD8+ T cell responses, coupling IL-21 production by CD4+ T cells to the induction of the gene program associated with CD8+ CTL differentiation via the action of T-bet.

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Disclosures
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