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Cutting Edge: IL-21 Is Not Essential for Th17 Differentiation or Experimental Autoimmune Encephalomyelitis

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Recent studies have suggested that IL-21 is a key factor in the development of IL-17-producing CD4 T cells (Th17) and that the induction of experimental autoimmune encephalomyelitis, which depends on mounting an efficient Th17 response, is reportedly impaired in the absence of IL-21 signaling. In this study, we provide supportive in vitro evidence that IL-21 can drive Th17 responses in conjunction with TGF-β. However, more importantly we also demonstrate, using IL-21- and IL-21R-deficient mice, that IL-21 is not essential for the differentiation of Th17 cells in vitro and in vivo. Moreover, we show that IL-21- and IL-21R-deficient mice are highly susceptible to experimental autoimmune encephalomyelitis with disease scores that were comparable, or even higher at the peak of disease, to those of control mice. Thus, our results challenge the notion that IL-21 is a key factor in driving Th17 immunity and disease. The Journal of Immunology, 2008, 180: 7097–7101.

 naïve CD4+ T cells, when activated in the presence of TGF-β and IL-6, develop into the recently discovered lineage of Th17 cells. This T effector lineage is defined by the production of IL-17A, IL-17F, IL-21, and IL-22 and the up-regulation of the IL-23R, which is essential for their expansion and maintenance (reviewed in Refs. 1 and 2). Th17 cells play an important role in enhancing the clearance of pathogens that are less efficiently resolved by the better-known Th1 and Th2 type responses. They have also been shown to be responsible for enhancing tissue destruction in autoimmune disease models such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis, diseases that were previously believed to be mediated by Th1-type responses (1, 2).

Recently, a number of studies have shown that IL-21, a common γ-chain cytokine with potent immunoregulatory effects (reviewed in Ref. 3), is capable of regulating Th17 development (4–8). In vitro culture of naïve CD4 T cells in the presence of IL-21 and TGF-β induced IL-17 production at levels similar to that induced by TGF-β and IL-6 (4–8). Moreover, four of these studies demonstrated that Th17 development was impaired in the absence of IL-21 signaling (4–7). In one study that used IL-21-deficient splenocytes, Th17 development in vitro and in vivo was almost completely abolished (5), whereas in two other studies using IL-21R-deficient mice, Th17 production was reduced (by ~50–70%) but clearly not abolished in the absence of IL-21 signaling (4, 6). Similarly, a 2- to 3-fold reduction in IL-17 production was observed when purified CD4+ T cells were cultured under Th17-inducing conditions in the presence of neutralizing anti-IL-21 Ab (7). Furthermore, IL-21-deficient mice immunized with myelin oligodendrocyte glycoprotein (MOG) peptide for the induction of EAE were protected from disease. The resistance to the development of EAE was attributed to the impaired Th17 response both in the periphery and CNS of IL-21-deficient mice compared with wild-type (WT) controls (5). These data are controversial. First, as mentioned, the extent to which IL-21 was required for Th17 development varied considerably between the different studies already described (4–6). Furthermore, an independent study reported that IL-21 and TGF-β did not induce Th17 development in vitro (9).

To independently assess the extent to which IL-21 was required for Th17 immunity, we examined IL-21- and IL-21R-deficient mice on the C57BL/6 background for in vitro and in vivo Th17 development and the Th17-mediated autoimmune disease EAE. Although we were able to verify a role for IL-21 in promoting Th17 differentiation in vitro, our data failed to support a major role for IL-21 in Th17 induction and, furthermore, in stark contrast to the earlier study (5), we found that

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5 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; WT, wild type.

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EAE was not IL-21 dependent and, if anything, was exacerbated in the absence of IL-21 signaling.

Materials and Methods

Mice

C57BL/6 mice were bred in house at the Peter MacCallum Cancer Centre (East Melbourne, Victoria, Australia). IL-21−/− (obtained from Lexicon Genetics via Zymogenetics) and IL-21R−/− (obtained from Dr. W. Leonard, National Heart, Lung, and Blood Institute, Bethesda, MD; Ref.10) were each backcrossed eight generations onto the C57BL/6 background and maintained at the Peter MacCallum Cancer Centre. All mice were used between ages 5 and 7 wk unless otherwise stated, and all experiments were conducted in accord with the animal ethics guidelines and were approved by the Peter MacCallum Cancer Centre Animal Ethics Committee.

Lymphocyte isolation

Lymphocytes were harvested from the spleen and lymph nodes (LNs) by gently grinding them between two frosted glass slides in PBS containing 2% FCS (JRH Biosciences). Splenocytes were further depleted of RBC by RBC lysis buffer (Sigma-Aldrich). CNS infiltrates were obtained from the brains and spinal cords of perfused mice. Briefly, brains and spinal cords were passed through 200-μm wire mesh sieves in PBS with 2% FCS and the resultant suspensions were centrifuged over a 33% isotonic Percoll (GE Healthcare) gradient at room temperature. Cells were washed twice before use in assays. To obtain naive CD4 T cells, splenocytes were stained with anti-CD25, anti-CD4, anti-CD62L, and anti-CD44 Abs and cell sorted on a FACSAnia (BD Biosciences) to obtain a pure population of CD4CD25−CD44highCD62L− T cells. Cells were then washed before being used in in vitro assays or for flow cytometric analysis.

Abs and flow cytometry

All Abs used are from BD Pharmingen. Abs include αβ TCR-FITC (clone H57-597), CD4-allophycocyanin-Cy7 (clone RM4-5), IL-17-PE (clone TC11-18H10), and IFN-γ-allophycocyanin (clone XMG1.2). For intracellular cytokine staining, cells were cultured in GolgiStop (BD Biosciences) before being fixed and permeabilized using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization kit. Flow cytometry was performed using either an LSR-II or FACSAnia apparatus and purification was performed on a FACSAnia cell sorter (BD Biosciences). Analysis was performed using FlowJo software (Tree Star).

CFA treatment

Mice were injected subcutaneously with 100 μl of PBS emulsified CFA, on each hind flank. Draining LNs and spleens were harvested 8 days postimmunization for flow cytometric analysis.

Induction and assessment of EAE

Mice were injected subcutaneously with 150 μg of MOG35–55 peptide (MEVQWYRSPFSRVVHLRNGK; Auspep) emulsified in CFA (Difco) supplemented with 400 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 in the presence of the IL-17-inducing conditions indicated. Seventy-two hours poststimulation, cells were harvested and immediately subjected to intracellular cytokine staining for IL-17. Figure 1. Data points represent mean percentages of IL-17+CD4 T cells ± SEM. Left-hand-side plots are from three experiments wherein samples were derived from n = 6–7 separate mice. Right-hand-side plots are from two experiments for WT and one experiment for IL-21/21R-deficient mice.

Results and Discussion

In vitro Th17 development does not require IL-21 signaling

Due to the potential importance of the role of IL-21 in regulating Th17 immunity and the inconsistency in the extent to which IL-21 deficiency affected the IL-17 response (4–7), we tested the significance of IL-21 in Th17 development by using WT, IL-21-deficient, and IL-21R-deficient mice that were backcrossed to the C57BL/6 background. Consistent with the majority of reports, we were able to verify that IL-21 did induce Th17 development in conjunction with TGF-β (Fig. 1). However, in sharp contrast to earlier studies using both IL-21- and IL-21R-deficient mice, we were unable to demonstrate a critical role for this cytokine in Th17 development (Fig. 1). Firstly, un fractionated splenocytes were cultured in the presence of TGF-β plus either IL-6 or IL-21 (plus anti-IFN-γ and anti-IL-4) (Fig. 1). As previously published, both the TGF-β/IL-6 and the TGF-β/IL-21 combinations induced comparable Th17 responses in vitro and, furthermore, the TGF-β/IL-21 combination failed to induce IL-17 when IL-21R-deficient splenocytes were used, confirming the IL-21R deficiency in these cells. However, in contrast to recent reports (4–6), the Th17 response following incubation with TGF-β and IL-6 was not diminished in the absence of IL-21 signaling. Because the use of unfractonated splenocytes may promote the expansion of previously differentiated Th17 cells and not reflect the true differentiation potential of IL-21, we examined cultures using sorted naive CD4+ T cells. We sorted naive CD4+CD44highCD25low CD4 T cells from WT, IL-21-deficient, and IL-21R-deficient mice and cultured them under IL-17-inducing conditions. Similar to our results using unfractonated splenocytes, we were able to detect IL-17 production from WT
and gene-targeted mice (Fig. 2). However, in this case, we detected a slight reduction in the IL-17 response in the IL-21- and IL-21R-deficient mice (15 and 30% respectively) compared with the WT controls (Fig. 4). Although this does not support the idea that IL-21 is a critical factor for Th17 development in vitro, it is important to point out that in other recent studies (4 – 6) the extent to which IL-21 was required for this process was quite variable, ranging from partial affects (50 – 70%) (4, 6, 7) to almost complete abolition (90%) of Th17 development (5).

In vivo Th17 development does not require IL-21 signaling

Given our inability to show that IL-21 plays a critical role in Th17 development in vitro, it is important to point out that in other recent studies (4 – 6) the extent to which IL-21 was required for this process was quite variable, ranging from partial affects (50 – 70%) (4, 6, 7) to almost complete abolition (90%) of Th17 development (5).

**FIGURE 2.** Naive CD4+ T cells differentiate into Th17 cells with IL-6 and IL-21, but IL-6-driven Th17 differentiation is largely IL-21 independent. Naive CD4+ T cells were sorted from B6, B6.IL-21-deficient (IL-21−/−), and B6.IL-21R-deficient (IL-21R−/−) spleens and cultured under the indicated IL-17-inducing conditions. Four days poststimulation, cells were stimulated with PMA and ionomycin (3 h) and subjected to intracellular cytokine staining for IL-17. Quadrant numbers represent mean percentage of IL-17+ cells ± SEM from four individual mice. Individual data points are depicted in scatter plots at the bottom of the figure and circles depict samples from individual mice.

The IL-17 responses in draining LNs and spleens of all mice were comparable, suggesting that IL-21 is not essential for the generation of Th17 responses in vivo (Fig. 3).

**FIGURE 3.** CFA induces a robust Th17 response in IL-21- and IL-21R-deficient mice. B6, B6.IL-21-deficient (IL-21−/−), and B6.IL-21R-deficient (IL-21R−/−) mice were injected s.c. with CFA. Eight days later, draining inguinal LNs and spleens were harvested. LN cells and splenocytes were stimulated with PMA and ionomycin (3 h) before intracellular cytokine staining for IL-17. Plots are gated on CD3+ T cells. Quadrant numbers represent mean percentage of IL-17+ CD4+ T cells ± SEM. B6, B6.IL-21-deficient, and B6.IL-21R-deficient percentages are from five, four, and three mice, respectively from one experiment. Individual data points for this experiment are also depicted in scatter plots at the bottom of the figure and circles depict samples from individual mice. Similar results were observed in another experiment with B6 and B6.IL-21-deficient mice, although B6.IL-21R-deficient mice were not included in that experiment.
support of this pathogenic role for IL-21, Nurieva et al. (5) showed that EAE (a Th17-mediated disease) was dramatically inhibited in IL-21-deficient mice.

Considering that we were unable to demonstrate an essential role for IL-21 signaling in Th17 development in vitro or in vivo using gene-targeted mice, we hypothesized that EAE onset would not be impaired in IL-21R-deficient mice. However, we were interested in determining whether IL-21 played an essential role in expanding and stabilizing the Th17 response during the course of disease. Indeed, immunization with MOG peptide induced EAE in both the IL-21- and IL-21R-deficient mice at least as efficiently as in the WT controls. Moreover, both gene-targeted strains demonstrated exacerbated disease phenotypes with higher mean maximal disease scores when compared with the WT controls during peak disease (from day 12 to day 17). The timing of disease onset however, was similar between WT, IL-21, and IL-21R-deficient mice. These data not only demonstrate that IL-21 is not essential in generating a Th17 response in vivo, it also suggests that IL-21 may normally be involved in limiting EAE once disease is underway. The data also indicate that Th17 cells that are incapable of making IL-21 are still potent mediators of inflammation and autoimmunity. This is consistent with a study that demonstrated that blocking IL-21 via administration of IL-21R.Fc exacerbated EAE in SJL mice (19). However, in that study the authors acknowledged that they could not discount the possibility that IL-21R.Fc was cross-linking and thereby enhancing IL-21 signaling, which made it difficult to reach a clear conclusion. Our results also resonate with a study of experimental autoimmune myocarditis (also Th17-mediated) that depicted IL-21 as not being important for the generation of Th17 autoimmunity by using IL-21R-deficient mice (20).

To examine the cellular response during EAE, brain and spinal cords (CNS) were harvested at day 11 postimmunization and mononuclear cells were assayed for IL-17 production (Fig. 4A). Because the extent of disease and amount of lymphocytic infiltration was higher in IL-21R-deficient mice compared with WT controls, it was difficult to directly compare the number of Th17 cells but, nonetheless, both IL-21- and IL-21R-deficient mice had robust IL-17 responses, supporting the notion that these responses were not abrogated in the absence of IL-21 signaling in vivo. In line with these findings, a description of some unpublished data in the study by Zhou et al. (6) stated that IL-17-producing cells were present in normal numbers in the lamina propria of IL-21R-deficient mice and that Th17 differentiation was partly defective in response to in vivo immunization. However, this is in contrast to reports (data not shown) from two other studies (4, 5) in which Nurieva et al. (5) stated that IL-17+ CD4+ T cells were completely absent in the lamina propria of IL-21-deficient mice and Korn et al. (4) stated that IL-17+ CD4+ T cells were markedly reduced in IL-21R-deficient mice.

Taken together, our results verify that IL-21 (in conjunction with TGF-β) is capable of driving the differentiation of Th17 cells; however, we found little evidence to suggest that IL-21 signaling is a critical factor for Th17 development in vitro or in vivo or that it is a key mediator of the Th17-mediated disease EAE, at least in C57BL/6 mice. To the contrary, our data suggest that IL-21 normally functions to limit disease in this model of EAE. It is not clear why our findings are inconsistent with those recently published (4–6), although the most likely factor may be the genetic background of the deficient mice. The IL-21- and IL-21R-deficient mice in the current study were originally generated using 129 embryonic stem cells and have both been backcrossed to the C57BL/6 strain for eight generations. The IL-21-deficient mice used in the study from Nurieva et al. (5) were (129 × C57BL/6)F2 mice whereas the IL-21R-deficient mice used by Zhou et al. (6) and Korn et al. (4) were stated as being on the C57BL/6 and BALB/c backgrounds, respectively, although in both cases the number of backcrosses was not stated and therefore the genetic backgrounds of these mouse lines may be responsible for the discrepancy between the studies. That said, different genetic backgrounds may not completely explain the conflicting results, because anti-IL-21 Abs also inhibited IL-17 induction in vivo from the naive CD4 T cells of C57BL/6 mice, although, again, the results only showed a partial

FIGURE 4. IL-21- and IL-21R-deficient mice demonstrate exacerbated EAE in comparison to WT mice. B6, B6.IL-21-deficient (IL-21−/−), and B6.IL-21R-deficient (IL-21R−/−) mice were injected s.c. with MOG35–55 emulsified in CFA. Mice also received pertussis toxin on days 0 and 2 and were monitored daily for clinical signs of paralysis. A, Results from B6 (●, n = 13), B6.IL-21−/− (■, n = 13), and B6.IL-21R−/− mice (▲, n = 14) were pooled from two independent experiments. Mice demonstrating early signs of forelimb paralysis were sacrificed due to ethical limitations. SEM is depicted and therefore the genetic backgrounds of these mouse lines may be responsible for the discrepancy between the studies.
(50–60%) defect (7). It is important to remember that IL-21 is a highly pleiotropic cytokine, known to influence many different components of the immune system and resulting in seemingly opposing outcomes, ranging from promotion to inhibition of immune responses (3, 21). This broad-ranging activity may also contribute to the seemingly conflicting observations that have been observed in different models when IL-21 function is inhibited.

In summary, our data support the concept that IL-21 signaling can contribute to IL-17 differentiation; however, we found little evidence to suggest that IL-21 is a critical factor driving this response in C57BL/6 mice. To the contrary, our data suggest that IL-21 signaling inhibits, rather than exacerbates Th17-mediated EAE.

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References