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IL-21 Counteracts the Regulatory T Cell-Mediated Suppression of Human CD4+ T Lymphocytes\textsuperscript{1}

Ilaria Peluso,* Massimo Claudio Fantini,* Daniele Fina,* Roberta Caruso,* Monica Boirivant,† Thomas T. MacDonald,‡ Francesco Pallone,* and Giovanni Monteleone\textsuperscript{2*}

High expression of IL-21 and/or IL-21R has been described in T cell-mediated inflammatory diseases characterized by defects of counterregulatory mechanisms. CD4\textsuperscript{+}CD25\textsuperscript{+} regulatory T cells (Treg) are a T cell subset involved in the control of the immune responses. A diminished ability of these cells to inhibit T cell activation has been documented in immune-inflammatory diseases, raising the possibility that inflammatory stimuli can block the regulatory properties of Treg. We therefore examined whether IL-21 controls CD4\textsuperscript{+}CD25\textsuperscript{+} T cell function. We demonstrate in this study that IL-21 markedly enhances the proliferation of human CD4\textsuperscript{+}CD25\textsuperscript{−} T cells and counters the suppressive activities of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells on CD4\textsuperscript{+}CD25\textsuperscript{−} T cells without affecting the percentage of Foxp3\textsuperscript{+} cells or survival of Treg. Additionally, CD4\textsuperscript{+}CD25\textsuperscript{+} T cells induced in the presence of IL-21 maintain the ability to suppress alloresponses. Notably, IL-21 enhances the growth of CD8\textsuperscript{+}CD25\textsuperscript{−} T cells but does not revert the CD4\textsuperscript{+}CD25\textsuperscript{+} T cell-mediated suppression of this cell type, indicating that IL-21 makes CD4\textsuperscript{+} T cells resistant to suppression rather than inhibiting CD4\textsuperscript{+}CD25\textsuperscript{+} T cell activity. Finally, we show that IL-2, IL-7, and IL-15, but not IL-21, reverse the anergic phenotype of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells. Data indicate that IL-21 renders human CD4\textsuperscript{+}CD25\textsuperscript{−} T cells resistant to Treg-mediated suppression and suggest a novel mechanism by which IL-21 could augment T cell-activated responses in human immune-inflammatory diseases. The Journal of Immunology, 2007, 178: 732–739.

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\textsuperscript{3}Abbreviations used in this paper: Treg, regulatory T cell; Ann V, annexin V.

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Ilterleukin-21 is a newly described cytokine produced by activated CD4\textsuperscript{+} T cells and known to mediate its effects through a class I cytokine family receptor, IL-21R. This receptor has homology to the shared \alpha-chain of the IL-2 and IL-15 receptors and interacts with the common \gamma cytokine receptor chain (1). Studies both in humans and mice have shown that IL-21 can enhance the growth and functional activity of T, B, and NK cells (1, 2). In line with this, enhanced expression of IL-21 and/or IL-21R has been documented in inflammatory diseases (3–5). Moreover, mice overexpressing IL-21 exhibit inflammatory infiltrates in several tissues (6), thus raising the possibility that IL-21 may play an important role in the induction and/or perpetuation of immune-inflammatory processes. In contrast, IL-21 exerts antitumor effects in vivo (1, 7), suggesting that this cytokine may have therapeutic potential.

Recent studies have led to the identification of a subpopulation of CD4\textsuperscript{+}CD25\textsuperscript{+} T lymphocytes, termed regulatory T cells (Treg) (3), that specifically express the forkhead transcription factor, forkhead winged-helix transcription factor gene (Foxp3) (8). Treg have important effects on the adaptive immune system but in a direction opposing that of IL-21. Indeed, Treg are highly specialized for the suppression of proliferation of autoreactive and effector T cells and therefore in the maintenance of immune homeostasis (8, 9). This function is further substantiated by the demonstration that the loss of Foxp3 results in a fatal autoimmune disorder (8, 9). Consistently, a decreased number and/or defective activity of Treg has been documented in autoimmune and allergic diseases (10). In contrast, Treg may sabotage effective immune responses against microbes and tumors (7, 8).

In addition to naturally occurring Treg that are produced by the thymus as a functionally distinct and mature population of T cells, Treg can arise in the periphery upon conversion of CD4\textsuperscript{+}CD25\textsuperscript{−} T cells into Foxp3\textsuperscript{+}CD4\textsuperscript{−}CD25\textsuperscript{+} cells in response to a variety of stimuli (8, 11), thus raising the possibility that the development and/or suppressor activity of Treg can be regulated. Indeed, recent studies have shown that molecules produced by inflammatory and tumor cells can either inhibit or enhance Treg function (12, 13). As IL-21 and Treg have opposite effects on CD4\textsuperscript{+} T cell activation, we hypothesized that IL-21 could make conventional CD4\textsuperscript{+}CD25\textsuperscript{−} T cells able to escape Treg-mediated suppression.

Materials and Methods

Cell isolation and culture

Human PBMC were isolated from enriched buffy coats of healthy volunteer donors and used to purify CD4\textsuperscript{+} T cells with the CD4 multisort magnetic microbeads kit (Miltenyi Biotech). The remaining CD4\textsuperscript{−} fraction of PBMC was then used to purify CD8\textsuperscript{+} T cells by using CD8 multisort magnetic beads (Miltenyi Biotech). Both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were subsequently fractioned in CD25\textsuperscript{−} and CD25\textsuperscript{+} cells by using CD25 magnetic beads (Miltenyi Biotech). In some experiments, CD3\textsuperscript{−} T cells were purified from enriched buffy coats using the CD3 magnetic beads (Miltenyi Biotech). Cell purity was routinely evaluated by flow cytometry and ranged between 96 and 98%. The study received approval from the local ethical committee.

Responder cells (CD4\textsuperscript{+}CD25\textsuperscript{−} T cells, CD8\textsuperscript{+}CD25\textsuperscript{−} T cells, or CD3\textsuperscript{+} T cells) were cultured in RPMI 1640 containing 10% FBS and standard
supplements (all from Sigma-Aldrich) in 96 U-bottom multwells (5 × 10^4 cells/well/200 μl) in the presence or absence of activating anti-CD3-bound beads (final concentration of 5 × 10^4 beads/well/200 μl) according to the manufacturer’s instructions (Miltenyi Biotec). In parallel, cell cultures were added with graded doses of IL-21 (50–200 ng/ml; BioSource International). For suppression experiments, CD4^+CD25^−, CD8^+CD25^−, or CD3^+ T cells (5 × 10^4 cells/well/200 μl) were cultured as indicated above in the presence of various concentrations of CD4^+CD25^− cells (1.25, 2.5, and 5 × 10^5 cells/well/200 μl). Cocultures were either left unstimulated or stimulated with activating CD3 beads in the presence or absence of IL-21 (50–200 ng/ml), IL-2 (100 ng/ml; R&D Systems), IL-7 (100 ng/ml), or IL-15 (100 ng/ml; both from PeproTech). When suppression experiments were performed using CD3^+ T cells as responder cells, the proliferation of both CD8^+ and CD4^+ T cells was evaluated by staining the cells with anti-CD3 and allophycocyanin (APC). To evaluate whether Treg suppress the growth of responder cells when added late into the culture, CD4^+CD25^− T cells were stimulated with anti-CD3, and CD4^+CD25^− T cells (1:1) were added at the same time or 24 h after the activation of CD4^+CD25^+ T cells.

In parallel experiments, cocultures of CD4^+CD25^+ T cells and CD4^+CD25^− T cells were used as indicated above in the presence of anti-CD3 for 0, 12, 24, and 48 h, followed by the addition of 100 ng/ml IL-21.

To examine whether the pretreatment of Treg with IL-21 altered the suppressive capability of these cells, CD4^+CD25^− T cells (5 × 10^4 cells/well/200 μl) were stimulated with activating CD3 and CD28 beads (both used at a final concentration of 5 × 10^4 beads/well/200 μl; Miltenyi Biotec) to enhance the percentage of Treg in vitro. These cell cultures were or were not added to 100 ng/ml IL-21. After 5 days of culture, an aliquot of cells was used for analysis of CD25 and Foxp3, whereas the remaining cells were extensively washed and then tested for their ability to inhibit alloresponses. For this purpose, Treg (5 × 10^4 cells/well/200 μl) generated either in the presence or absence of IL-21 (10 × 10^4 cells/well/200 μl) were cocultured with allogenic CFSE-labeled CD4^+CD25^− T cells in the presence of activating CD3 beads. Cell proliferation was evaluated at the indicated time points.

CFSE labeling
To track the proliferation of responder cells, CD4^+CD25^−, CD8^+CD25^−, or CD3^+ T cells were incubated in 0.2 μM CFSE (Invitrogen Life Technologies) at 37°C for 30 min, and CD4^+CD25^− T cells were either left unlabelled or labeled with 2 μM PKH26 (Sigma-Aldrich) at room temperature for 3 min and extensively washed before culture. In some experiments, responder cells were labeled with PKH26 and CD4^+CD25^− T cells were labeled with CFSE. CFSE fluorescence was evaluated with the FL1 detector and PKH26 with the FL2 detector. Flow cytometric data were analyzed with the proliferation Wizard module in ModFit LT Macintosh software. The proportion of cells undergoing divisions was determined.

Annexin V (Ann V) staining
To evaluate whether IL-21 affects the survival of CD4^+CD25^− T cells, this cell type was cultured either alone or with CD4^+CD25^− T cells in the presence of anti-CD3 and/or IL-21. In these cocultures, CD4^+CD25^− T cells were labeled with CFSE. After 1, 2, 3, and 5 days of culture, the fraction of Ann V^+ cells was evaluated by flow cytometry using a commercially available kit (Beckman Coulter).

Cell phenotype analysis
Anti-CD4 FITC, anti-CD8 allophycocyanin, anti-CD25 PE (all from Beckman Coulter), anti-CD3 PerCP (BD Biosciences), anti-IL-21R PE (Biolegend), anti-CD4 peripheral blood mononuclear cells (Societa Chimici Italiana), and control isotype Abs were used for analysis of relative Ags at the indicated time points according to the manufacturer’s instructions.

Cytokine assays
At the end of the cell culture, supernatants were collected and analyzed for the content of IFN-γ by ELISA using a commercially available kit (R&D Systems).

Statistics
Two-way ANOVA, followed by pairwise multiple comparison procedures (Student-Newman-Keuls method), were performed. Data are expressed as mean ± SEM.

Results
IL-21 enhances the proliferation of CD4^+CD25^− cells, even in the presence of CD4^+CD25^+ T cells
Since inflammatory stimuli block the ability of Treg to inhibit T cell activation (12, 14), we assessed whether IL-21 could alter Treg-mediated immunosuppression. We first examined the proliferative effect of IL-21 on purified CFSE-labeled CD4^+CD25^− T cells. The addition of IL-21 to CD4^+CD25^− T cells resulted in no change in cell growth (Fig. 1A). In contrast, IL-21 enhanced the proliferation of anti-CD3-stimulated CD4^+CD25^− T cells (Fig. 1, A and B). The IL-21-induced mitogenic effect was dose dependent, with significant proliferation seen at 100 ng/ml IL-21 (p < 0.001; Fig. 1A), a concentration similar to that used by other authors to assess the biological activities of IL-21 in vitro (5).

We have also attempted to examine the effect of IL-21 on purified CD4^+CD25^+ T cells with no CD4^+CD25^- T cells in the culture. However, in the absence of CD4^+CD25^- T cells, >50 and 80% of CD4^+CD25^+ T cells underwent apoptosis by 24 and 72 h of culture, respectively, regardless of whether cells were stimulated with anti-CD3 or anti-CD3 plus IL-21.

Subsequently, we assessed the effect of CD4^+CD25^− T cells on the proliferation of CD4^+CD25^− T cells cultured in the presence of anti-CD3 with or without IL-21. To this end, we used the intracellular fluorescent label CFSE to tag the proliferating CD4^+CD25^− T cells, and therefore to determine the proliferation history of specific cell populations. Proliferation of anti-CD3-activated CFSE-labeled CD4^+CD25^− T cells was inhibited by coculture with CD4^+CD25^+ T cells (1:1; Fig. 1, A and B; p < 0.01). By contrast, the addition of unlabeled CD4^+CD25^− T cells to CFSE-labeled CD4^+CD25^− T cells (1:1) did not alter the proliferation of responder cells (Fig. 1A), thus excluding the possibility that the inhibition of CD4^+CD25^− T cell growth seen in cocultures with CD4^+CD25^+ T cells is simply related to the number of cells per well. The addition of IL-21 to cocultures of CD4^+CD25^− T cells and CD4^+CD25^− T cells restored the proliferation of responder cells. Notably, the percentage of proliferating CD4^+CD25^− T cells in coculture with CD4^+CD25^+ T cells and 200 ng/ml IL-21 was almost similar to that measured in cells cultured with the same dose of IL-21 and no CD4^+CD25^− T cells, indicating that IL-21 completely blocks the CD4^+CD25^− T cell-mediated suppression of CD25^− T cell growth (Fig. 1A). To examine whether the inhibitory effect on responder cells was observed at lower concentrations of CD4^+CD25^− T cells, we conducted cocultures of CFSE-labeled CD4^+CD25^− T cells and CD4^+CD25^− T cells (1:0, 1:0.25, 1:0.5, and 1:1) in the presence or absence of IL-21. As shown in Fig. 1C, CD4^+CD25^− T cells inhibited the growth of responder cells at any concentration, even though their inhibitory effect was significant only at high CD4^+CD25^+; CD4^+CD25^- T cell ratios (i.e., 1:1; p < 0.05). Although this ratio appears to be unphysiologic, it is noteworthy that only 50% of our CD4^+CD25^+ T cells were positive for Foxp3, thus implying that the remaining were indeed activated T cells. Therefore, we used this ratio in the subsequent experiments. Notably, the capacity of IL-21 to counteract the inhibitory effect of CD4^+CD25^− T cells on CD4^+CD25^− T cell growth was seen independently on the concentration of CD4^+CD25^− T cell used.

Consistent with the above data, we also showed that the addition of IL-21 to cocultures of CD4^+CD25^− and CD4^+CD25^+ T cells prevented the inhibition of IFN-γ secretion (Fig. 1D).

Analysis of the kinetics of Treg activity in vitro has revealed that murine CD4^+CD25^− T cells mediate their suppressive activity within the first 12 h of T cell activation, and that responding T cells become refractory to suppression if Treg are added 12 h after
IL-21 enhances the growth of anti-CD3-activated CD4\(^+\)CD25\(^+\) T cells and restores proliferative responses of CD4\(^+\)CD25\(^-\) T cells in coculture with CD4\(^+\)CD25\(^-\) T cells. A, CFSE-labeled CD4\(^+\)CD25\(^+\) T cells (5 \times 10\(^5\) cells/well) were either left unstimulated or stimulated with anti-CD3 in the presence or absence of graded doses of IL-21, with or without the initial addition of unlabelled CD4\(^+\)CD25\(^-\) T cells or CD4\(^+\)CD25\(^+\) T cells (5 \times 10\(^5\) cells/well). After 5 days, the percentage of proliferating cells was evaluated by flow cytometry. Data indicate mean ± SEM of six different experiments (anti-CD3 vs anti-CD3 + IL-21; †, p < 0.05; CD4\(^+\)CD25\(^-\) T cells vs CD4\(^+\)CD25\(^+\) T cells and CD4\(^+\)CD25\(^+\) T cells; *, p < 0.05; **, p < 0.01). B, Representative histograms of CFSE-labeled CD4\(^+\)CD25\(^+\) T cells cultured in the presence or absence of CD4\(^+\)CD25\(^-\) T cells (1:1 ratio) for 5 days with the initial addition of anti-CD3 or anti-CD3 + IL-21 (100 ng/ml). Numbers above lines indicate the percentages of proliferating cells. C, CFSE-labeled CD4\(^+\)CD25\(^+\) T cells (5 \times 10\(^5\) cells/well) were stimulated with anti-CD3 in the presence or absence of 100 ng/ml IL-21, with or without the initial addition of various concentrations of unlabelled CD4\(^+\)CD25\(^-\) T cells (CD4\(^+\)CD25\(^-\) T cells/CD4\(^+\)CD25\(^+\) T cells ratio, 1:0; 1:0.25; 1:0.5; 1:1). After 5 days, the percentage of proliferating cells was evaluated by flow cytometry. Data indicate mean ± SEM of four different experiments. (†, p < 0.05; CD4\(^+\)CD25\(^-\) T cells vs CD4\(^+\)CD25\(^+\) T cells; *, p < 0.05; **, p < 0.01). D, CD4\(^+\)CD25\(^-\) T cells (2 \times 10\(^5\) cells/well) were stimulated with anti-CD3 in the presence or absence of IL-21 (100 ng/ml), with or without the initial addition of CD4\(^+\)CD25\(^+\) (2 \times 10\(^5\) cells/well). After 5 days, the cell-free culture supernatants were collected and analyzed for the content of IFN-γ by ELISA. Data are expressed as picograms per milliliter and indicate the mean ± SD of three separate experiments. CD4\(^+\)CD25\(^-\) T cells significantly inhibit the synthesis of IFN-γ by CD4\(^+\)CD25\(^+\) T cells cultured in the presence of anti-CD3 alone, although no inhibitory effect is seen when cocultures were added with anti-CD3 and IL-21 (†, p < 0.05; **, p < 0.01).

**FIGURE 1.** A, CD4\(^+\)CD25\(^+\) T cells do not suppress the proliferation of activated CD4\(^+\)CD25\(^-\) T cells when added 24 h after the activation of responders. CD4\(^+\)CD25\(^+\) T cells were added to cultures of CFSE-labeled CD4\(^+\)CD25\(^-\) T cells 0 or 24 h after the activation with anti-CD3. Five days later, the cells were harvested and analyzed by flow cytometry. Data indicate mean ± SEM of four different experiments (CD4\(^+\)CD25\(^-\) T cells vs CD4\(^+\)CD25\(^-\) T cells and CD4\(^+\)CD25\(^+\) T cells at day 0 (**, p < 0.01). B, CFSE-labeled CD4\(^+\)CD25\(^+\) T cells were cocultured with or without CD4\(^+\)CD25\(^-\) T cells in the presence of anti-CD3 for 0, 12, 24, and 48 h, followed by the addition of IL-21 (100 ng/ml). The percentage of proliferating cells was evaluated at day 5 by flow cytometry. Data indicate mean ± SEM of three different experiments (CD4\(^+\)CD25\(^+\) T cells and CD4\(^+\)CD25\(^-\) T cells vs CD4\(^+\)CD25\(^+\) T cells and CD4\(^+\)CD25\(^+\) T cells at 0; 12; 24; and 48 h).

IL-21 neither diminishes the percentage of Foxp3\(^+\) T cells nor directly inhibits the suppressive properties of Treg

The above results suggest different mechanisms by which IL-21 could exert its effects in the cocultures of responder and Treg cells. First, IL-21 could promote Treg apoptosis. Second, IL-21 could directly antagonize the Treg activity. Third, IL-21 could make responder cells resistant to Treg-mediated suppression. Finally, IL-21 could enable responder cells to resist the antiproliferative activity of Treg, leaving other functions of responder cells compromised. To begin to address this issue, we first assessed which IL-21R expression ranged from 0.5 to 8% in freshly isolated Treg. To this end, IL-21 was added 0, 12, 24, and 48 h after the activation of CFSE-labeled CD4\(^+\)CD25\(^+\) T cells. By contrast, IL-21 did partially, but not significantly, block the CD4\(^+\)CD25\(^+\) T cell-mediated suppression of CD4\(^+\)CD25\(^-\) T cells when added 24 h after the activation of CFSE-labeled CD4\(^+\)CD25\(^+\) T cells. As expected, CD4\(^+\)CD25\(^+\) T cells significantly inhibited the proliferation of anti-CD3-stimulated CD4\(^+\)CD25\(^+\) T cells when these two cell types were cocultured simultaneously. In contrast, no inhibitory effect was seen when CD4\(^+\)CD25\(^+\) T cells were added 24 h after the activation of responder cells (Fig. 2A). Subsequently, we evaluated whether IL-21 exerts its effects in already established cocultures of responders and Treg. To this end, IL-21 was added 0, 12, 24, and 48 h after the anti-CD3 activation of cocultures of CFSE-labeled CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\) T cells. Data in Fig. 2B show that IL-21 was able to significantly counteract the suppressive effect of CD4\(^+\)CD25\(^+\) T cells on CD4\(^+\)CD25\(^-\) T cell proliferation when added at the same time or 12 or 24 h after the activation of the cocultures. By contrast, IL-21 did partially, but not significantly, block the CD4\(^+\)CD25\(^+\) T cell-mediated suppression of CD4\(^+\)CD25\(^-\) T cell growth if it was added 48 h after the activation of CD4\(^+\)CD25\(^-\) T cells (Fig. 2B).

**FIGURE 2.** A, CD4\(^+\)CD25\(^+\) T cells do not suppress the proliferation of activated CD4\(^+\)CD25\(^-\) T cells when added 24 h after the activation of responders. CD4\(^+\)CD25\(^+\) T cells were added to cultures of CFSE-labeled CD4\(^+\)CD25\(^-\) T cells 0 or 24 h after the activation with anti-CD3. Five days later, the cells were harvested and analyzed by flow cytometry. Data indicate mean ± SEM of four different experiments (CD4\(^+\)CD25\(^-\) T cells vs CD4\(^+\)CD25\(^-\) T cells and CD4\(^+\)CD25\(^+\) T cells at day 0 (**, p < 0.01). B, CFSE-labeled CD4\(^+\)CD25\(^+\) T cells were cocultured with or without CD4\(^+\)CD25\(^-\) T cells in the presence of anti-CD3 for 0, 12, 24, and 48 h, followed by the addition of IL-21 (100 ng/ml). The percentage of proliferating cells was evaluated at day 5 by flow cytometry. Data indicate mean ± SEM of three different experiments (CD4\(^+\)CD25\(^+\) T cells and CD4\(^+\)CD25\(^-\) T cells vs CD4\(^+\)CD25\(^+\) T cells and CD4\(^+\)CD25\(^+\) T cells at IL-21; *, p < 0.05; **, p < 0.01).
CD4^+CD25^+ T cells. However, the simultaneous evaluation of Foxp3 and IL-21R in these cells revealed that the receptor was undetectable in freshly isolated Treg (Fig. 3A). Moreover, IL-21R was barely detectable in CD4^+CD25^- T cells (Fig. 3A). We also examined whether IL-21R was differentially modulated in cocultures of CD4^+CD25^+ and CD4^+CD25^- T cells. As indicated above, the two cell populations were clearly distinguishable because CD4^+CD25^- T cells were labeled with CFSE. In anti-CD3-stimulated cocultures, 45 ± 10% of CD4^+CD25^- and 60 ± 10% of CD4^+CD25^+ T cells were positive for IL-21R. The addition of IL-21 to the cocultures did not increase the expression of IL-21R in CD4^+CD25^- T cells (60 ± 5%), although it increased expression in CD4^+CD25^+ T cells (63 ± 7%, p = 0.034). Moreover, CFSE dilutions revealed that proliferating CD4^+CD25^- T cells in culture with IL-21 expressed the IL-21R (Fig. 3B).

Subsequently, we examined whether IL-21 affected the survival and/or proliferation of CD4^+CD25^- T cells. To this end, we performed a time-course analysis of Annex V in cocultures of CFSE-labeled responders and CD4^+CD25^+ T cells stimulated with anti-CD3 in the presence or absence of IL-21. As shown in Fig. 4A, the addition of IL-21 to the anti-CD3-stimulated cocultures did not affect the percentage of Annex V^+ cells. This was evident for both CFSE^+ and CFSE^- cells at each time point. Similarly, IL-21 did not affect the percentage of PKH26^+CD4^+CD25^- T cell growth (Fig. 4B). To confirm further that the IL-21-mediated inhibitory effect on CD4^+CD25^- T cell suppression was not secondary to a reduction of Treg, we assessed the percentage of Foxp3^+ cells in cocultures of CD4^+CD25^- and CD4^+CD25^+ T cells after 5 days of stimulation with IL-21. In these experiments, CD4^+CD25^- T cells were labeled with CFSE. Data in Fig. 5A show that the percentage of Foxp3^+, CFSE-labeled cells was not changed by IL-21 (34 vs 35%). Similarly, Foxp3 was only marginally increased by IL-21 in CFSE-negative cells (3 vs 1%). Because Foxp3^+ cells can be generated in vitro from CD4^+CD25^- T cells (16, 17), we also evaluated whether IL-21 regulated the induction of Foxp3^+ cells in cultures of CD4^+CD25^- T cells stimulated with anti-CD3/CD28. The percentage of Foxp3-bearing cells was 0.7 ± 0.26% in unstimulated cultures and was slightly, but not significantly, increased by anti-CD3 (3.1 ± 1%) or anti-CD3 and IL-21 (6.5 ± 1.8%; Fig. 5B). In line with previous studies (16), activation of CD4^+CD25^- T cells with anti-CD3 and CD28 significantly enhanced the percentage of Foxp3^+ cells (37.5 ± 4.3, p < 0.001), but no further increase was seen when cultures were also added with IL-21 (Fig. 5B).

To assess whether IL-21 suppresses Treg inhibitory functions, CD4^+CD25^- T cells were stimulated with anti-CD3 and anti-CD28 in the presence or absence of IL-21. After 5 days, cells were harvested and an aliquot was immediately analyzed for CD25 and Foxp3 by flow cytometry. CD25 expression ranged from 92 to 100% while Foxp3-positive cells were 40–55%, and these percentages were not changed by IL-21 (93–100% for CD25 and 41–53% for Foxp3). The remaining cells were then tested for their suppressive functions in cocultures with anti-CD3-stimulated CFSE-labeled allogeneic CD4^+CD25^- T cells. As controls, cocultures of CD4^+CD25^- T cells and allogeneic CD4^+CD25^- T cells or freshly isolated allogeneic CD4^+CD25^- T cells (1:1 ratio) were also conducted. Data in Fig. 5C show that freshly isolated CD4^+CD25^- T cells significantly suppressed the anti-CD3-induced proliferation of CD4^+CD25^- T cells (p < 0.05). Similarly, the proliferation of anti-CD3-stimulated CD4^+CD25^- T cells was inhibited by CD4^+CD25^- T cells (Treg) induced in vitro by activation of CD4^+CD25^- T cells with anti-CD3/CD28. This inhibitory effect was seen regardless of whether these cells were induced in the presence or absence of IL-21 (Fig. 5C; p < 0.05).

IL-21 enhances CD8^+ T cell growth but does not counteract the Treg-mediated suppression of CD8^+ T cell proliferation

Because it is known that Treg abrogate the proliferation of CD8^+ T cells (18), we then examined whether IL-21 also counters CD4^+CD25^- T cell-mediated inhibition of CD8^+ T cell growth. Initially, we evaluated the expression of IL-21R on freshly isolated
CD8⁺ T cells. IL-21R was, however, barely detectable on these cells. We also examined whether IL-21R expression on CD8⁺ CD25⁺ T cells was modified in cocultures with CD4⁺ CD25⁺ T cells by anti-CD3 and/or IL-21. To distinguish the two cell types, CD8⁺ CD25⁺ T cells were labeled with CFSE. In anti-CD3-stimulated cocultures, 41 ± 11% of CD8⁺ T cells were positive for IL-21R, and such an expression was not significantly affected by IL-21 (39 ± 9%; Fig. 6A). Subsequently, we evaluated whether IL-21 blocked the CD4⁺ CD25⁺ T cell-mediated suppression of CD8⁺ CD25⁺ T cell growth. For this purpose, freshly isolated, CFSE-labeled, CD8⁺ CD25⁺ T cells were cultured with or without CD4⁺ CD25⁺ T cells (1:1 final ratio), and the cultures were then stimulated with anti-CD3 in the presence or absence of IL-21 for 5 days. As observed with CD4⁺ CD25⁺ T cells, the addition of IL-21 to cocultures of CD8⁺ CD25⁺ T cells did not enhance the cell growth (Fig. 6B). However, IL-21 significantly augmented the proliferation of anti-CD3-activated CD8⁺ CD25⁺ T cells at doses of 100 and 200 ng/ml (Fig. 6B; p < 0.05). The addition of CD4⁺ CD25⁺ T cells to anti-CD3-activated CD8⁺ CD25⁺ T cells resulted in a marked suppression of CD8⁺ T cell growth regardless of
were harvested and the percentage of proliferating CD8$^+$ T cells was analyzed. Data indicate mean ± SEM of four different experiments ($p$ values, differences of proliferation of CD4$^+$ CD25$^+$ T cells cocultured with CD4$^+$ CD25$^+$ T cells in the presence or absence of cytokines: †††, $p < 0.001$; ††, $p < 0.01$). B, Cells were cultured as indicated above, but at the end of culture, the percentage of proliferating CD4$^+$ CD25$^+$ T cells was analyzed. Data indicate mean ± SEM of four different experiments ($p$ values, differences of proliferation of CD4$^+$ CD25$^+$ T cells cocultured with CD4$^+$ CD25$^+$ T cells in the presence or absence of cytokines: †††, $p < 0.001$). C, Representative dot plots of cocultures of CFSE-labeled CD4$^+$ CD25$^+$ T cells (5 x 10$^4$ cells/well) and PKH26-labeled CD4$^+$ CD25$^+$ T cells (5 x 10$^4$ cells/well) in the presence of anti-CD3, anti-CD3 + IL-21 (100 ng/ml), anti-CD3 + IL-2 (100 ng/ml), anti-CD3 + IL-7 (100 ng/ml), or anti-CD3 + IL-15 (100 ng/ml). Numbers above arrow indicate the percentage of cells in the designated gates. One of three representative experiments is shown. D, CFSE-labeled CD8$^+$ CD25$^+$ T cells (5 x 10$^4$ cells/well) were cultured with or without CD4$^+$ CD25$^+$ T cells in the presence of or absence of PHK26-labeled CD4$^+$ CD25$^+$ T cells (5 x 10$^4$ cells/well) in the presence of anti-CD3, anti-CD3 + IL-21 (100 ng/ml), anti-CD3 + IL-2 (100 ng/ml), or anti-CD3 + IL-7 (100 ng/ml). After 5 days, cells were harvested and the percentage of proliferating CD8$^+$ CD25$^+$ T cells was analyzed by flow cytometry. Data indicate mean ± SEM of four different experiments ($p$ values, differences of proliferation of CD8$^+$ CD25$^+$ T cells cocultured with CD4$^+$ CD25$^+$ T cells in the presence or absence of cytokines: †††, $p < 0.001$).

whether cocultures were performed in the presence or absence of IL-21 (Fig. 6, B and C; $p < 0.05$).

To further confirm these findings, we examined whether IL-21 differently regulates the proliferation of CD8$^+$ and CD4$^+$ T cells when both of these cell types are cocultured with CD4$^+$CD25$^+$ T cells. To this end, unfractionated, CFSE-labeled, CD3$^+$ T cells were cultured with or without CD4$^+$ CD25$^+$ T cells and stimulated with anti-CD3 in the presence or absence of IL-21. After 5 days of culture, cells were harvested and incubated with a CD8 Ab to discriminate the CD8 and CD4 T cell responses. As shown in Fig. 6D, activation of CD3$^+$ T cells with anti-CD3 significantly increased the percentage of both proliferating CD8$^+$ and CD8$^+$ T cells (11 ± 3 and 16 ± 3.9%, respectively) in comparison to unstimulated cells (2 ± 0.4 and 1.8 ± 0.6%, respectively; $p < 0.03$). Moreover, the addition of IL-21 to such cultures significantly enhanced the growth of both CD8$^+$ and CD8$^+$ T cells (27 ± 4 and 26 ± 3.9%, $p < 0.05$). When anti-CD3-activated CD3$^+$ T cells were cocultured in the presence of CD4$^+$ CD25$^+$ T cells, a marked inhibition in the percentage of proliferating CD8$^+$ and CD8$^+$ T cells was seen (1.8 ± 0.5 and 7 ± 1%, respectively; $p < 0.01$). Notably, the addition of IL-21 to these cocultures significantly blocked the CD4$^+$ CD25$^+$ T cell-mediated suppression of CD8$^+$ (16 ± 5%; $p = 0.04$), but not CD8$^+$ T cell growth (9 ± 5%; Fig. 6C). Overall, these results suggest that IL-21 does not inhibit the intrinsic regulatory capacity of CD4$^+$ CD25$^+$ T cells, but rather makes responding CD4$^+$ T cells resistant to suppression.

**FIGURE 7.** A, CFSE-labeled CD4$^+$ CD25$^+$ T cells (5 x 10$^4$ cells/well) were cultured in the presence or absence of PKH26-labeled CD4$^+$ CD25$^+$ T cells (5 x 10$^4$ cells/well) with anti-CD3, anti-CD3 + IL-21 (100 ng/ml), anti-CD3 + IL-2 (100 ng/ml), anti-CD3 + IL-7 (100 ng/ml), or anti-CD3 + IL-15 (100 ng/ml). After 5 days, cells were harvested and the percentage of proliferating CD4$^+$ CD25$^+$ T cells was analyzed by flow cytometry. Data indicate mean ± SEM of four different experiments ($p$ values, differences of proliferation of CD8$^+$ CD25$^+$ T cells cocultured with CD4$^+$ CD25$^+$ T cells in the presence or absence of cytokines: †††, $p < 0.001$; ††, $p < 0.01$). B, Cells were cultured as indicated above, but at the end of culture, the percentage of proliferating CD4$^+$ CD25$^+$ T cells was analyzed. Data indicate mean ± SEM of four different experiments ($p$ values, differences of proliferation of CD4$^+$ CD25$^+$ T cells cocultured with CD4$^+$ CD25$^+$ T cells in the presence or absence of cytokines: †††, $p < 0.001$). C, Representative dot plots of cocultures of CFSE-labeled CD4$^+$ CD25$^+$ T cells (5 x 10$^4$ cells/well) and PKH26-labeled CD4$^+$ CD25$^+$ T cells (5 x 10$^4$ cells/well) in the presence of anti-CD3, anti-CD3 + IL-21 (100 ng/ml), or anti-CD3 + IL-2 (100 ng/ml). Numbers above arrow indicate the percentage of cells in the designated gates. One of three representative experiments is shown. D, CFSE-labeled CD8$^+$ CD25$^+$ T cells (5 x 10$^4$ cells/well) were cultured with or without CD4$^+$ CD25$^+$ T cells in the presence or absence of PKH26-labeled CD4$^+$ CD25$^+$ T cells (5 x 10$^4$ cells/well) in the presence of anti-CD3, anti-CD3 + IL-21 (100 ng/ml), anti-CD3 + IL-2 (100 ng/ml), or anti-CD3 + IL-7 (100 ng/ml). After 5 days, cells were harvested and the percentage of proliferating CD8$^+$ CD25$^+$ T cells was analyzed by flow cytometry. Data indicate mean ± SEM of four different experiments ($p$ values, differences of proliferation of CD8$^+$ CD25$^+$ T cells cocultured with CD4$^+$ CD25$^+$ T cells in the presence or absence of cytokines: †††, $p < 0.001$).

IL-21, IL-2, IL-7, and IL-15 differ in their ability to overcome the CD4$^+$ CD25$^+$ T cell-mediated immunosuppression

Studies in other systems have shown that the block of the Treg-induced immunosuppression by activating molecules, such as IL-2, IL-7, and IL-15, associates with the ability of these stimuli to reverse the anergic phenotype of suppressors (19, 20). We therefore compared the effect of IL-21 and the other common γ-chain-related cytokines on the proliferation of both CD4$^+$ CD25$^+$ and CD4$^+$ CD25$^+$ T cells. Freshly isolated CD4$^+$ CD25$^+$ and CD4$^+$ CD25$^+$ T cells were labeled with CFSE and PKH26, respectively, and then cocultured in medium containing anti-CD3 in the presence or absence of IL-21 (100 ng/ml), IL-2 (100 ng/ml), IL-7 (100 ng/ml), or IL-15 (100 ng/ml) for 5 days. Data in Fig. 7A show that all of these cytokines were effective in counteracting the CD4$^+$ CD25$^+$ T cell-mediated suppression of anti-CD3-activated CD4$^+$ CD25$^+$ T cells ($p < 0.01$), even though the proliferative effect of IL-2, IL-7, and IL-15 on CD4$^+$ CD25$^+$ T cells was more marked than that of IL-21. By contrast, in the same cell cultures, IL-2, IL-7, and IL-15, but not IL-21 significantly enhanced the growth of CD4$^+$ CD25$^+$ T cells (Fig. 7B; $p < 0.001$). The representative experiment depicted in Fig. 7C shows that IL-2 was able to accelerate the division of both CD4$^+$ CD25$^+$ and CD4$^+$ CD25$^+$ T cells, whereas IL-21 enhanced the proliferation of CD4$^+$ CD25$^+$ T cells but not the growth of CD4$^+$ CD25$^+$ T cells (2.5 vs 1.4% in CD3-stimulated cells). Finally, we examined the ability of IL-21, IL-2, IL-7, and IL-15 to counteract the CD4$^+$ CD25$^+$ T cell-mediated suppression of anti-CD3-activated CD8$^+$ CD25$^+$ T cells. As indicated above, IL-21 did not overcome the CD4$^+$ CD25$^+$ T cell-mediated block of CD8$^+$ CD25$^+$ T cell growth. By contrast, IL-2, IL-7, and IL-15 were effective in reversing the CD4$^+$ CD25$^+$ T cell-mediated suppression of activated CD8$^+$ CD25$^+$ T cell proliferation (Fig. 7D).

**Discussion**

This study was undertaken to examine whether the recently described T cell-derived cytokine IL-21 is able to overcome Treg-mediated immunosuppression. Indeed, recent studies have shown that high IL-21 levels occur in inflammatory diseases that have been associated with a defective capacity of counterregulatory mechanisms to dampen T cell-mediated inflammatory diseases (3–5).
Collectively, our data indicate that IL-21 counteracts the regulatory effects of CD4+CD25+ T cells by providing human CD4+CD25+ T cells signals that raise their threshold for suppression by CD4+CD25+ T cells. While this manuscript was in preparation, Comes et al. (7) showed that IL-21 partially reverts the immunosuppressive activity of CD4+CD25+ Treg isolated from tumor-draining lymph nodes, and that such cells fail to proliferate in response to IL-21 and alloantigen or anti-CD3 stimulation.

The ability of IL-21 to interfere with the CD4+CD25+ T cell-mediated suppression of CD4+CD25+ T cell growth was also seen when IL-21 was added 12 and 24 h later in already established cocultures of responder cells and Treg. This finding and the demonstration that Treg are efficient in inhibiting responses only up to 12 h after activation (15) suggest the possibility that IL-21 exerts directly its effects on the responding T cells and not on the Treg. This is also substantiated by the fact that the IL-21-induced block of CD4+CD25+ T cell suppression does not occur in CD8+ T cells. Our data, therefore, conform and expand on previous studies showing that other signals, such as those provided by IL-2, IL-7, IL-15, and CD28 engagement, revert the Treg-mediated suppression of CD4+CD25+ but not CD8+ T cells (20, 21). The reason why IL-21 does not protect CD8+ T cells from the suppressive action of CD4+CD25+ T cells remains unknown. It is unlikely that this simply relies on a different responsiveness of CD4+ and CD8+ T cells to IL-21, because our data clearly indicate that IL-21 enhances the proliferation of both cell types. A possibility is that IL-21 selectively induces on CD4+ T cells specific molecules that may, in turn, antagonize the activity of CD4+CD25+ T cells. A potential candidate could be the glucocorticoid-induced TNFR family-related receptor ligand, as it was recently shown that such a protein can be also expressed by T cells and provide signals that make CD4+CD25+ T cells resistant to CD25+ T cell-mediated suppression (22). Studies are now in progress to address this issue.

The results presented herein also indicate that IL-21 neither enhances the percentage of Foxp3+ cells nor augments the proliferation of CD4+CD25+ T cells in cocultures with CD4+CD25+ T cells. In contrast, IL-2, IL-7, and IL-15 were effective in increasing CD4+CD25+ T cell growth in accordance with previously published data (18–21). In this context, it is also noteworthy that in the absence of CD4+CD25+ T cells, that are known to be an important source of IL-2, almost all of the CD4+CD25+ T cells undergo apoptosis by 24 h of culture with anti-CD3+ IL-21, clearly indicating that IL-21 is not a survival factor for Treg. These data, along with the demonstration that, in other cell systems, IL-2 and IL-4, but not IL-13, are growth factors for Treg (11, 23), suggest that signaling through the common γ-chain that is shared by all of these cytokines is not sufficient for maintaining/prolonging CD4+CD25+ T cell survival.

In line with previously published studies, we demonstrate that IL-21 is an important growth factor for both CD4+CD25+ and CD8+CD25+ T cells (1), thus emphasizing the possible contribution of IL-21 to immune-mediated diseases. Indeed, we recently showed that IL-21 is produced in excess in the inflamed gut of patients with inflammatory bowel diseases, such as Crohn’s disease and ulcerative colitis, and that IL-21 helps sustain the ongoing Th1 cell response in the gut of patients with Crohn’s disease (3). Moreover, enhanced expression of IL-21 and IL-21R has been described in patients with systemic sclerosis (4), and up-regulation of IL-21R also occurs in synovial macrophages and fibroblasts of patients with rheumatoid arthritis (24). Studies in experimental models of autoimmune diseases have also shown that administration of IL-21 into mice enhances the recruitment of leukocytes into inflamed tissues and increases the severity of the inflammation (25). Finally, increased production of IL-21 has been described in NOD mice that are known to develop diabetes due to β cell destruction by activated T cells (26). Overall, these findings suggest that IL-21 may activate the autoreactive T cell repertoire and trigger the effector phase of immune-mediated diseases. These effects could be also facilitated by the ability of IL-21 to counteract the suppressor function of Treg.

Since their discovery, Treg have been found to play important roles in the control of immune responses. Although they were initially described to modulate self-tolerance and to protect against autoimmunity, more recent studies have suggested that Treg may also be implicated in suppressing immune responses in infective and neoplastic diseases (7, 10). Concomitantly, several authors have shown that Treg activity can be differently modulated by inflammatory molecules, bacterial products, and tumor-derived molecules, thus raising the possibility that we can either enhance or inhibit Treg function for therapeutic purposes. In this context, our data suggest that manipulation of IL-21 activity may be a promising way to differently regulate the effect of Treg on immune cells. Therefore, in immunoinflammatory disorders in which high IL-21 associates with defective Treg function, blocking IL-21 may not only decrease bystander T cell activation, but also reconstitute the suppressor function of Treg, thus leading to the resolution of ongoing inflammatory processes. In contrast, in circumstances in which the Treg response could be detrimental rather than protective for the host, such as during viral infections and malignancies (27), administration of IL-21 could antagonize Treg, thereby contributing to relieve the virus- or tumor-induced Treg-mediated immunosuppression.

Disclosures

The authors have no financial conflict of interest.

References


