Introduction

T regulatory cells (Tregs) are CD4^+CD25^High T cells identified by their expression of forkhead transcription factor (Foxp3).\(^1\) Foxp3 is responsible for the acquisition of immunosuppressive function of Tregs and for their anergy. Tregs exhibit immune-regulatory properties and their dysfunction has been implicated in the development of allergic and autoimmune diseases.\(^2\) The increased numbers of Tregs are observed in several malignancies and have been implicated in additional mechanisms reducing immunity, which may contribute to poor prognosis and responses to chemotherapy.\(^3\)\(^4\) The number and inhibitory functions of the Tregs can be decreased by chemotherapeutic agents such as fludarabine therapy in chronic lymphocytic leukemia (CLL).\(^5\) Tregs can develop both in the thymus and periphery. Tregs that develop in the thymus are called natural Tregs (nTregs) and those that are actively generated in the periphery through the conversion of CD4^+CD25^- T cells into Foxp3^+ cells are known as induced Tregs.\(^6\) Although increased Tregs is a characteristic feature in many tumors, the molecular and cellular basis for their increase and maintenance is not clearly understood.\(^3\)

Interleukin (IL)-2 is a member of the common gamma chain family of cytokines with potent T cell growth promoting activity that has been used clinically to enhance T cell immunity in patients with acquired immune deficiency syndrome or cancer. Interestingly, disruption of the IL-2 pathway results in lymphoid hyperplasia and autoimmunity rather than immune deficiency, indicating that the major physiological function of IL-2 is to limit rather than enhance T cell responses. This is mediated by the critical role of IL-2 in the development and peripheral expansion of CD4^+CD25^+ regulatory T cells. The role of common gamma chain containing cytokines such as IL-2 and IL-21 on...
Treg biology is diverse. Several studies have suggested that IL-2 has a non-redundant function in the generation of Treg in the thymus. In its absence, Tregs failed to survive and expand in the periphery, and so IL-2 signaling in these cells appears to be critical for their homeostasis. IL-21, a novel common gamma chain family member, is also a Type I cytokine secreted by activated T cells. IL-21 causes a broad range of effects, including enhancement of the proliferation of T cells, differentiation of B cells into plasma cells and augmentation of the cytolytic function of natural killer cells. It also plays a role in the development of autoimmunity and exhibits anti-tumor activity. We and others have shown direct cytotoxic effect of IL-21 on a subpopulation of primary B cells from CLL patients through activation of STAT1 signaling events and upregulation of the BH3-only containing, pro-apoptotic protein BIM. Although IL-2 and IL-21 share a common gamma chain receptor and its downstream signaling pathways, they exhibit differential effects on diverse immune cells. Thus, in addition to its activities in normal lymphoid cells, IL-21 is an in vitro growth factor for myeloma and acute-T cell leukemia cells; however, it induces apoptosis of CLL B cells.

In the present study, we demonstrate that CD4⁺CD25⁺ Treg cells from CLL patients express the IL-21 receptor. Interestingly, in contrast to IL-2, we report that IL-21 does not expand the Treg cell population or increase the expression of Foxp3 in CD4⁺CD25Intermediate T cells derived from whole blood of CLL patients. In contrast to their differential effects on Treg expansion, IL-2 and IL-21 mediate redundant roles in CD4⁺CD25High Treg mediated suppression of natural killer cell (NK) mediated antibody-dependent cellular cytotoxicity (ADCC). These observations suggest that IL-21 is not an essential cytokine to Treg cell expansion, but may have a redundant role in Treg cell function. Given the infusion related toxicities and pro-survival effect of IL-2 in CLL, these studies provide a rationale to explore IL-21 as an alternate gamma chain cytokine in CLL therapy.

**Results**

CD4⁺CD25⁺ regulatory T cells from CLL patients express the IL-21 receptor. We and others have recently demonstrated the direct cytotoxic effect of IL-21 on CD19⁺ primary B cells from CLL patients. While common gamma chain cytokines such as IL-2 and IL-21 have been shown to exert diverse effects on multiple cell types including B cells, T cells and NK cells, little is known of their effect on CD4⁺CD25Intermediate Tregs from CLL patients. As the IL-21 receptor expression has been observed in B cells and CD4⁺ T cells, we first examined whether the CD4⁺CD25⁻ Treg cells isolated from CLL patients express IL-21 receptor. Of the six CLL patient samples examined, the IL-21 receptor was observed in five patients. Interestingly, differential levels of IL-21 receptor expression were observed in CD4⁺CD25Intermediate and CD4⁺CD25Dim⁻ populations. Thus, majority of CD4⁺CD25Intermediate regulatory T cells (68% to 95%) expressed high levels of IL21 receptor (MFI > 400; ranging from 400–900), while fewer CD4⁺CD25Intermediate (13–29%) and CD4⁺CD25Dim⁻ (1–5%) T cells expressed high levels of IL-21 receptor (Fig. 1).

IL-2 but not IL-21 causes expansion of the CD4⁺CD25⁺ T cells. Several studies have revealed an essential role for IL-2...
Figure 2. IL-2 but not IL-21 induces expansion of regulatory T cells. Whole blood samples from four independent CLL patients were treated with IL-2 or IL-21 (10 ng/ml) for 48 Hours. The cells were analyzed by tricolor staining using anti-CD4 (APC), anti-CD25 (FITC) and anti-Foxp3 (PE) antibodies. The changes in (A) CD4^+CD25^{high}Foxp3^+ cells (B) Right Panel shows CD4 and CD25 staining profiles with the boxed regions indicating CD25^{high}, CD25^{intermediate}, CD25^{dim/low} Cells that are further analyzed for Foxp3 expression. The histogram on the left panel shows the Foxp3 expression in CD4^+CD25^{high}, cells in untreated, IL-2 (10 ng/ml) or IL-21 (10 ng/ml) treated PBMC. (C and D) CD4^+CD25^{intermediate}Foxp3^+ (E and F) CD4^+CD25^{dim/low} Foxp3^+ normalized to media treated controls is shown. IL-2 and IL-21 effects when compared independently to untreated control, the expansion of CD4^+CD25^{high} cells was significant only with IL-2 (Using Holms procedure estimated difference 1.39, CI 0.63, 2.14, p = 0.0057) in contrast to IL-21 (estimated difference 0.75, CI -0.01, 1.15 and p = 0.1048).
in the survival, expansion and homeostasis of Treg cells. IL-21 is a member of the common gamma chain receptor family of cytokines that shares structural and signaling features through the JAK/STAT pathway. One of the impediments identified in clinical use of IL-2 was ascribed to expansion of Treg populations. In order to determine if IL-21 modulates Treg homeostasis in CLL patients, CLL patient derived whole blood was treated with varying concentrations of IL-2 or IL-21 for 48 hours. The PBMCs were then isolated and examined by three color flow cytometry for CD4+CD25+ T cells expressing intracellular Foxp3. Pretreatment of whole blood with concentrations of IL-2 or IL-21 with as low as (10 ng/ml) resulted in expansion of CD4+CD25(high)Foxp3+ cells selectively with IL-2, but not with IL-21 (Fig. 2A and B). Even increasing the concentration of IL-21 to ten fold failed to result in increased CD4+CD25(high)Foxp3+ cells (data not shown).

To investigate if IL-2 and IL-21 differentially modulated Foxp3 expression in CD4+CD25(Intermediate) and CD4+CD25(Dim/-) T cell populations, we analyzed the expression of Foxp3 in these populations by multi-color flow cytometry. IL-2 had moderate effect on the increase in Foxp3 expressing cells in the CD4+CD25(intermediate) cohort (Fig. 2C and D). In the CD4+CD25(Dim/-) T cells (Fig. 2E and F) IL-2 increased the Foxp3 expressing cells by two fold at both the low and high concentrations. In contrast, IL-21 failed to increase the number of CD4+CD25(Intermediate)Foxp3+ or CD4+CD25(Dim/-) Foxp3+ T cells. When the IL-2 and IL-21 effects were compared independently to the untreated controls, the expansion of CD4+CD25(high) cells was significant only with IL-2, (estimated difference 1.39, CI 0.63, 2.14, p = 0.0057), but not with IL-21 treatment (estimated difference 0.75, CI -0.01, 1.15 and p = 0.1048).

IL-2 and IL-21 mediate redundant roles in CD4+CD25(high) Treg mediated suppression of natural killer cell mediated antibody dependent cellular cytotoxicity. Several studies have demonstrated the ability of Tregs to suppress direct killing by NK cells. However, no evidence has been linked to suppressive activity of NK mediated ADCC by Tregs. Given the importance of ADCC mediated by therapeutic antibodies such as IL-2 and IL-21 on B, T and NK cells, we examined the effects IL-2 and IL-21 on regulatory T cell homeostasis and NK mediated ADCC function. We observed that regulatory T cells from majority of CLL patients variably express the IL-21 receptor. In contrast to IL2, IL-21 failed to induce the expansion of regulatory T cells as evidenced by lack of increase in CD4+CD25(Foxp3)T cells with IL-21. In contrast to their differential effect on expansion of the CD4+CD25(Foxp3)T cells, IL-2 and IL-21 exhibited a redundant role in suppression of NK cell mediated ADCC function.

The differential effects of IL-2 and IL-21 is an interesting phenomenon that has been observed previously in NOD mice, wherein CD8+ autoreactive T cells are expanded with high serum IL-21 levels while the CD4+CD25+ regulatory T cells are decreased in these mice, suggesting that the IL-21, but not IL-2, is an essential cytokine for Treg cells. At higher concentration of IL-21 we have observed expansion of Tregs in some patients, which is probably related to the co-culturing of the regulatory cells with other T cells and B cells in whole blood wherein IL-21 could have activated the CD8+CD25−T cells to secrete IL-2, resulting in expansion of Treg cells in some patient samples. The novel method of studying the effects of IL-2 and IL-21 in an in vitro setting using whole blood, thus simulating physiological conditions where there is complex interaction of immune cells and CLL cells, is highly relevant and clinically applicable. In this context, we speculate that IL-21 is likely to mediate limited effects on Treg cell growth and expansion in CLL patients in vivo. Further, in line with the other studies, IL-21, in contrast to IL-2, did not expand the CD4+CD25(intermediate) population or enhance Foxp3 expression in the CD4+CD25(Dim/-) T cells, suggesting that the IL-21 is not critical for the homeostasis of Tregs, and thus may not result in the increased inducible Treg cells in periphery observed with IL-2.

Treg cells suppress the proliferation of cytotoxic T cells and inhibit the secretion of interferon gamma by NK cells. However the role played by Tregs in inhibition of NK cell mediated ADCC has not been previously described. The studies described here demonstrate that Tregs can actively inhibit the ADCC function of NK cells when both are co-cultured for 24 hours. Interestingly, the naive Treg cells exhibited suppressive effects even when not pre-activated with cytokines or anti-CD3 antibody (Fig. 3A). The presence of plate bound anti CD3 significantly enhanced this suppressive effect (Fig. 3B). This is consistent with studies where Treg cells are required to be activated through the CD3 and CD28 to exhibit suppressive function. Freshly separated naive Tregs have been shown to suppress direct NK cytotoxicity.

**Discussion**

We and others have recently demonstrated potential use for IL-21 as a cytotoxic agent in CLL. Given the pleiotropic effects of the common gamma chain family of cytokines such as IL-2 and IL-21 on B, T and NK cells, we examined the effects IL-2 and IL-21 on regulatory T cell homeostasis and NK mediated ADCC function. We observed that regulatory T cells from majority of CLL patients variably express the IL-21 receptor. In contrast to IL2, IL-21 failed to induce the expansion of regulatory T cells as evidenced by lack of increase in CD4+CD25(Foxp3)T cells with IL-21. In contrast to their differential effect on expansion of the CD4+CD25(Foxp3)T cells, IL-2 and IL-21 exhibited a redundant role in suppression of NK cell mediated ADCC function.

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when cultured together for 4 hours. In our experiments, the Treg mediated suppression of the ADCC function of the NK cell line was evident at 1:1 ratio without CD3 activation, but required 72 hours of culture. In previously published results, these cells have been shown not to secrete any cytokines and their suppression to be mediated through TGFbeta. Interestingly, the suppression of ADCC by Tregs was enhanced by both IL-2 and IL-21 to a similar extent, and by CD3 activation. This is most likely ascribed to the fact that both these cytokines that belong to the common gamma chain and may share redundant signaling features. Despite these similarities, and in contrast to IL-2, IL-21 lacks the ability to expand CD4+CD25High cells, and importantly, prevents the intracellular Foxp3 acquisition by CD4+CD25-T cells that is essential for suppressive function. Furthermore, the high concentrations of IL-21 with reduced Treg cell population in NOD mice further support our findings that IL-21 may not be a critical cytokine for the homeostasis of Treg cells in these mice.

The studies herein, which show that IL-21 does not induce expansion of Treg cells in CLL patients, have tremendous clinical relevance. Thus, recent observations on an IL-21 mediated direct apoptotic effect that functions through induction of pro-apoptotic Bim protein and activation of granzyme B in CLL cells while activating and recruiting the innate immune system for tumor clearance has tremendous clinical implications. Clinically, IL-21 therapy as single agent, or in combination with rituximab, or rituximab and fludarabine in patients with CLL, is highly feasible. Given the tolerable toxicities with IL-21, in contrast to IL-2 which has significant infusional toxicities and pro-survival effect on CLL cells, our results provide a compelling
to explore the combination of IL-21 with other chemoinmunotherapeutic agents to augment their efficacy.23

Materials and Methods

Antibodies, cytokines and reagents. Surface staining with CD4-APC CD25-FITC (both from BD Pharmingen, San Diego, CA) and biotin labeled IL21R-α (kindly provided by ZymoGenetics, Inc., Seattle, WA) followedby Streptavidin-FITC (BD Pharmingen) was performed according to the manufacturer’s suggested protocols. Foxp3 PE intracellular staining was performed using a kit (ebioscience) according to the manufacturer’s instructions. Data were acquired on BD Facscan Aria. The data was subsequently analyzed on the WinMDI program. Recombinant human IL-21 was kindly provided by ZymoGenetics. IL-2 was obtained from commercial vendor (Peprotech, Rockhill, NJ) and used at 10 ng/ml or 100 ng/ml.

Cell isolation and separation. Blood was obtained from CLL patients with informed consent under a protocol approved by the hospital internal review board. All patients examined in this series had immunophenotypically defined CLL, as outlined by the modified 96 National Cancer Institute criteria.24 Whole blood was treated with IL-2 and IL-21 at indicated doses for 48 hours on a rocker at room temperature. The peripheral blood mononuclear cells (PBMC) were then isolated by ficoll density gradient centrifugation (Ficoll-Paque Plus, Amersham Biosciences, Piscataway, NJ).

Cell purification and culture. CD4+CD25+ cells were purified from leukopaks of healthy volunteer donors using a Treg separation kit (Stem Cell Technologies, Vancouver, BC) according to the manufacturer’s directions. Briefly, the CD4+ T cells were enriched by incubation with RosetteSep T cocktail followed by ficoll density gradient centrifugation. The enriched CD4+ T cells were incubated with bixin anti-CD25+ (25 μg/10^8 cells) followed by streptavidin conjugated nanoparticles (50 μg/10^6 cells) and subjected to magnetic columns. Purity of the enriched cells ranged from 85–90%. The cells were activated in RPMI 1640 supplemented, with penicillin (56 U/ml), streptomycin (56 μg/ml) (all from Invitrogen, Carlsbad, CA) and 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) in the presence or absence of indicated concentrations of IL-2 or IL-21 at 37°C, in an atmosphere of 5% CO₂ for indicated time points.

Antibody dependent cellular cytotoxicity (ADCC) inhibition assay. The Treg cells from normal healthy volunteer donor were co-incubated in fresh media without the cytokines with NK-92 cells, at a 1:1 concentration for 24 hours. The NK-92 cell line is an IL-2 dependent human NK cell clone derived from human non-Hodgkin’s lymphoma with large granular lymphocyte phenotype (CD56+CD16) which has been transfected with CD16.25 For the ADCC experiments these cells were starved of IL-2 for 48 hours prior to use. Raji cells were used as the targets in the standard ADCC assay at indicated effector: target (E:T) ratios. ADCC activity was determined by standard 4-hour 51Chromium-release assay as described previously.26 Effector cells (NK-92 cells) were co-cultured with CD4+CD25+ Treg cells for 24 hours at 1:1 ratio. 51Cr-labeled target cells (Raji cell line) were incubated with indicated antibodies for 20 minutes, washed off excess antibody and the cells were placed in 96-well plates at the indicated effector to target ratio (E:T). After 4-hour incubation, supernatants were harvested and counted on a gamma counter. The percentage of relative lysis was determined by: % lysis = 100 x (ER-SR)/(MR-SR) where ER, SR and MR represent experimental, spontaneous and maximum 51Chromium release respectively.

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