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Lenalidomide induces interleukin-21 production by T cells and enhances IL21-mediated cytotoxicity in chronic lymphocytic leukemia B cells

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Abstract

The immunomodulatory drug lenalidomide has demonstrated efficacy in chronic lymphocytic leukemia (CLL) patients, despite a lack of direct cytotoxic effect *in vitro*. The mechanism of lenalidomide efficacy *in vivo* is thought to occur via a combination of enhanced immune activity and an alteration of tumor cell–microenvironment interactions. We demonstrate in whole blood from CLL patients that lenalidomide significantly depletes malignant B cells. Lenalidomide also induced production of interleukin-21 (IL21) and its mRNA in T cells from CLL patients. Lenalidomide also enhanced upregulation of functional IL21 receptor (IL21R) on the cell surface and increased receptor mRNA *in vitro*. The *in vitro* combination of IL21 and lenalidomide enhanced IL21-mediated cytotoxicity toward CLL cells through a variety of mechanisms. We show association of cell death with upregulation of Bid by IL21, enhanced upregulation of Bid by the combination therapy, and diminished Lck and downstream BCR signaling activation of Syk and PLCG2. Collectively, we demonstrated an immune cell–tumor cell interaction through lenalidomide-mediated induction of IL21 and IL21R, with enhanced IL21-mediated cytotoxicity, which provides justification for this combination in clinical trials for CLL patients.

Introduction

Chronic lymphocytic leukemia (CLL) is the most prevalent adult leukemia and is characterized by an accumulation of functionally incompetent malignant B cells (1, 2). B cell defects and hypogammaglobulinemia, coupled with dysfunction in other immune cells, often result in profound immune suppression, making infection a major cause of morbidity and mortality in CLL patients (3–5). Unfortunately, common first-line cytotoxic therapies

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can exacerbate immune dysfunction. Identification of alternative therapies that can improve the patient's immune system function are therefore of great interest.

One such potential therapy is lenalidomide, an immunomodulatory agent that has shown promise in CLL. Lenalidomide is FDA approved for treatment of multiple myeloma, mantle cell lymphoma, and myelodysplastic syndrome. In multiple myeloma, lenalidomide has direct cytotoxic effects against the tumor cells (6, 7), presumably through the increased targeting and degradation of the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) by the Cul4–Rbx1–DDB1–Cereblon E3 ubiquitin ligase complex (8–10).

The precise mechanism of lenalidomide action in CLL has not been fully elucidated. It is thought to function in an indirect manner by reversing immune defects and promoting an antitumor responses by the patient's immune system, because lenalidomide as a single agent does not have cytotoxic activity against CLL cells *in vitro*, in contrast to multiple myeloma. Lenalidomide acts as an immune stimulant for T cells and can activate natural killer (NK) and natural killer T (NKT) cells (11–16). Lenalidomide can correct the defects in immune synapse between T cells and CLL cells, reversing T-cell dysfunction and enhancing the immune recognition of tumor cells (17–19). Lenalidomide increases expression of costimulatory molecules, such as CD40, CD80, CD86, and CD154 (CD40L) expression on CLL cells (20, 21).

Another potential immune-stimulating therapy for CLL is interleukin-21 (IL21). IL21 is a pleiotropic immunomodulatory cytokine produced mainly by T follicular helper cells, T_H17 cells, and NKT cells(22). In addition to augmenting the activity and proliferation of cytotoxic CD8⁺ T cells (22, 23), IL21 enhances antibody-dependent cellular cytotoxicity (ADCC) of NK cells against rituximab-coated CLL cells (24). Phase I trials have focused on using IL21 in solid tumors or in conjunction with monoclonal antibody therapy. However, in addition to possessing immune-stimulating properties, IL21 is also capable of direct cytotoxicity, in proportion to expression of the IL21 receptor (IL21R) on CLL cells (24–26). Combination of IL21 with agents that increase IL21R expression, such as CpG oligodeoxynucleotides or CD40 ligand, improves the antitumor activity of IL21 and could enhance response in patients with low baseline expression of IL21 (25, 26).

Here, we examine the effects of lenalidomide on T-cell production of IL21, and demonstrate that lenalidomide induces IL21R on CLL B cells. In addition, this work shows that lenalidomide enhances the cytotoxic effect of IL21 in CLL cells through multiple distinct pathways.

Materials and Methods

Cell isolation, cell culture, and reagents

Patient samples were obtained following written informed consent in accordance with the Declaration of Helsinki and under a protocol reviewed and approved by the Institutional Review Board of the Ohio State University. All patients had CLL as defined by the IWCLL 2008 criteria(27). CLL cells were selected using RosetteSep Human B Cell Enrichment reagent (Stem Cell Technologies, Vancouver, BC, Canada) and isolated by Ficoll density

gradient centrifugation (Ficoll-Paque Plus, Amersham Biosciences, Piscataway, NJ). CLL patient T cells were isolated in a similar manner using RosetteSep Human T cell Enrichment Cocktail. Normal T cells were isolated in the same manner from Red Cross leukopaks (Red Cross). Cells were suspended in RPMI 1640 medium (Gibco, Life Technologies, Grand Island, NY) with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and penicillin (100 U/ml)/streptomycin (100 µg/ml; Sigma-Aldrich, St. Louis, MO) at 37°C in an atmosphere of 5% CO₂. For CD3 stimulation of T cells, cells were cultured on anti-human CD3 T-cell activation plates (BD Biosciences, San Jose, CA). CD28 stimulation of T cells was performed using soluble CD28 (BD Biosciences) at 2.5 µg/mL. CD40 blocking antibody HCD-122 was kindly provided by Novartis (Cambridge, MA). Lenalidomide was obtained and extracted as described (28). IL21 was provided by ZymoGenetics or purchased from Peprotech (Rocky Hill, NJ).

Whole blood depletion assay

Whole blood depletion assays were performed on blood from CLL patients that was treated with 0.5 µM or 1 µM lenalidomide or an equivalent volume of DMSO vehicle control and kept on continuous rotator. A fixed volume of blood was stained with CD3-FITC (BD Biosciences 555332), CD19-PE (BD Biosciences 555413), and LIVE/DEAD fixable near-IR cell stain (Invitrogen) for exclusion of dead cells. Red cells were lysed with Cal-Lyse (Invitrogen). Flow cytometry was performed using a Beckman Coulter FC500 cytometer (Beckman Coulter, Indianapolis, IN). Prior to flow analysis, AccuCheck counting beads (Invitrogen) were added in order to obtain absolute cell counts. Absolute count of cell populations was determined by: Absolute count (cell/µl) = number of cells counted/total number of beads counted × number of beads/µl (as obtained from lot insert).

Immunoblotting

Immunoblotting was performed using standard procedures as described (29). Blots were probed using the following primary antibodies: mouse anti-human total STAT1 (9H2), rabbit anti-human phospho-STAT1^{Y701} (D4A7), mouse anti-human total STAT3 (124H6), rabbit anti-human phospho-STAT3^{Y705} (#9131), rabbit anti-human Lck (73A5), rabbit anti-human phospho-ZAP70^{Y319}/phospho-SYK^{Y352} (65E4), rabbit anti-human SYK (#2712), rabbit anti-human phospho-PLCγ2^{Y759} (#3874), rabbit anti-human PPLCγ2 (#3872), rabbit anti-human BTK (D3H5), and rabbit anti-human Bid (#2002) (Cell Signaling, Danvers, MA); goat anti-human IL21R (#L-20), rabbit-anti-human p21 (N-20) (#469), mouse anti-human GAPDH (#0411) (Santa Cruz); mouse anti-human ZAP70 (#2F3.2; Millipore, Billerica, MA); rabbit anti-human phospho-Lck^{Y394} (SAB4300118; Sigma Aldrich, St. Louis, MO); rabbit anti-human phospho-BTK^{Y223} (ab101233; abcam, Cambridge, MA).

Cell viability

Cell viability of primary CLL cells was determined by Annexin V-FITC/propidium iodide (PI) according to the manufacturer's protocol (BD Pharmingen, San Diego, CA) using a Beckman Coulter FC500 cytometer (Beckman Coulter, Indianapolis, IN). Unstained and Annexin V-FITC or PI only stained cells were processed for compensation. Cell viability was determined by annexin V and PI double negative population.

Enzyme-linked immunosorbent assay (ELISA)

IL21 ELISA was performed using the human IL21 ELISA Ready-SET-Go! kit (eBioscience, San Diego, CA) according to the manufacturer's instructions.

RNA extraction, cDNA, and quantitative RT-PCR

Patient cells were suspended in TRIzol reagent (Life Technologies, Grand Island, NY). Following chloroform extraction using 200 µl chloroform per 1 ml TRIzol, RNA was isolated with the mirVana kit (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Isolated RNA was eluted with RNase-, DNase-free water and stored at -80°C. Reverse transcription was performed using Superscript First Strand System (Invitrogen). Quantitative RT-PCR was performed using the IL21R (Hs00222310_m1) and IL21 (Hs00222327_m1) Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA). Transcript levels were determined from threshold cycle numbers and normalized to internal control CD52 (Taqman Gene Expression Assay Hs00174349_m1, Applied Biosystems).

Microarray

RNA extracted from primary CLL cells treated with vehicle, lenalidomide, vehicle and IL21, or lenalidomide and IL21, was quality assessed by Agilent bioanalyzer. Gene expression profiling was performed in the Microarray Shared Resource in the OSU Comprehensive Cancer Center. Gene expression was analyzed by Affymetrix GeneChip Human Genome U133 plus 2.0 (Affymetrix, Santa Clara, CA). Microarray data for this study were deposited in the GEO repository and can be found with accession number GSE79660.

Statistics

For microarray data, signal intensities were analyzed by Affymetrix Expression Console software. Background correction, quantile normalization, and gene expression summarization were performed by the RMA method (30). A filtering method based on percentage of arrays below background cutoff was applied to filter out low expression genes. A linear model was employed to detect differentially expressed genes. In order to improve the estimates of variability and statistical tests for differential expression, a variance smoothing method with fully moderated *t*-statistic was used for this study(31). The significance level was adjusted by controlling the mean number of false positives(32). For the experiments with repeated measures, data were analyzed by using mixed effect models incorporating observational dependencies within each subject. For the experiments involving independent groups, data were analyzed by analysis of variance. Multiplicities were adjusted by Holm's method to control the family-wise error rate at 0.05. Statistical software SAS 9.3 (SAS, Inc; Cary, NC) and R was used for analysis.

Results

Lenalidomide depleted CLL cells in ex vivo whole blood assay

Although lenalidomide does not have a direct cytotoxic effect on CLL cells(33), its clinical activity is attributed to immune modulation and alteration of microenvironment interactions.

To determine whether lenalidomide could deplete CLL cells *in vitro* in an environment containing other immune cells, we evaluated the absolute number of B cells by flow cytometry on a fixed volume of blood following treatment with lenalidomide or vehicle. Baseline B-cell counts varied widely between patients. At 72 hours, both 0.5 μ M and 1 μ M lenalidomide significantly decreased the CD19⁺ B cell population ($P = 0.02$ and $P = 0.0004$, respectively; Fig. 1A and B). We chose the 0.5 μ M concentration which is pharmacologically obtainable for all subsequent studies.

Lenalidomide induced production of IL21 by T cells

Th17 cells in the IKZF1 knockout mouse have increased production of IL21 (34). As Ikaros degradation results in IL2 production in T cells (9), we sought to establish whether lenalidomide treatment leads to production of IL21 mRNA and protein by T cells. In initial experiments, donor T cells from healthy volunteers were isolated from leukopaks and stimulated with vehicle or 0.5 μ M lenalidomide, with no additional stimulation or with anti-CD3, CD28, or anti-CD3 + antiCD28. Although lenalidomide alone showed a modest increase in IL21 mRNA expression (decreased Ct indicating increased mRNA expression), IL21 mRNA was significantly increased in CD3-activated T cells given lenalidomide as compared to vehicle (Fig. 2A). At the protein level without lenalidomide, CD3 stimulation was necessary to detect the small amounts of induced IL21 protein in culture supernatants, as without it, IL21 protein was below detectable limits of the ELISA. However, lenalidomide greatly increased production of IL21 by CD3-activated T cells from healthy volunteers (Fig. 2B). The addition of CD28 to lenalidomide and CD3 did not significantly affect production of IL21 compared to lenalidomide and CD3 without CD28 ($P = 0.1$). This was suggestive of lenalidomide acting as a “second signal”, as has been reported by others (11).

Because of known T-cell defects in CLL patients, we assessed whether T cells isolated from CLL patients were similarly responsive to stimulation with lenalidomide. Although IL21 mRNA production showed a trend of increasing with lenalidomide or lenalidomide+CD3 stimulation, as compared to vehicle for comparable conditions, it did not reach statistical significance (Fig. 2C). However, at the protein level, T cells from CLL patients significantly increased production of IL21 upon lenalidomide/anti-CD3 stimulation, as assessed by ELISA ($p < 0.0001$ for lenalidomide vs. vehicle or lenalidomide+CD3 vs. vehicle+CD3, Fig. 2D). In contrast to the observation with T cells from normal donors, those from CLL donors produced additional IL21 protein when anti-CD28 was added to the lenalidomide+anti-CD3 stimulation. Production of IL21 protein varied across patients, but all samples showed an increase with the addition of lenalidomide and anti-CD3, demonstrating that CLL patient T cells could produce IL21 in response to lenalidomide. When whole blood from CLL patients was treated with lenalidomide *ex vivo*, the T cells isolated from it also showed a significant increase in IL21 mRNA, even, even without addition of anti-CD3 (Fig. 2E). Consistent with the finding of Huang et al. (35), IL21 protein was not detectable by ELISA (data not shown); this is likely due to a high IL21 receptor density on other cells within the whole blood setting.

Lenalidomide induced functional IL21R on CLL cells

Having established that lenalidomide can induce production of IL21 by T cells, we next examined the effects of lenalidomide on the expression of the IL21 receptor (IL21R) on the surface of CLL cells. Treatment of CLL cells with lenalidomide led to increased IL21R mRNA and protein, with peak protein expression 48 hours following initiation of treatment (Fig. 3A–C).

Treatment of CLL cells with IL21 induces STAT1 and STAT3 phosphorylation (24, 25). To determine the functional competency of lenalidomide-induced IL21R on CLL cells, we treated these cells with IL21. IL21 treatment increased both phosphorylated STAT1 and STAT3 in these CLL cells, compared to vehicle control (Fig. 3D–G), providing evidence that this receptor was functional relative to ligand-mediated downstream signaling.

In addition, IL21R expression increased modestly on peripheral blood CLL cells from patients receiving low-dose lenalidomide (2.5 mg) in a clinical trial of genetically high-risk, asymptomatic, previously untreated patients. This provided evidence that lenalidomide is capable of upregulating IL21R *in vivo* (Fig. 3H).

Lenalidomide enhanced IL21-mediated cytotoxicity in CLL B cells

Lenalidomide is generally not directly cytotoxic toward CLL cells *in vitro* (33). Our previous report indicating a positive correlation of IL21-mediated cytotoxicity with IL21R density (24) led us to test if lenalidomide-induced IL21R would enhance IL21-mediated cytotoxicity against CLL cells. CLL cells were treated with lenalidomide for a period of 48 hours, the optimum time for induction of IL21R, followed by 72 hours of treatment with combined lenalidomide and IL21. As expected, lenalidomide alone did not have a significant effect on the viability of the cells *in vitro* in the absence of accessory cells. However, lenalidomide enhanced the cytotoxic efficacy of IL21, as compared with vehicle with IL21 (Fig. 4A). IL21-mediated cytotoxicity was time dependent, with or without lenalidomide (both P values < 0.0001, slope tests), and as a single agent did not demonstrate significant cytotoxicity until the 72 hour time point (Fig. 4b).

Lenalidomide, IL21, and the combination, induced different transcriptional profiles

Ikaros and Aiolos can act as repressors or transcription enhancers and are each degraded by lenalidomide through a cereblon-mediated pathway (9, 10). We first confirmed a previous report that IKZF1 and IKZF3 are significantly depleted in CLL following lenalidomide treatment (36) (Supplemental Fig. S1). We and others have also shown that IL21 leads to STAT1 and STAT3 activation. As these four transcription factors potentially activate and repress different transcription pathways and, when increased together, produce a unique feature of enhanced apoptosis, we hypothesized that each therapy and the combination would produce a distinct gene expression pattern. We therefore treated samples chosen from three different patients who demonstrated synergistic apoptosis with both agents. From a vehicle, vehicle+IL21, lenalidomide, or lenalidomide+IL21 sample we isolated RNA, and examined gene expression. Supplemental Fig. S2 shows a Venn diagram depicting genes increased or decreased at least a 2-fold change compared to vehicle, with a cutoff of $P=0.001$. IL21 and lenalidomide promote change in 173 and 105 genes, respectively, with this

criteria, and the combination modulates 369 genes, with 226 of these genes being unique to the combination treatment of IL21 and lenalidomide. Relevant genes up- or down-regulated by each treatment and selected for further study are summarized in Supplemental Table 1.

Induction of p21 by combined lenalidomide and IL21

One gene of interest identified by the microarray data was p21, which showed an increase with lenalidomide but much more so when lenalidomide was combined with IL21. Lenalidomide induces p21 in lymphoma, multiple myeloma, and CLL through a p53-independent mechanism (37). Induction of p21 in CLL cells by lenalidomide inhibits proliferation induced by coculture with CD40L-expressing fibroblasts (36). For assessment of protein changes over time, CLL cells were treated with lenalidomide for 48 hours, then treated with a combination of IL21 and lenalidomide for a subsequent 72 hours. Protein was collected at 48 hours, 48+24 hours, 48+48 hours, and 48+72 hours. Although a small induction of p21 was observed with lenalidomide alone in some time course patient samples, it did not achieve statistical significance at any point in the time course. Significant induction of p21 protein was observed in patient samples treated with the combination of lenalidomide and IL21 (Fig. 5A and B). (lenalidomide+IL21 vs. vehicle, 48+24 h, $P=0.02$; 48+48 h, $P=0.045$; 48+72 h, $P=0.02$). As expected, we did see degradation of Ikaros and Aiolos with lenalidomide treatment, but this was not significantly enhanced by the addition of IL21 (Supplementary Fig. S1).

Induction of Bid after combined lenalidomide and IL21 treatment

We previously showed that the cytotoxic effect of IL21 against CLL cells is mediated by the BH3-only Bcl-2 family member Bim (24). Our microarray data here showed that another BH3-only family member, Bid, was increased by the combination of lenalidomide and IL21. Immunoblots of additional patient samples confirmed that although lenalidomide or IL21 alone induced Bid expression, the combination of IL21 and lenalidomide led to much higher expression (data not shown). This difference was evident in the time course samples as well (Fig. 5C and D). The combination of lenalidomide and IL21 induced more Bid protein at an earlier time point (at 48+24 hours, lenalidomide vs. lenalidomide+IL21, $P=0.007$; lenalidomide+IL21 vs. vehicle, $P<0.0001$), at which point neither lenalidomide nor IL21 have induced significant Bid protein.

Decreased Lck and reduced phospho-SYK and -PLC γ 2 in combination-treated CLL cells

Microarray results also indicated changes to Lck, which is expressed at significant levels in CLL cells (38) and is identified as a potential therapeutic target in CLL (39). In T cell receptor signaling, Lck is responsible for phosphorylation of ZAP70, but in CLL it appears to phosphorylate Syk, which is involved in B cell receptor (BCR) signaling(39). As BCR signaling is a major survival pathway for CLL cells, disruption of this pathway is therapeutically beneficial. Supplemental Table 1 indicates some decrease of Lck with IL21 or lenalidomide alone, with much greater decrease upon combination of both IL21 and lenalidomide. These results were verified on the protein level in the time-course patient samples (Fig. 6A). As total Lck was diminished, so was activated phospho-Lck^{Y394} (Fig. 6A–C).

We hypothesized that decreased Lck may disrupt BCR signaling in CLL cells. We observed diminished phosphorylation of SYK at 48+24 hour time point (vs. vehicle at 48+24 hours, $P = 0.01$ and 48 + 72 hours, $P = 0.003$) (Fig. 7). Phosphorylation of PLC γ 2 was also significantly decreased with the combination of lenalidomide and IL21, at the 48+48 hour and 48+72 hour time points ($P = 0.01$) (Figure 7). Collectively, these studies provide mechanistic data why IL21 and lenalidomide are synergistic in CLL, providing justification for future clinical trials.

Discussion

Lenalidomide does not have direct cytotoxic effects on CLL cells *in vitro*, but shows clinical efficacy in CLL patients, presumably by affecting the function of other immune cells and modulating microenvironment interactions. Although lenalidomide in our *in vitro* studies of CD19-selected tumor cells had no activity, we show here that lenalidomide can deplete CLL cells in whole blood *in vitro*, suggesting that its indirect effects may depend on the presence of other cell populations. We also showed that lenalidomide could induce production of IL21 by T cells from both healthy volunteers and CLL patients. Ikaros acts as a repressor of IL21 expression in CD4⁺ T cells (34). Therefore, it is likely that lenalidomide is inducing IL21 indirectly through cereblon-mediated degradation of Ikaros, in a manner similar to the induction of IL2 by lenalidomide (9). We could detect significantly more IL21 in supernatants from the T cells of both healthy volunteers and CLL patients when the isolated T cells were treated with lenalidomide+IL21. However, after treating whole blood samples from CLL patients with lenalidomide+IL21, we were unable to detect IL21 by ELISA in the plasma, despite significant induction of mRNA. This may be due to capture of plasma IL21 by the IL21Rs present on other cells, which thereby act as an “IL21 sink”. This is consistent with a report showing undetectable IL21 production by T cells within a mixed peripheral blood mononuclear cell population by ELISA, unless a blocking antibody was added to prevent the receptor/cytokine interaction (35).

As we showed here that lenalidomide induces expression of the IL21R in CLL cells, we previously showed that IL21 cytotoxicity corresponds to IL21 receptor expression (24), we hypothesized that lenalidomide would enhance the cytotoxic effects of IL21. Initial assessments showed increased cytotoxicity with the combination of lenalidomide and IL21. Microarray data identified other potential pro-apoptotic pathways. Contrary to expectation, we observed enhanced cytotoxicity even in patient samples that failed to show an upregulation of IL21R, suggesting contributions of other pathways to cytotoxicity. Unfortunately, it was not possible to perform detailed mechanistic studies with shRNA or overexpressing constructs typically utilized in molecular biologic studies as we could not identify any CLL cell lines, including OSU-CLL, MEC-1, and MEC-2, that recapitulated the finding of enhanced cytotoxicity with the combination of lenalidomide and IL21 that was observed in primary CLL cells. In lieu of cell line studies, we performed time course studies in primary CLL cells.

We observed that the combination of lenalidomide and IL21 induced much more p21 in CLL cells than did lenalidomide treatment alone. Fecteau et al. published that the induction of p21 by lenalidomide abrogated the proliferation of CLL cells induced by coculture with

CD40L-expressing fibroblasts, suggesting that one potential mechanism of action for lenalidomide is prevention of microenvironment-induced proliferation through induction of p21(36). These findings indicate that the strong induction of p21 by the combination of lenalidomide and IL21 we observed has potentially interesting implications for patient treatment, even in patient samples that failed to show enhanced cytotoxicity *in vitro* in response to the treatment. Although a patient sample may fail to respond to the combination of lenalidomide and IL21 *in vitro*, that same patient may still benefit from combination therapy *in vivo* due to greater disruption of survival and proliferation signals from the microenvironment.

The microarray analysis of combined therapy led us to Lck as an interesting candidate for promoting synergistic cytotoxicity as it showed significant decrease with the combination of lenalidomide and IL21. Time course data revealed that Lck protein decreased with the combination of lenalidomide and IL21. Phosphorylation of Syk and PLC γ 2, targets of the Lck activation pathway in CLL cells, were decreased with the combination treatment, although the decrease in phospho-PLC γ 2 was not apparent at the latest time points, perhaps due to alternative activation from upstream molecules, or selection of the cells least responsive to the combination treatment. These findings suggest that the combination of lenalidomide and IL21 may diminish BCR signaling, which is a major pro-survival pathway in CLL and a clinically relevant therapeutic target (39, 40). Future studies should focus on examining the influence of lenalidomide on IGHV un-mutated CLL, the subtype that is more dependent upon B-cell receptor signaling.

Taken together, these studies show that lenalidomide and IL21 offer a potential therapeutic combination for CLL that may be relevant for other malignancies as well. They also give insight into the mechanism of lenalidomide in CLL, showing that lenalidomide promotes CLL cell apoptosis in a whole-blood setting and enhances IL21 production by T cells. Future experiments that block IL21 production in the whole blood assay are warranted, as our data as presented in Fig. 1 does not confirm a direct or indirect role of IL21 promoting killing in this setting. However, these data can contribute to the development of rational therapeutic combinations for CLL that enhance immune activity, indirectly targeting the malignant cells, while also having a direct cytotoxic effect on the tumor cell population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Lenalidomide depleted CLL cells in whole blood

A and B, Absolute CD19⁺ cell counts in CLL whole blood after 72 h treatment with vehicle, 0.5 μM lenalidomide, or 1 μM lenalidomide. AccuCheck flow beads were included to obtain absolute counts. (**A**) is depicted as data from each individual patient, whereas (**B**) shows mean ± SD. (0.5 μM lenalidomide, $P = 0.02$. 1 μM lenalidomide, $P = 0.004$.)

Figure 2. Lenalidomide with CD3 stimulation induced production of IL21 in T cells from healthy volunteers and CLL patients

A) Expression of IL21 mRNA in T cells from healthy volunteers. Data are represented as $\Delta CT (IL21-CD52)$. Each point represents an individual patient sample. (Lenalidomide+CD3 vs. vehicle+CD3, $P = 0.001$; $n = 8$) B) Production of IL21 protein in T cells from healthy volunteers as measured by ELISA. Data are represented as mean $\pm$ SEM. ($n = 8$) C) Expression of IL21 mRNA in T cells from CLL patients. Data are represented as $\Delta CT (IL21-CD52)$. Each point represents an individual patient sample (lenalidomide+CD3 vs. vehicle+CD3, $P = 0.09$; $n = 4$). D) Production of IL21 protein in T cells from CLL patients as measured by ELISA. Data are represented as mean $\pm$ SEM. ($n = 4$) E) Treatment of CLL whole blood with lenalidomide led to increased IL21 mRNA in T cells. Each point represents an individual patient sample. (lenalidomide vs. vehicle, $P < 0.0001$; lena+CD3 vs. vehicle+CD3, $P < 0.0001$). ** indicates $P < 0.01$, *** indicates $P < 0.001$ in all figure parts.

Figure 3. Treatment of CLL cells with lenalidomide leads to upregulation of IL21R mRNA and protein

A) Representative immunoblot showing upregulation of IL21R protein in CLL cells after 48 h treatment with 0.5 μ M lenalidomide. Blots were reprobbed with GAPDH for loading control. B) Average fold change of IL21R protein in CLL cells treated with lenalidomide. IL21R/GAPDH ratio was normalized to vehicle condition. Data are represented as mean $\pm$ SEM. C) Quantitative RT-PCR assessment of IL21R mRNA in CLL cells treated with 0.5 μ M lenalidomide for 48 hours. Fold changes were calculated using Ct(IL21R-CD52) normalized to vehicle control. Data are represented as mean $\pm$ SEM. D) Representative blot of phospho- and total STAT1 in CLL cells treated with lenalidomide or vehicle for 48 h,

followed by a 15 min stimulation with IL21 (100 ng/ml). Blots were reprobed with GAPDH for loading control. E) pSTAT1 in CLL cells treated with lenalidomide or vehicle prior to IL21 stimulation. Data were normalized to vehicle control and are represented as mean $\pm$ SD. F) Representative blot of phospho- and total STAT3 in CLL cells treated with lenalidomide or vehicle for 48 hours, followed by 15 minute stimulation with 100 ng/ml IL21. Blots were reprobed with GAPDH for loading control. G) pSTAT3 in CLL cells treated with lenalidomide or vehicle prior to IL21 stimulation. Data were normalized to vehicle control and are represented as mean $\pm$ SD. H) IL21R protein values in peripheral blood mononuclear cells isolated from CLL patients receiving daily low dose lenalidomide. By cycle 1 day 8 (C1D8), 5/8 patients showed an increase in IL21R protein of 20% or more as compared to baseline (pre-treatment) sample C1D1. IL21R protein was normalized to actin. For all graphs, * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, and **** indicates $P < 0.0001$.

Figure 4. Lenalidomide significantly enhances IL21-mediated cytotoxicity in primary CLL cells

A) CLL cells were treated with vehicle or 0.5 μ M lenalidomide for 48 hours, followed by combined lenalidomide or vehicle with 100 ng/ml IL21 for 72 h. Viability was assessed by AnnexinV-FITC/PI flow, with Annexin/PI negative cells considered the viable population. Data are represented as mean $\pm$ SEM. For IL21 vs. vehicle, $P < 0.05$. For lenalidomide+IL21 vs. IL21 alone, $P = 0.0005$. For lenalidomide+IL21 vs. vehicle or lenalidomide alone, $P < 0.0001$. $n = 53$. B) Viability assessed at each 24 hour time point post addition of IL21. $n = 17$. Time dependence of IL21-mediated cytotoxicity $P < 0.0001$ whether lenalidomide is present or absent. The respective lines connect means of cytotoxicity at each time point.

Figure 5. Combined lenalidomide and IL21 leads to induction of p21 and BID in primary CLL cells

A) Representative immunoblot of p21 protein induction in time course samples. CLL cells were treated with lenalidomide or vehicle for 48 h, then with combined lenalidomide or vehicle and IL21 for 72 h, with protein collected every 24 h after addition of IL21 to culture. Blot was reprobed with GAPDH for loading control. B) Mean values of p21 protein induction in time course samples. p21 protein values were normalized to GAPDH and then normalized to vehicle control. Data are represented as mean $\pm$ SD. * indicates $P < 0.05$. C) Representative immunoblot of BID induction in time course samples. CLL cells were treated with lenalidomide or vehicle for 48 h, then with the combination of lenalidomide or vehicle and IL21 for 72 h, with protein collected every 24 h after addition of IL21 to culture. Blot was reprobed with GAPDH for loading control. D) Mean values of BID induction in time course samples. BID protein values were normalized to GAPDH and then normalized to vehicle control. Data are represented as mean $\pm$ SD. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

Figure 6. The combination of lenalidomide and IL21 decreases Lck expression in CLL cells

a) Representative immunoblot of Lck and pLck (pTyr394) in time course samples. CLL cells were treated with lenalidomide or vehicle for 48 h, then with the combination of lenalidomide or vehicle and IL21 for 72 h, with protein collected every 24 h after addition of IL21 to culture. Blot was reprobed with GAPDH for loading control. b and c) Mean values $\pm$ SD are shown of Lck and pLck in time course samples. Lck or pLck protein values were normalized to GAPDH and then normalized to vehicle control.

Data are represented as mean $\pm$ SD. * indicates $P < 0.05$, ** indicates $P < 0.01$.

Figure 7. The combination of lenalidomide and IL21 decreases phosphorylation of SYK and PLC γ 2 in CLL cells

Representative blot of PLC γ 2, pPLC γ 2^{Y759}, SYK, and pSYK^{Y352} in time course samples. CLL cells were treated with lenalidomide or vehicle for 48 hours, then with the combination of lenalidomide or vehicle and IL21 for 72 hours, with protein collected every 24 h after addition of IL21 to culture. Blot was reprobed with GAPDH for loading control. For decreased phosphorylation of PLC γ 2, the combination of lenalidomide and IL21 vs. vehicle at the 48+24 h timepoint $P = 0.01$, $n = 17$. For decreased phosphorylation of SYK, lenalidomide+IL21 vs. vehicle at 48+24 h $P = 0.01$ and 48 + 72 h, $P = 0.003$, $n = 18$.