CD126 and Targeted Therapy with Tocilizumab in Chronic Lymphocytic Leukemia

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Abstract

Purpose: IL6 promotes tumor growth and signal transduction via both its membrane-bound (CD126) and soluble receptors (sCD126). We aimed to study whether the levels of CD126 expression in chronic lymphocytic leukemia (CLL) cells can predict in vitro and in vivo treatment response.

Experimental Design: The levels of membrane-bound CD126 expression were determined on freshly isolated CLL B cells (n = 58) using flow cytometry. These CLL cells were treated with chlorambucil or fludarabine with or without anti-CD126 antibody tocilizumab for 24 hours and IL6-mediated STAT3 transcriptional activity and cell-cycle alteration were evaluated.

Results: CD126 surface expression was found in all cases and positively correlated with the levels of in vivo constitutive STAT3 activity. The levels of CD126 expression were significantly and positively correlated with the resistance of CLL cells to in vitro treatment with chlorambucil or fludarabine and poor in vivo treatment response of CLL patients. Blocking IL6 signaling with the anti-CD126 antibody, tocilizumab, had profound effects on STAT3-mediated survival and growth signals: decreased Mcl-1 and Bcl-xl, favoring an apoptotic profile; and decreased p27 with increased cyclin E and CDK2 expression, leading to cell-cycle shift from G0–G1. These tocilizumab-mediated changes induced chemoresensitization in resistant CLL cells, with the greatest effect seen in cells with higher CD126 expression (P < 0.001).

Conclusions: CLL cells with higher CD126 expression are more resistant to treatment in vivo and in vitro via IL6–CD126–STAT3 axis. Blocking CD126 using tocilizumab sensitizes CLL cells to chemotherapy. Clin Cancer Res; 22(10); 2462–9. © 2015 AACR.

Introduction

Chronic lymphocytic leukemia (CLL) is a highly variable disease and the clinical outcomes are significantly different in individual patients: some patients will live for decades and never require treatment, while others have aggressive disease requiring treatment at initial presentation (1). Although the standard treatment includes first-line chemoimmunotherapy approaches, which are associated with a prolonged survival, effective therapies for the more frail elderly patients or those who relapse after previous treatment are urgently needed (2).

CD126, also known as IL6-Rα, is a cytokine receptor. Anti-CD126 antibodies, such as tocilizumab, have been used to inhibit IL6 signaling in several diseases. In CLL, IL6 expression is correlated with clinical outcomes, and IL6 signaling is involved in cell survival and proliferation. In this study, we investigated the role of CD126 expression in CLL and its correlation with clinical outcomes. We also evaluated the effects of tocilizumab on CLL cells in vitro and in vivo.

Methods

We retrospectively analyzed 58 CLL patients who were treated at our institution. Freshly isolated CLL B cells were treated with chlorambucil or fludarabine with or without anti-CD126 antibody tocilizumab for 24 hours. The levels of membrane-bound CD126 expression were determined using flow cytometry. IL6-mediated STAT3 transcriptional activity and cell-cycle alteration were evaluated.

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Conclusions

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Translational Relevance

Increased levels of both IL6 and sCD126 in a variety of cancers are associated with an aggressive disease course and poor prognosis. However, the role of membrane-bound CD126 and its clinical impact are poorly defined. The current work shows that chronic lymphocytic leukemia (CLL) B cells express higher levels of CD126 compared with normal B cells. Higher levels of CD126 significantly correlate with poor treatment response in vitro and in vivo. Tocilizumab, an anti-CD126 antibody, is currently used for the clinical treatment of rheumatoid arthritis. We found that combined treatment of tocilizumab with chemotherapy can overcome chemoresistance of CLL cells, with the greatest effect seen in CLL cases with greatest CD126 expression. CD126 expression could be used to predict treatment response in a two-step approach with chemosensitization by tocilizumab followed by a second hit in CLL.

Tocilizumab, a humanized anti-CD126 mAb, approved by the FDA for the treatment of rheumatoid arthritis (21), targets membrane-bound and soluble CD126 and blocks IL6 classical and trans-signaling (22). IL6 in the microenvironment plays an important role in cancer cell progression (13) by constitutively activating STAT3 (23). Recent reports have highlighted the potential of tocilizumab for the treatment of a variety of cancers (24, 25). In the current study, we investigated whether the levels of CD126 in CLL have prognostic value and the effects of blocking CD126 by tocilizumab on CLL cells.

Materials and Methods

Patients, cell separation, and culture

The protocol was approved by the National Research Ethics Service (East London, United Kingdom), and the City HIA Local Research Ethical Committee for in vitro studies on blood samples from CLL patients. Fifty-eight patients were enrolled in this study and all the cases were either untreated ($n = 20$) or had not received chemotherapy or steroids for over 6 months (Supplementary Table S1). Peripheral blood was obtained after informed consent from patients with CLL in accordance with the Declaration of Helsinki. After informed consent, control blood samples were taken from normal health donors. Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation over Ficoll-Paque (GE Healthcare Life Science), the cells were used immediately or cultured in complete RPMI1640 containing 10% fetal calf serum, 2 mmol/L l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma) for the further experiments. The normal B lymphocytes from healthy donors were purified by selection of CD19+ cells using a column purification system (MiltenyiBiotec, ref. 18) and were stored in liquid nitrogen.

Antibody and reagents

Anti-IL6R antibody (tocilizumab) was kindly donated by Chugai Pharmaceutical Co Ltd. The antibodies of p-STAT3 (phospho-STAT3-Ser-727), Mcl-1, and STAT3 were purchased from Cell Signaling Technology; Bel-2, Bel-xl, Bax, p27, CDK2, and cyclin E were purchased from Santa Cruz Biotechnology; and β-actin was purchased from Sigma.

Measurement of membrane-bound IL6 receptor CD126

PBMCs from CLL patients were incubated with anti CD126-PE and CD19-PerCP antibody (BD Biosciences) for 15 minutes at room temperature in the dark, and then the CD19+/CD126+ dual positive cells were determined by FACSCanto I (Becton Dickinson).

Treatment and cell death assays

Cell viability was determined by Annexin V and propidium iodide (PI) dye exclusion after treatment with 20 μg/mL chlorambucil or 20 μg/mL of fludarabine or 10 μg/mL of tocilizumab (donated by Chugai Pharmaceutical Co Ltd) for 24 hours. After wash, cells were incubated with 100 μL of binding buffer, containing 5 μL of Annexin V–FITC, and 10 μL of PI for 15 minutes at room temperature in the dark. Viable cells were determined by flow cytometry. For Ki67 expression, cells were permeabilized and fixed, then washed with PBS. Cells were incubated with anti-Ki67-FITC (Dako) for 1 hour at 4°C. Before analysis, 10 μg/mL of PI was added. Nuclear DNA content was assessed with PI and analyzed by flow cytometry.

Measurement of autocrine IL6

CLL cells were isolated immediately after phlebotomy and used post-Ficoll as PBMC. If ≥95% of cells were CD19+, but if not, B-CLL cells were purified using a CD19+ Cell Isolation Kit (Miltenyi Biotec), with a purity of ≥97%. A total of 5 × 10^7/mL cells were cultured in RPMI1640 medium at 37°C in a humidified incubator with 5% CO₂. The supernatants were stored at −80°C until assayed for IL6 by ELISA, according to the manufacturer’s instructions (R&D Systems).

Western blotting

Proteins were extracted with CellLytic M cell lysis Reagent (Sigma) supplied with protease inhibitor and phosphatase inhibitor cocktails (Sigma). Proteins were subjected to 4%–20% NuPAGE gels (Invitrogen) and transferred onto PVDF membrane (Sigma) at 20 V for 1 hour by semi-dry transfer, as described previously (26). PVDF membrane was blocked with 5% nonfat milk in TBST for 1 hour and then incubated with indicated primary antibodies overnight at 4°C, visualized by GeneSnap (SynGene) after adding ECL plus (GE Healthcare Life Science).

Immunoprecipitation

Immunoprecipitation was performed using Dynabeads protein A (Invitrogen). Dynabeads in 50 μL of PBST was incubated with anti-p27 antibody or anti-IgG for 1 hour at room temperature on a rotator. Washing three times with PBST, Dynabeads protein A coated with antibody or IgG was mixed with 500 μg proteins and incubated for 1 hour at room temperature on the rotator. Protein complex was eluted by a loading buffer and detected by Western blot analysis.

Transfection of siRNAs

Human STAT3 and control siRNA A (Santa Cruz Biotechnol, sc-29493; sc-37007) were used for knocking down gene expression. Fresh primary CLL cells (5 × 10^6/mL) were suspended in 100 μL of Human B Cell Nucleofector (R) reagent (Lonza) and 2 μg of STAT3 siRNA or control siRNA A was added into the mixture. The transfection was performed using Nucleofector II apparatus with the program U-015 (Lonza). Transfection efficiency and cell death were determined by flow cytometry after
transfection with PMAX-GFP plasmid for 24 hours. Protein expression was determined after 24 hours transfection (27).

Statistical analysis

Statistical analysis was performed with GraphPad Prism and SPSS IBM version 22 software. Data are shown as either mean ± SEM or mean ± SD; comparisons between groups were analyzed with the two-tailed paired or unpaired Student t test. Correlation of multiple variables was analyzed by Spearman correlation using SPSS software. GelScan V5.1 software was used to quantify protein expression by Western blotting.

Results

CD126 expression on CLL cells correlates with STAT3 activity and ZAP70 positivity

The levels of CD126 expression in CLL and its prognostic impact are unknown. We investigated CD126 expression on 58 fresh CLL samples. The clinical characteristics of the patients are shown in Supplementary Table S1. Membrane-bound CD126 expression on normal B cells was negative or very low (mean = 1.7%; range from 0% to 6.3%). However, all cases of CLL cells have surface CD126 expression, and this was highly variable in 58 CLL cases (mean = 20.1%; range from 4.5% to 59%; Fig. 1A; Supplementary Fig. S1A). In cultured CLL cells, CD126 expression was stable over 48 hours compared with in vivo expression (Supplementary Fig. S1B). These data indicate that CLL B cells have significantly greater levels of CD126 compared with normal B cells.

The constitutive activation of STAT3 in cancer results from chronic stimulation by extracellular signals in the microenvironment, including IL6 (23), and overexpression of CD126 enhanced IL6/STAT3 responses (16, 28). In CLL cells, in vivo constitutively activated STAT3 involves phosphorylation of serine 727 residues (p-STAT3S727) but not tyrosine 705 residues (19). Constitutively activated p-STAT3S727 was found in all CLL cases, but not in normal B cells (Fig. 1B). In CLL cells, there was a significant correlation between the levels of constitutive activation of STAT3 and membrane-bound CD126 expression (Fig. 1C; P < 0.001), indicating the importance of CD126 in STAT3 activation.

Many prognostic markers have been identified in CLL, of which the best described are clinical stage, the expression of CD38 or ZAP70, and cytogenetic status measured by FISH (3–7). The levels of CD126 expression were evaluated in each of these prognostic subgroups of CLL and between treated and untreated patient groups (Fig. 1D). CD126 expression was significantly higher in ZAP70 positive versus negative cases of CLL (P < 0.01), whereas there were no statistically significant differences in CD126 expression based on any of the other studied parameters. These results demonstrate that higher levels of CD126 expression may be associated with worse clinical outcome in patients with CLL. In 11q- or 17p- CLL cells, higher expression of CD126 exhibited more resistance to chlorambucil-induced cell death and poor in vivo chemoresponsiveness compared with the lower expression of CD126 cases (Supplementary Fig. S2).

CD126 levels significantly correlate with poor treatment response in vitro and in vivo

In vivo, CLL cells are long-lived with survival dependent on external signals from the microenvironment and internal expression of antiapoptotic proteins (16, 17), which contribute to the chemoresistance of CLL cells and poor clinical outcomes (2). Chlorambucil, a chemotherapy agent which targets the G1 phase of the cell cycle, induced variable levels of cell death in CLL cells (median = 18%, range 1%–69.5%, Fig. 2A). Cells with higher STAT3 activation were more resistant to chlorambucil (P < 0.001, Fig. 2B), which reflects the role of STAT3 in poor response of CLL cells to treatment. CLL cells with greater resistant capacity (using the median level of chlorambucil-induced death as a cut-off) had significantly higher CD126 expression (P < 0.0001, Fig. 2C). The inverse correlation between chlorambucil-induced cell death and CD126 expression was statistically significant for both chlorambucil (R = −0.57, P < 0.0001, Fig. 2D), and fludarabine-induced cell death (R = −0.746, P < 0.01, Supplementary Fig. S3). These
data indicate that higher CD126 expression is associated with the chemoresistance of CLL cells.

Patients were divided into two groups based on their response to in vivo treatment: achieving a complete response (CR) versus not achieving a CR (i.e., a partial response, stable disease, and progressive disease). CD126 expression in the CR group was significantly lower than in the non-CR group (Fig. 2E, \( P < 0.001 \)).

Blocking CD126 with tocilizumab induces cell-cycle redistribution in CLL cells

CLL remains incurable and this is partly attributable to cells being in the G0 phase of the cell cycle and having high levels of antiapoptotic Bcl-2 family proteins (30). It was previously reported that IL6-mediated G0–G1 cell-cycle arrest is through JAK2/STAT3 pathway (31). Ki67 is a cellular maker for proliferation, which is expressed throughout the cell cycle, except for G0 phase (32). In CLL cells, both Ki67 and nuclear DNA staining demonstrated that most cells were in G0 phase (Fig. 3A), a factor contributing to chemoresistance (33). Treatment with the humanized anti-CD126 blocking antibody, tocilizumab, led to a marked increase in G1 phase cells, as detected by Ki67 positivity, without further progression through the cell cycle to G2/M/S phases (Fig. 3A and B), as detected by Ki67 positivity, without further progression through the cell cycle to G2/M/S phases (Fig. 3A, top), as numbers of cells in S/G2/M phases were not altered (Fig. 3A, bottom). In resting cells, cell-cycle control is achieved by binding of p27 with cyclin E/CDK2 complexes to prevent entry into G2 phase (34). Progressive decreases in p27 or overexpression of cyclin E/CDK2 lead to G0 exit and G1 progression, allowing G0–S-phase transitions (34). In cultured CLL cells, the expression of p27, cyclin E, and CDK2 were stable up to 24 hours (Fig. 3C, right). However, treatment of CLL cells with tocilizumab led to increased expression of cyclin E and CDK2 and decreased expression of p27 (Fig. 3C). Using immunoprecipitation, we found that treatment with tocilizumab significantly decreased binding between p27 with cyclin E and CDK2 (Fig. 3D). These data demonstrate that blocking CD126 by tocilizumab promotes cell-cycle reentry by decreasing binding between p27 and cyclin E/CDK2.

Tocilizumab restores the chemosensitivity of resistant CLL cells by inhibiting STAT3 transcriptional activity

In many cancer cells, constitutive activation of resistant CLL cells by inhibiting STAT3 transcriptional activity

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Figure 2.
Correlation between expression of CD126 and chemosensitivity of CLL. Fresh CLL cells were incubated with 20 µg/mL of chlorambucil for 24 hours. A, cell death was measured as PI+ cells by FACS (\( n = 58 \)). B, the correlation between p-STAT3 and chlorambucil-induced cell death (\( n = 34 \), \( P < 0.001 \)). CD126 expression in chlorambucil-sensitive (cell death > median) and chlorambucil-resistant (cell death < median) cases, \( n = 58 \), \( P < 0.0001 \). D, the correlation between CD126 expression and chlorambucil-induced cell death (\( n = 48 \), \( P < 0.001 \)). E, CD126 expression in patients achieving a CR (\( n = 13 \)) following treatment versus patients achieving a partial remission, stable, or progressive disease (non-CR, \( n = 25 \)). F, CD126 expression in patients with a lymphocyte doubling time (LDT) of >12 months (\( n = 13 \)) versus <12 months (\( n = 20 \)). ** \( P < 0.01 \); *** \( P < 0.001 \).
proteins, and promoting cell survival and chemoresistance (23, 35). The autocrine IL6 itself maintains STAT3 activity, thereby creating a positive feedback loop. CLL cells can produce IL6 (23, 35). The autocrine IL6 itself maintains STAT3 activity, thereby promoting cell survival and chemoresistance (36), with autocrine IL6 detectable at 6 hours and then increasing over 48 hours (Supplementary Fig. S4). In cultured CLL cells, the constitutively activated p-STAT3S727 and its targeted Bcl-2 family proteins, and promoting cell survival and chemoresistance (23, 35). The autocrine IL6 itself maintains STAT3 activity, thereby creating a positive feedback loop. CLL cells can produce IL6 (23, 35). The autocrine IL6 itself maintains STAT3 activity, thereby promoting cell survival and chemoresistance (36), with autocrine IL6 detectable at 6 hours and then increasing over 48 hours (Supplementary Fig. S4). In cultured CLL cells, the constitutively activated p-STAT3S727 and its targeted Bcl-2 family proteins showed stable expression up to 48 hours (Fig. 4A and B). Inhibition of CD126 by tocilizumab led to decreased STAT3 phosphorylation (detectable at 6 hours) and downregulation of Mcl-1 and Bcl-xl expression (Fig. 4A and B); whereas, Bcl-2 and Bax expression were unaffected (Fig. 4B). Knockdown of STAT3 decreased expression of Mcl-1 and Bcl-xl is caused by inhibition of STAT3 activity. Interestingly, tocilizumab did not induce cell death (Supplementary Fig. S5), suggesting that apoptotic cell death of CLL is regulated by multiple signal pathways.

CLL cells with the highest expression of CD126 were the most resistant to chlorambucil-induced cell death (Fig. 2D; Fig. 4D, CLL 1017, 1042, 1046, 1066, and 1003), but also showed the greatest tocilizumab-induced chemosensitization to chlorambucil with marked increases in cell death (Fig. 4D, CLL 1017, 1042, 1046, 1066, and 1003). In contrast, those cases with lower CD126 expression not only showed greater chemosensitivity to chemotherapy alone, but also were least responsive to the combination of chlorambucil and tocilizumab (Fig. 4D, CLL 1048, 1045, 1024, 1054, and 1043). The increased percentage of cell death induced by tocilizumab with chlorambucil over chlorambucil alone showed that there was no effect on this increased chemosensitization of increasing doses of chlorambucil (Supplementary Fig. S6), whereas there was a highly statistically significant direct correlation with CD126 expression ($P < 0.0001$, Fig. 4E). Collectively, these data indicate that combination of tocilizumab with chemotherapeutic drugs can overcome chemoresistance of CLL cells with higher (>10%) expression of CD126. In CLL cases with CD126 expression <10%, the average increase of apoptosis by tocilizumab was 3.6%, as compared with a mean increase of 13% in those cases with >10% CD126 expression ($P < 0.001$, Fig. 4F).

Discussion

Here we demonstrate for the first time that CLL cells have increased expression of CD126, as compared with normal B cells, which is significantly correlated with the constitutive activity of...
CD126 and Chemoresistance

The expression profile of IL6 membrane-bound receptor, CD126, is limited to normal hepatocytes, CD4+ or CD8+ T cells (15), certain inflammatory cells, and some cancer cells (16, 17). Signal transduction can still occur in the absence of surface CD126 via the IL6 trans-signaling pathway, where IL6 binds with the soluble form of CD126 (15). We found that CLL cells are positive for surface CD126 and the level of expression was tightly correlated with constitutive activity of STAT3 and response to chemotherapy. The clinical relevance of these findings was evaluated by LDT and response to treatment. CLL cases with a longer LDT (≥12 months) had lower CD126 expression versus cases with a more aggressive course (LDT < 12 months). Similarly, CLL cells had lower expression of CD126 in patients who achieved a CR versus those who did not. The correlation of chemosensitivity in vitro and in vivo was significant; the chemoresistance was greater in cases with higher CD126 expression and STAT3 activation. We found that CD126 was not correlated with known prognostic risk factors, such as stage of disease, CD38 expression, IgHV mutation status, and cytogenetic abnormalities. However, higher levels of CD126 were found to correlate with ZAP-70 positivity, although a larger study is required to fully evaluate the pathologic significance of membrane-bound CD126 in CLL.

In vivo, CLL cells are long-lived in peripheral blood, secondary lymphoid organs, and bone marrow (29) with survival dependent on external signals from the microenvironment and internal expression of antiapoptotic proteins (29, 37), which contribute to the chemoresistance of CLL cells and poor clinical outcomes (2). In cancer cells, aberrant IL-6 signaling and constitutive activation of STAT3 are closely related to cell proliferation and drug resistance (13), with increased IL6 levels associated with chemoresistance and poor prognosis (9). IL6 can induce STAT3 activation and increased production of IL6 (13, 24). CD126 mediates IL6-dependent STAT3 activation, while decreased CD126 (both membrane-bound and soluble) diminishes

Figure 4. Tocilizumab increases chemosensitivity in CLL cells. CLL cells were cultured with or without 10 μg/mL of tocilizumab for the indicated times. A, STAT3 phosphorylation. B, Mcl-1, Bcl-xl, Bcl-2, and Bax expression were determined by Western blotting. C, after knocking down STAT3 for 24 hours, Mcl-1, Bcl-xl, Bcl-2, and Bax expression were determined by Western blotting. D, association between cell death (PI+ cells) and CD126 expression and the tocilizumab-mediated increased cell death (tocilizumab plus chlorambucil-induced cell death versus chlorambucil alone, n = 33, P < 0.0001). E, tocilizumab increased chlorambucil-induced apoptosis in CLL divided by surface CD126 expression of <10% (n = 13) or >10% (n = 20; ***, P < 0.0001).
expression of phosphorylated STAT3 proteins (38). We demonstrate that tocilizumab can interrupt the IL6/CD126/STAT3 feedback loop. CD126 blockade by tocilizumab in CLL cells leads to decreased STAT3 phosphorylation and downregulation of Mcl-1 and Bcl-xl expression.

IL6 is a pleiotropic cytokine that regulates diverse cell functions including proliferation, differentiation, and tumorgenesis (13, 14, 24). However, in IgG-bearing human B lymphoblastoid cells, IL6 induces the cell-cycle inhibitor p18 (39); in hepatocellular carcinoma cells, IL6 downregulates CDK2, CDK4, cyclin D, and cyclin E expression (31), and IL6 also induces upregulation of p27 mRNA and protein in melanoma cells (40), resulting in cell-cycle arrest (31, 40). IL6-mediated cell-cycle arrest is via activation of STAT3 (31, 41). In CLL cells, inhibition of the IL6/CD126 interaction by tocilizumab decreases p27 expression and reduces p27/CDK2 or p27/cyclin E complexes formation, leading to cell exit from G0 and entry into the cell cycle (G1). Most of chemotherapeutic drugs are cell-cycle dependent. The cell-cycle quiescent cancer cells can escape from the killing induced by chemotherapeutic drugs. Stimulating the quiescent cells, cell-cycle entry has been proposed as a powerful approach to increase the chemosensitivity of cancer stem cell (42). The mechanisms by which IL6 mediates cell-cycle regulation in CLL are under further evaluation.

mAbs have become an increasingly important component of therapeutic regimens in oncology (43). IL6 pathway blockade with tocilizumab inhibits angiogenesis, cell proliferation, and tumor growth in cancer (24, 25) and anti-IL6 strategies have been investigated for various cancers, but not CLL. We have shown here that tocilizumab promotes cell-cycle shift of CLL cells from G0 to G1 phase, while p-STAT3, Mcl-1, and Bcl-xl decrease, and these changes are associated with increased chemosensitivity. This suggests that the IL6/CD126/STAT3 axis may represent a new therapeutic target for CLL in a novel approach to chemosensitization of quiescent CLL cells. Patient selection for tocilizumab therapy could be individualized to those cases with high CD126 expression on CLL cells, as these cases show the greatest chemosensitization both in vitro and in vivo, as well as the greatest response to CD126 blockade.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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CD126 and Chemoresistance
CD126 and Targeted Therapy with Tocilizumab in Chronic Lymphocytic Leukemia

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