CD126 and Targeted Therapy with Tocilizumab in Chronic Lymphocytic Leukemia

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Running Title: CD126 and chemoresistant

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Conflict of interest: The authors have declared that no conflict of interest exists.
Abstract

**Purpose:** Interleukin (IL)-6 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 leukemic (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 (CBL) or Fludarabine with or without anti-CD126 antibody Tocilizumab for 24 hours and IL-6-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 CBL or Fludarabine and poor *in vivo* treatment response of CLL patients. Blocking IL-6 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 to G1. These Tocilizumab-mediated changes induced chemo-sensitization in resistant CLL cells, with the greatest effect seen in cells with higher CD126 expression (P<0.001).

**Conclusion:** CLL cells with higher CD126 expression are more resistant to treatment *in vivo* and *in vitro* via IL-6-CD126-STAT3 axis. Blockade CD126 using Tocilizumab sensitizes CLL cells to chemotherapy.
Translational Relevance

Increased levels of both IL-6 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 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 chemoresistant 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 chemo-sensitization by Tocilizumab followed by a second hit in CLL.
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 require treatment at initial presentation (1). Although the standard treatment includes first-line chemo-immunotherapy 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). CLL prognostication at diagnosis is possible with clinical and biological parameters, such as deletions at chromosome 17p or 11q (3), absence of mutations in the immunoglobulin heavy chain gene (IgHV) (4,5), expression of protein tyrosine kinase zeta-associated protein 70 (ZAP-70) (6) the cell surface glycoprotein CD38 (7), and advanced clinical stage (Binet stage B/C) (8). Serum IL-6 levels have also been correlated with clinical outcome in CLL, with higher levels of IL-6 associated with a worse prognosis (9); similar results have been found in other cancers (10,11). Although IL-6 is elevated in the serum of humans suffering from breast, prostate, lung, liver and colon cancer, raised serum IL-6 is also seen in a wide variety of non-malignant diseases (12).

IL-6 is a multi-function cytokine that is produced by a variety of cell types, including T-cells, B-cells, dendritic cells, macrophages, fibroblasts, endothelial cells, monocytes and keratinocytes (13,14). However, the IL-6 membrane-bound receptor, CD126, is expressed only on normal hepatocytes, CD4+ or CD8+ T-cells (15), certain inflammatory cells, and some cancer cells (16,17). If cells do not express sufficient cell-surface CD126, signal transduction can still occur via the IL-6 trans-signaling pathway when IL-6 binds with the soluble form of CD126 (15).
The constitutive phosphorylation of STAT3 on tyrosine 705 residues (p-STAT3\textsuperscript{Y705}) is found in a wide variety of human cancer cells, whereas it is absent in fresh CLL cells, which in contrast show constitutively phosphorylated STAT3 on serine 727 residues (p-STAT3\textsuperscript{S727}) (18,19). However, in CLL cells STAT3 phosphorylation on both Tyr705 and Ser727 can be induced by IL-6 (19). There is limited data on the function of p-STAT3\textsuperscript{S727}: nuclear p-STAT3\textsuperscript{S727} mediates gene transcription and mitochondrial p-STAT3\textsuperscript{S727} regulates mitochondrial respiration (19,20).

Tocilizumab, a humanized anti-CD126 monoclonal antibody, approved by Food and Drug Administration for treatment of rheumatoid arthritis (21), targets membrane-bound and soluble CD126 and blocks IL-6 classical and trans-signaling (22). IL-6 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 present 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, and the City HA Local Research Ethical Committee for in vitro studies on blood samples from CLL patients. 58 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 1). Peripheral blood was obtained after written 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), the cells were used immediately or cultured in complete RPMI-1460 containing 10% fetal calf serum, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin (Sigma) for the further experiments. The normal B lymphocytes from health donor were CD19 positive selection by a column purification system with anti-CD19 beads (Milteny Biotec, Bergish-Gladbach, Germany) (18), the purified CD19+ normal B lymphocytes were stored in liquid nitrogen.

Antibody and reagents

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

Measurement of membrane-bound IL-6 receptor CD126
PBMC from CLL patients were incubated with anti CD126-PE and CD19-PerCP antibody (BD Biosciences) for 15 minutes at room temperature in the dark, then the CD19+/CD126+ dual positive cells were determined by FACS Canto I (Becton Dickinson).

Treatment and cell death assays

Cell viability was determined by Annexin V and propidium iodide (PI) dye exclusion - after treatment by 20µg/ml Chlorambucil or 20µg/ml of Fludarabine or 10µg/ml of Tocilizumab (donated by Chugai Pharmaceutical Co Ltd, Japan) 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 IL-6

CLL cells were isolated immediately after phlebotomy and used post-ficoll as PBMC. If greater than 95% of cells were CD19+, but if not, B-CLL cells were purified using a CD19+ cell isolation kit (MiltenyiBiotec, Bergish-Gladbach, Germany), with a purity of >97%. 5x10^6/ml cells were cultured in RPMI-1640 medium at 37°C in a humidified incubator with 5% CO2. The supernatants were stored at -80°C until assayed for IL-6 by ELISA, according to the manufacturer’s instructions (R&D systems, Abingdon, UK).

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% non-fat milk in TBST for 1 hour and then incubated with indicated primary antibodies overnight at 4°C, visualized by GeneSnap (SynGene, Cambridge, UK) after adding ECL plus (GE Healthcare Life Science).

**Immuno-precipitation**

Immuno-precipitation 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.

**Transfection of siRNAs**

Human STAT3 and control siRNA A (Santa Cruz, sc-29493; sc-37007) were used for knocking down gene expression. Fresh primary CLL cells (5 × 10⁷/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’s t test. Correlation of multiple variables was analyzed by Spearman’s 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 1. 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 has surface CD126 expression, although was highly variable in 58 CLL cases (Mean=20.1%, range from 4.5% to 59%) (Figure 1A and Supplementary Figure 1A). In cultured CLL cells, CD126 expression was stable over 48 hours compared with in vivo expression (Supplementary Figure 1B). This 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 IL-6 (23), and overexpression of CD126 enhanced IL-6/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 (Figure 1B). In CLL cells, there was a significant correlation between the levels of constitutive activation of STAT3 and membrane-bound CD126 expression (Figure 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 ZAP-70 and cytogenetic status measured by fluorescent in situ hybridization (3-7). The levels of CD126 expression
were evaluated in each of these prognostic subgroups of CLL and between treated and untreated patient groups (Figure 1D). CD126 expression was significantly higher in ZAP-70 positive versus negative cases of CLL (P<0.01), whereas there were no statistic 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 CBL-induced cell death and poor in vivo chemo-responsiveness compared with the lower expression of CD126 cases (Supplementary Figure 2).

**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 anti-apoptotic proteins (16,17), which contribute to the chemoresistance of CLL cells and poor clinical outcomes (2). Chlorambucil (CBL), 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%, Figure 2A). Cells with higher STAT3 activation were more resistant to CBL (P<0.001, Figure 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 CBL-induced death as a cut-off) had significantly higher CD126 expression (P<0.0001, Figure 2C). The inverse correlation between CBL-induced cell death and CD126 expression was statistically significant for both CBL (R= -0.57, P<0.0001, Figure 2D), and Fludarabine-induced cell death (R= -0.746, P<0.01, Supplementary Figure 3).
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 (ie, a partial response, stable disease and progressive disease). CD126 expression in the CR group was significantly lower than in the non-CR group (Figure 2E, P<0.01). In vivo, CLL cells are typically long-lived, accumulate, but have a low proliferation rate (29), which can be evaluated clinically by the lymphocyte double time (LDT). Cases with a longer LDT (>12 months) had lower CD126 expression compared to cases with a shorter LDT (<12 months) (Figure 2F, P<0.01). These results suggest that CLL patients with lower CD126 expression may have good response to the treatment and less aggressive disease.

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 anti-apoptotic Bcl-2 family proteins (30). It was previously reported that IL-6-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 (Figure 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 (Figure 3A, B), as detected by Ki67 positivity, without further progression through the cell cycle to G2/M/S phases (Figure 3A, upper
panel), as numbers of cells in S/G2/M phases were not altered (Figure 3 A, bottom panel). In resting cells, cell cycle control is achieved by binding of p27 with cyclin E/cyclin dependent kinase 2 (CDK2) complexes to prevent entry into G1 phase (34). Progressive decreases in p27 or overexpression of cyclin E/CDK2 lead to G0 exit and G1 progression, allowing G0 to S phase transitions (34). In cultured CLL cells, the expression of p27, cyclin E and CDK2 were stable up to 24 hours (Figure 3 C, right-hand panel). However, treatment of CLL cells with Tocilizumab led to increased expression of cyclin E and CDK2, and decreased expression of p27 (Figure 3 C). Using immunoprecipitation, we found that treatment with Tocilizumab significantly decreased binding between p27 with cyclin E and CDK2 (Figure 3 D). These data demonstrate that blocking CD126 by Tocilizumab promotes cell cycle re-entry 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 STAT3 results in up-regulating secretion of IL-6, expression of anti-apoptotic proteins, and promoting cell survival and chemoresistance (23,35). The autocrine IL-6 itself maintains STAT3 activity, thereby creating a positive feedback loop. CLL cells can produce IL-6 in vitro (36), with autocrine IL-6 detectable at 6 hours and then increasing over 48 hours (Supplementary Figure 4). In cultured CLL cells, the constitutively activated p-STAT35727 and its targeted Bcl-2 family proteins showed stable expression up to 48 hours (Figure 4 A, B). Inhibition of CD126 by Tocilizumab led to decreased STAT3 phosphorylation (detectable at 6 hours) and down regulation of Mcl-1 and Bcl-xL expression (Figure 4 A, B); whereas, Bcl-2 and Bax expression were unaffected
Knockdown of STAT3 reduced Mcl-1 and Bcl-xL expression (Figure 4 C), indicating that decreased expression of Mcl-1 and Bcl-xL is caused by inhibition of STAT3 activity. Interestingly, Tocilizumab did not induce cell death (supplementary Figure 5), 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 CBL-induced cell death (Figure 2 D; Figure 4 D, CLL 1017, 1042, 1046, 1066 and 1003), but also showed the greatest Tocilizumab-induced chemosensitization to CBL with marked increases in cell death (Figure 4 D, 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 CBL and Tocilizumab (Figure 4 D, CLL 1048, 1045, 1024, 1054 and 1043). The increased percentage of cell death induced by Tocilizumab with CBL over CBL alone showed that there was no effect on this increased chemosensitization of increasing doses of CBL (Supplementary Figure 6), whereas there was a highly statistically significant direct correlation with CD126 expression (P<0.0001, Figure 4 E). Collectively, these data indicate that combination of Tocilizumab with chemotherapeutic drugs can overcome chemoresistance of CLL cells with higher (more than 10%) expression of CD126. In CLL cases with CD126 expression less than 10%, the average increase of apoptosis by Tocilizumab was 3.6%, as compared to a mean increase of 13% in those cases with great than 10% CD126 expression (P<0.001, Figure 4 F).
Discussion

Here we demonstrate for the first time that CLL cells have increased expression of CD126, as compared to normal B cells, which is significantly correlated with the constitutive activity of STAT3 and chemoresistance. Blocking CD126 with Tocilizumab sensitized CLL cells to chemotherapy.

The expression profile of IL-6 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 IL-6 trans-signaling pathway, where IL-6 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<12months). 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, cytogenetic abnormalities. However, higher levels of CD126 was found to correlate with ZAP-70 positivity, although a larger study is required to fully evaluate the pathological 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 anti-apoptotic 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 IL-6 levels associated with chemoresistance and poor prognosis (9). IL-6 can induce STAT3 activation and increased production of IL-6 (13,24). CD126 mediates IL-6-dependent STAT3 activation, while decreased CD126 (both membrane-bound and soluble) diminishes expression of phosphorylated STAT3 proteins (38). We demonstrate that Tocilizumab can interrupt the IL-6/CD126/STAT3 feedback loop. CD126 blockade by Tocilizumab in CLL cells leads to decreased STAT3 phosphorylation and down-regulation of Mcl-1 and Bcl-xL expression.

IL-6 is a pleiotropic cytokine that regulates diverse cell functions including proliferation, differentiation and tumorigenesis (13,14,24). However, in IgG-bearing human B lymphoblastoid cells, IL-6 induces the cell cycle inhibitor p18 (39); in hepatocellular carcinoma cells, IL-6 down-regulates CDK2, CDK4, Cyclin D and Cyclin E expression (31); and IL-6 also induces up-regulation of p27 mRNA and protein in melanoma cells (40), resulting in cell cycle arrest (31, 40). IL -6 mediated cell cycle arrest is via activation of STAT3 (31, 41). In CLL cells, inhibition of the IL-6/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 chemotherapy drugs are cell cycle-dependent. The cell cycle quiescent cancer cells can escape from the killing induced by chemotherapeutic
drugs. Stimulating the quiescent cells entry cell cycle has been proposed as a powerful approach to increase the chemosensitivity of cancer stem cell (43). The mechanisms by which IL-6 mediates cell cycle regulation in CLL are under further evaluation.

Monoclonal antibodies (mAbs) have become an increasingly important component of therapeutic regimens in oncology (42). IL-6 pathway blockade with Tocilizumab inhibits angiogenesis, cell proliferation and tumor growth in cancer (24,25) and anti-IL-6 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 IL-6/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 chemoresistance both in vitro and in vivo, as well as the greatest response to CD126 blockade.

Acknowledgments

We thank the all patients with CLL who kindly donated their blood samples for this study and Chugai Pharmaceutical Co Ltd kindly offer Tocilizumab.

Funding support

This work was supported by the National Natural Science Foundation (NNSF) of China (81172109) to FL.
References


22. Nishimoto N, Terao K, Mima T, Nakahara H, Takagi N, Kakehi T. Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease. Blood 2008;112(10):3959-64.


Figure legends

Figure 1. Correlation between expression of CD126 and STAT3 activity in CLL. (A) CD126 expression in CLL and normal B cells (CLL, n=58, normal B-cells [NBC], n=20). (B) In vivo constitutive activity of STAT3 (anti-phospho-STAT3-Ser-727 antibody, Cell Signaling Technology) was detected in fresh CLL cells and CD19+ normal B cells from health donors. (C) The correlation between levels of p-STAT3 and CD126 expression (n=17, P<0.001). (D) CD126 expression in different prognostic groups: Binet stage at presentation (A vs B/C, n=58), treated vs untreated, CD38 (negative or positive; cut-off 20%, n=58), cytogenetic (absence or presence of 11q and 17p deletions, n=58), ZAP70 (negative or positive; cut-off 20%, n=48) and IgHV mutated vs unmutated (n=20) cases. *P<0.05, **P<0.01, ***P<0.001.

Figure 2. Correlation between expression of CD126 and chemosensitivity of CLL. Fresh CLL cells were incubated with 20 µg/ml of CBL for 24 hours. (A) Cell death was measured as PI+ cells by FACS (n=58). (B) The correlation between p-STAT3 and CBL-induced cell death (n=14) P<0.001. (C) CD126 expression in CBL sensitive (cell death > median) and CBL resistant (cell death < median) cases, n=58, P<0.0001. (D) The correlation between CD126 expression and CBL-induced cell death (n=48), P<0.001. (E) CD126 expression in patients achieving a complete remission (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 month (n=20). *P<0.05, **P<0.01, ***P<0.001.
**Figure 3: Tocilizumab-induced cell cycle redistribution of CLL cells.** CLL cells were cultured with or without 10 µg/ml of Tocilizumab for the indicated times. (A) Cell cycle distribution was analyzed by expression of Ki67 and Propidium iodide (PI) for DNA content in CLL cells after 16 hours in culture, isotype control cells (IgG), untreated control cells (Control) and Tocilizumab-treated cells. (B) Ki67 expression in Tocilizumab treated or untreated CLL cells (n=9). (C) The expression of p27, CDK2 and Cyclin E in CLL cells with or without Tocilizumab treatment at indicated times. (D) Fresh CLL cells were treated with 10 µg/ml of Tocilizumab for 12 hours. The interaction between p27 with CDK2 or Cyclin E was analyzed by Western blotting following immune-precipitation with anti-p27 antibody. **P<0.01.

**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 was determined by Western blotting. β-actin were used as loading control. (D) Association between cell death (PI+ cells) and CD126 expression. CLL cells were treated with CBL with or without Tocilizumab for 24 hours. (E) The correlation between CD126 expression and the Tocilizumab-mediated increased cell death (Tocilizumab plus CBL induced cell death versus CBL alone, n=33, P<0.0001). (F) Tocilizumab increased CBL-induced apoptosis in CLL divided by surface CD126 expression of less than 10% (n=13) or more than 10% (n=20, *** P<0.001).
Figure 1

A

B

C

D

**R=0.731  p < 0.001**
Figure 2
**Figure 3**

A) **IgG Control**

![Graphs showing Ki67 expression](image)

**DNA Content**

B) **Ki67 expression (%)**

![Bar graph showing Ki67 expression](image)

C) **Tocilizumab treated vs. Untreated**

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D) **Immunoprecipitation with p27**

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![Immunoprecipitation graphs](image)

**Relative expression (AU)**

![Bar graph showing relative expression](image)
Figure 4
Clinical Cancer Research

CD126 and targeted therapy with Tocilizumab in Chronic Lymphocytic Leukemia

Fengting Liu, Li Jia, Ping Wang, et al.

Clin Cancer Res Published OnlineFirst December 28, 2015.

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