

Chronic lymphocytic leukemia nurse-like cells express hepatocyte growth factor receptor (c-MET) and indoleamine 2,3-dioxygenase and display features of immunosuppressive type 2 skewed macrophages

Paolo Giannoni,¹ Gabriella Pietra,¹ Giorgia Travaini,³ Rodolfo Quarto,¹ Genti Shyti,¹ Roberto Benelli,⁴ Laura Ottaggio,⁵ Maria Cristina Mingari,^{1,2,4} Simona Zupo,⁵ Giovanna Cutrona,⁵ Ivana Pierri,⁶ Enrico Balleari,⁷ Alessandra Pattarozzi,⁸ Marco Calvaruso,⁹ Claudio Tripodo,⁹ Manlio Ferrarini,¹⁰ and Daniela de Toter³

¹Department of Experimental Medicine, University of Genoa; ²Excellence Center for Biomedical Research, University of Genoa; ³Transfer Gene Laboratory and ⁴Immunology, Department of Integrated Oncology Therapies, IRCCS AOU San Martino-IST, Genoa; ⁵Pathology and Molecular Diagnostic Laboratory, Department of Service, IRCCS AOU San Martino-IST, Genoa; ⁶Clinical Oncohematology, University of Genoa; ⁷Clinic of Internal Medicine 3, Department of Hematology, IRCCS AOU San Martino-IST, Genoa; ⁸Section of Pharmacology, Department of Internal Medicine, Genoa; ⁹Pathological Anatomy, Tumor Immunology Unit, Department of Science for Health Promotion and Maternal Infantile "G. D'Alessandro", Palermo; and ¹⁰Direzione Scientifica IRCCS AOU San Martino-IST, Genoa, Italy

©2014 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2013.091405

Manuscript received on May 14, 2013. Manuscript accepted on February 17, 2014.

Correspondence: daniela.detotero@istge.it / daniela.detotero@hsanmartino.it

Cell preparation:

After written informed consent and in agreement with the Helsinki declaration peripheral blood mononuclear cells (PBMCs) or Bone marrow (BM) cells were withdrawn from CLL patients. Patients' characteristics are summarized in Supplementary Table I. IgVH mutational status, ZAP70, CD38 expression and cytogenetic studies were performed as described (de Toter, Blood 2006, 107:3708; Ottaggio L, Hematol Oncol 2008, 26:39). Samples were isolated by density gradient centrifugation (Ficoll, Biochrom, Berlin Germany), resuspended in RPMI 1640 + 10% FBS (Lonza, BioWhittaker, Verviers, Belgium) and preliminarily characterized after staining with fluorochrome-conjugated anti-CD19,-CD5,-CD23,-CD3,-CD4,-CD8,-CD38 monoclonal antibodies (moAbs) (Immunotools, Friesoythe, Germany) and subsequent flow cytometric analysis . Peripheral blood of healthy donors were kindly provided by the blood bank of AUO San Martino-IST, Genoa, Italy.

Nurse-like cells (NLCs) were derived by culturing 12×10^6 cells of PBMCs from CLL patients in 2 ml of complete medium in 6 well plates. After 14 days CLL B cells were removed by vigorously washing each well with medium-culture. The adherent cells were thus detached by trypsin and further analyzed by flow cytometry or subjected to RNA extraction or treated with growth factors for signaling experiments. Some experiments were further performed by staining directly NLCs cultured for 14-days in 8 well chambered cover glass (BD Biosciences Europe, Erembodegem, Belgium). To this aim 2×10^6 PBMCs of CLL patients were cultured in a total volume of 300 μ l of complete medium for 14 days and B cells were further washed out. Purified monocytes CD14+ from PBMCs of CLL patients or of healthy donors were obtained by positive selection with CD14-conjugated magnetic beads following the manufacturer's instructions (Milteny Biotec, Bergish Gladbach, Germany).

The human acute leukemic cell line THP-1 was from the ICLC cell Bank (Genoa, Italy: www.iclc.it) THP-1 cell line was maintained in RPMI 1640 medium supplemented with 10% FBS. In selected experiments THP-1 cell were stimulated with recombinant human (rh) HGF (PeproTech EC, London, UK) or PMA (Sigma-Aldrich, Milan, Italy) or rhIFN γ (Milteny Biotec , Calderara di

Reno, Bo Italy) and LPS (Sigma-Aldrich), or rhIL-4 (Pepro Tech). Moreover in selected experiments PMA-induced THP-1 cells were also pre-treated with the neutralizing humanized anti-HGF moAb L2G7 obtained under a Material Transfer Agreement with Galaxy Biotech (Cupertino, CA,USA).

Fluorescence microscopy and flow cytometry

Nurse-like cells (NLCs) were cultured in 8 well chambered cover glass. After 14 days B cells were vigorously washed out while adherent cells were fixed with PBS+ 4% PFA and permeabilized with PBS+ 0,1% saponin. Cells were thus stained with primary antibodies anti-c-MET (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IDO (Vinci-Biochem, Firenze, Italy), anti-vimentin (DakoCytomation,A/S; Glostrup, Denmark) or anti-CD68 (Biolegend Inc, San Diego, CA, USA) for 30 min and after two washes with a secondary moAb goat anti-rabbit alexa488-conjugated (Molecular Probes, Oregon USA) or a secondary goat anti-rabbit FITC or PE-conjugate or a goat anti-mouse-PE-conjugated (Jackson ImmunoResearch Laboratories, Baltimore, USA) moAb. To study IL-10 expression NLCs were preventively treated with monensin (Biolegend Inc, San Diego, CA, USA) for 4 hours. After this time cells were washed, fixed as described above and stained with a directly PE-conjugated anti-IL-10 antibody (Biolegend Inc). Fluorescein-conjugated anti-CD14,-CXCR4-(Immunotools) or the anti-CD163PE (Biolegend Inc) moAbs were also utilized to directly stain NLCs cultured in chamber slides or in 6-well plates. To analyze STAT3 activation NLCs derived in chamber slides were treated with HGF in sera-free medium for 40 min and further fixed, permeabilized and stained with a specific anti-pSTAT3 PE-conjugated moAb (Santa Cruz Biotechnology). Nuclear counterstainings were performed with DAPI (Invitrogen, Milan,Italy) Images were acquired on a Nikon Digital Sight DS-5Mc camera mounted on an Olympus BX51 fluorescence microscope, and recorded by the NIS-Element F software, release 2.20.

c-MET and IDO expression in NLCs or in fresh monocytes from PBMCs of CLL patients or from normal donors was also analyzed by flow cytometry (FacScan BD Biosciences, San Jose, CA,

USA). In this case the cells were first detached by plates with trypsin, preventively fixed and permeabilized and further stained with primary unconjugated anti-c-MET or anti-IDO antibodies for 45 min at 4°C. After two washes permeabilized cells were stained with goat anti-rabbit alexa-488-conjugated or goat-anti-rabbit PE- or FITC-conjugated secondary antibodies. For analysis cells were gated on the basis of Forward Scatter (FSC)/Side Scatter (SSC) parameters and CD14 expression, and at least 3000 gated events were acquired. Only gated cells that were at least 95% CD14+ were analyzed for c-MET and IDO expression.

To quantify the pSTAT3 immunopositive cells area discriminating between HGF-treated or – untreated cells we performed image analysis using the National Institute of Health Image J freeware (release 1.38X; <http://rsb.info.nih.gov/ij/>). Photographs of untreated or HGF-stimulated cells, acquired under the same conditions, enlargement and resolution, were set to the identical color threshold; the boundaries of cell areas, in each image, were defined by a computerized procedure that automatically grouped contiguous thresholded pixels, immunopositive to PE-conjugated anti-p-STAT3 moAb, in the largest continuous close-curved outlines; the procedure tagged each outline and calculated its integrated density, i.e. the product of its area –in square pixels- and of its mean gray value –i.e. the average gray value of all pixels in the selection (for RGB images an automatic feature of the software allows conversion of colored pixels to relative gray values). The procedure provided a mirror image of all outlines in each photograph; only outlines that included DAPI-positive nuclei, upon superimposing the mirror image to its respective original one, were included in measurement calculations and subsequent analysis. Average values of integrated areas in HGF-treated cells were normalized to the values obtained in untreated cells from the same patient.

Analysis of STAT3 and pSTAT3 activation by Western Blot.

STAT3, pSTAT3 and β Actin were also evaluated by Western Blotting in purified monocytes from normal donors, or from CLL patients or from NLCs at basal conditions or after HGF treatment as well as in THP1 cells as controls. NLCs and monocytes were cultured overnight in 24 well plates

with RPMI+FCS2%. The day after the cells were treated with HGF (100ng/ml) for 40 min. Cells were thus gently scraped and total protein were extracted. THP1 cell line was utilized as positive control and also to choose the best time of treatment. Total proteins were extracted using the M-PER® Protein extraction reagent (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA). Protein content was assessed by the Pierce BCA assay kit (Thermo Scientific). An equal amount of total protein lysates for each couple of sample (untreated and HGF treated) were subjected to 4%-12% SDS-PAGE separation and transferred on PVDF membranes by standard Western blotting. Membranes were blocked for 2h in TBS-T buffer (20 mM Tris/HCl; 0.5 M NaCl; 0.1% Tween20) containing 5% dry milk powder, washed three times in TBS-T and incubated overnight at 4°C with anti-STAT3 (Cell Signaling Technologies Inc., Danvers, MA, USA), anti-P-STAT3 primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Ca., USA) or anti-βactina (cat. n. A2228, Sigma –Aldrich Milan, Italy) in 5% low fat milk in TBS-T buffer. Immunodetection was performed adding specific anti-rabbit or anti-mouse HRP-linked secondary for 1h at room temperature, followed by visualization by means of ECL™ Prime Western Blotting Detection Reagent on Amersham Hyperfilm™ ECL chemiluminescence film (antibodies, detection kit and film were from Amersham-GE Healthcare Ltd.; Little Chalfont, UK) according to the manufacturer's instructions. The relative pSTAT3 levels were determined by densitometric analysis using a ChemiDoc XRS apparatus (BIO-RAD laboratories, Milan, Italy) equipped with ImageLab software for the quantification chemiluminescence band intensities.

Immunohistochemistry and immunofluorescence analysis of lymphonodal and bone marrow biopsies.

For in situ single-marker immunohistochemical analysis sections were deparaffinized with xylene and rehydrated to water through a graded alcohol series. Antigen retrieval was performed using 10 mmol citrate buffer (pH 9.0) at high temperature for 20 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Sections were incubated with anti-human c-MET (Santa

Cruz Biotechnology) or anti-human IDO (Vinci-Biochem) at room temperature for 1 hour. Staining was performed with the Novolink Max Polymer Detection System (Leica Microsystems) using DAB (3,3'-Diaminobenzidine) substrate chromogen (Leica Microsystems). Finally, sections were counterstained with hematoxylin.

For immunofluorescence analysis tissue sections were deparaffinized with xylene and rehydrated to water through a graded alcohol series. Following antigen retrieval, the samples were incubated, by sequential immunostaining, with primary antibodies anti-human c-MET (Santa Cruz Biotechnology), anti-human IDO (Vinci-Biochem), anti-human CD163 (Biolegend Inc.), anti-human vimentin, and anti-human CD68 (Novocastra, Newcastle Upon Tyne, UK). After Fc blocking, Alexa 488 and Alexa 568 Fluor-conjugated secondary Abs (Invitrogen) were used. Slides were evaluated using a Leica DM2000 microscope equipped with a Leica DFC320 digital camera.

RNA isolation and quantitative Real time PCR

Total messenger RNA was extracted from each cell type used according to the instructions of the PerfectPure RNA Cultured Cell Kit (5-Prime GmbH, Hamburg, Germany); generation of the cDNA pools, for each each sample, was carried out by using the SuperScriptTM III First-strand synthesis system for RT-PCR Kit (Invitrogen). Primer sets for each gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH; hepatocyte growth factor, HGF; HGF receptor, c-MET; and stromal derived factor 1, SDF-1) were derived from published sequences (23,14), or purposely designed (CD-68 forward: 5'-gATTCATgCAGgACCTCCAgC, reverse: 5'-gAATgTCCACTgTgCTgCgTg; SDF-1 receptor, CXCR4 forward: 5'-gAgACTCATAATCCAAGTg, reverse: 5'-AgCATAcAgTTTCTTgCAg; Transforming Growth Factor β -1, TGF- β -1 forward: 5'-ACgTggAgCTgTACCAgAAAT, reverse: 5'-CCggTAgTgAACCCgTTgAT; Interleukin-10, IL-10, forward: 5'-ggAggACTTTAAgggTTACCT, reverse: 5'-TCTCTTggAgCTTATTAAGgC). Relative expression of each gene of interest was assessed by syber-green real time quantitative RT-PCR; samples of cDNAs were amplified with the RealMasterMix SYBR ROX 2,5X (5'-Prime) in

an Eppendorf Mastecycler Realplex² apparatus, performing quadruplicate reactions for each sample as follows: 95°C for 3 minutes; 35 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 40 sec, and a final step at 72°C for 7 min. The annealing step for c-MET was instead performed at 52°C. The specificity of the reaction products was counterchecked by melting curve analysis. Gene expression in each sample was normalized to the endogenous control gene GAPDH.

Evaluation of c-MET and IDO expression on normal monocytes following their co-culture with CLL B cells.

Purified monocytes (90×10^3) from different healthy donors (n=4) were cultured alone or co-cultured with purified B cells (2×10^6) from different CLL patients (n=7) or with B cells from normal donors (n=2). After 10 days CLL cells or normal B cells were washed out, attached monocytes were scraped off and stained with anti-c-MET and anti-IDO antibodies to be analyzed by flow cytometry as above described.

Quantification of HGF in different media cultures.

HGF production was detected by enzyme linked immunosorbent assay (ELISA) kit (Quantikine Sandwich ELISA kit; R&D System Europe Ltd, Abingdon,UK) according to manufacturer's instructions. We examined supernatants obtained from fresh purified monocytes (CD14+) of CLL patients or from CLL cells cultured for 48h, or from purified monocytes/NLCs plus CLL cells co-cultured for 14 days or NLCs re-cultured alone for 48h after B cells removal. Briefly, monocytes were first purified by CD14+magnetic beads and further cultured alone (90×10^3 cells/ well) for 48h or with CLL cells (3×10^6 cells/well) in a 24 well plate for 14 days. In a separate well the same number of monocytes and CLL cells were dispensed and co-cultured for 12 days. At day 12 CLL cells were recovered and adherent differentiated NLCs were re-cultured in fresh culture medium for 48h more. All the media from the different cultures were kept frozen at -20°C until use. HGF expression was also evaluated on CLL cells or on monocytes/NLCs either fresh or following their

co-culture (4, 8, 11, 14 days) by flow cytometry: CLL cells were co-cultured as described above in the Elisa assay section and the HGF expression was assessed by 4-colour staining (CD19/CD5/CD14/HGF) in CLL cells. Briefly CLL cells, recovered from the co-cultures at different time points, were stained with directly conjugated anti-CD19-PECY7,-CD14FITC (BD Biosciences), -CD5APC (Dakocytomation) for 30 min. After two washes with PBS+2%FCS, cells, fixed and permeabilized with FIX/PERM buffers (Invitrogen), were stained with anti-HGF antibody (Santa Cruz) for 30 min. Following two washes the cells were further counterstained with a secondary anti-Rabbit PE antibody (Jackson), incubated at 4°C for 30 min more and further analyzed by flow cytometry. Percentage and mean fluorescence intensity of HGF expression was assessed on CD19+/CD5+ gated CLL cells. HGF expression on monocytes/NLCs was determined by staining with anti-HGF moAb and anti-CD14 moAbs: adherent cells were recovered by gentle scraping, fixed/permeabilized, stained with anti-HGF or anti-CD14 moAbs and incubated for 30 min at 4°C at dark. Cells were further counterstained with secondary anti-rabbit or anti mouse PE-conjugated secondary antibody to be analyzed by flow cytometry.

Blocking of CLL survival by anti-HGF and anti-CXCR4 neutralizing antibody.

Purified monocytes (30×10^3) were cultured with CLL cells (3×10^6) in 24 well plates for 12 days to induce NLCs differentiation. CLL cells were thus recovered, washed, counted and replated with or without NLCs in presence or absence of the neutralizing anti-HGF moAb (L2G7) alone or in association with an anti-CXCR4 moAb (R&D System). Apoptosis of CLL B cells were then evaluated by DiOC6 (3,3' dihexylocarbocyaniniodide; Sigma-Aldrich) staining, as previously described (de Totero D. et al Blood 2006,107:3708), at different times of re-culture (2, 4, 6 days).

Determination of the inhibition of T cell proliferation by monocytes or NLCs from CLL patients.

In order to determine potential suppression of T cell proliferation, peripheral blood mononuclear cells (PBMCs), separated from normal donors, were preventively labeled with carboxylfluorescein-diacetatesuccinimidyl-ester (CFSE) (Sigma). CFSE labeling will allow to quantify the number of cell division relative to T cell proliferation. Briefly PBMCs were resuspended in pre-warmed PBS containing 5 μ M CFSE and incubated at 37°C for 8 min. After two washes with RPMI +FCS10% the cells were counted and further plated in 24 well/ plates with anti-CD3 supernatant (UCHT1) and IL-2 (15U/ml). At the same time purified monocytes, obtained from peripheral blood mononuclear cells of normal donors or CLL patients by positive selection with anti-CD14-conjugated magnetic microbeads (Milteny Biotec), were seeded at graded concentration (30000, 60000, 90000, 120000 cells/well) in 24 well plates. After 24 hours of incubation pre-stained CFSE and pre-activated T cells were recovered, washed, counted and added at a concentration of 3x10⁵/well to monocytes either from normal donors or from CLL patients and cocultured together. After 4-5 days of co-culture T cells were harvested, washed and stained with an anti-TCR $\alpha\beta$ -PC5 moAb (BD Biosciences). Cells were then analyzed by a FACSCalibur flow cytometer (BD) and the reduced intensity of CFSE staining, related to the numbers of T cell divisions, was evaluated on gated TCR $\alpha\beta$ + lymphocytes. To further investigate whether NLCs were capable of inhibiting T cell proliferation we preliminarily cultured purified monocytes from each CLL patients at a concentration of 90 x 10³ cells /well in 24 well plates with 6x10⁶ autologous B cells/well for 14 days. After this time leukemic-B cells were vigourously washed out while the attached NLCs were co-cultured with 3x10⁵ CFSE pre-stained/ pre-activated T cells and after 4-5 days T cell proliferation was determined as above described. In selected experiments neutralizing anti-IL-10 (2 μ g/ml) (Biolegend), or anti-TGF β (400ng/ml)(R&D System, Minneapolis, MN, USA) moabs or IDO inhibitors (1-methyl-L-tryptophan + 1-methyl-D-tryptophan, (Vinci-Biochem) were utilized to identify potential cytokines responsible of the inhibition of T cell proliferation.

Determination of Treg expansion in long-term co-cultures of enriched CLL-monocytes and autologous PBMCs.

PBMCs (3×10^6) from CLL patients (N=2) and autologous purified monocytes (90×10^3) were cultured together in 24 well plates. The presence of Treg was evaluated in fresh PBMCs and after some days of cultures (8, 11, 14 days). To this aim we recovered cells in suspension and performed 3-color staining with anti-CD4FITC,-CD25APC (BD Biosciences), -FOXP3PE (Biolegend Inc) moAbs. After previous membrane staining with anti-CD4 FITC and anti-CD25 APC moAbs, the cells were fixed, permeabilized and further stained by the directly conjugate FOXP3-PE moAb or isotypic control. Percentage of FOXP3 expression on CD4+/CD25^{high}+ cells identified the percentage of T reg cells (Baecher-Allan C et al; Journal of Immunol 2001, 167:1245).

LEGENDS to SUPPLEMENTAL FIGURES:

Supplemental Figure 1:

Western Blot analysis of STAT3 phosphorylation, STAT3 and β Actin, of THP1 cell line or representative healthy monocytes (n=3), CLL-monocytes (n=2 as pool of monocytes or n=4 as single cases examined) and NLCs (n=3) before and after HGF treatment (+HGF). For THP1 a time course (0, 10', 40', 90') experiment of HGF treatments is presented. Membranes were subjected to densitometric analysis and pSTAT3 normalization was performed versus total STAT3 protein for each sample. Additionally the same normalization was also performed versus β ACTIN protein, yielding essentially the same results. Histograms represent the normalized ratio of densitometric values between pSTAT3/STAT3 (upper graphs) or pSTAT3/ACTIN (lower graphs), in untreated (CTR) or treated (+HGF) samples for each cell type. Standard deviation refers to 3 separate readings of each blot.

Supplemental Figure 2

Real time PCR expression ratios of IL10, c-MET, HGF, CD68, SDF-1 and CXCR4 with the housekeeping gene GAPDH.

PCR analysis show that IL-10, c-MET, HGF, CD68 and SDF-1 were relatively more expressed on Nurse-like cells than on B cells while CXCR4 was expressed at high levels on B leukemic cells, as previously reported (Burger JA et al.: Blood 1999, 94(11): 3658-3667). n= number of cases assessed; each panel depicts the relative p values determined by Student t test; n.s.= not significant.

Supplemental Figure 3

Immunofluorescence analysis of c-MET and IDO expression on CD68+ or CD14+ Nurse-like cells derived from Bone Marrow aspirates.

c-MET was expressed on CD68+ or on CD14+ large cells with morphology of NLCs derived from 14-days cultures of Bone marrow aspirates; one representative case (#3) is shown of two studied. CD14+ large cells also scored positively for IDO as shown in merged images. Presented images are 20X and inserts 40X. BF=bright field.

Supplemental Figure 4

Immunofluorescence study of the expression of CD163 and CXCR4 on 14-days derived large cells showing typical nurturing features.

Cells cultured in chamber slides were challenged with monoclonal anti-CD163-PE-conjugated antibody or with anti-CXCR4/goat-anti-mouse-FITC-conjugated and anti-CD68-PE-conjugated antibodies. Counterstaining was performed with DAPI. As shown in brightfield (BF) or in merged images large CD163+, CXCR4+/CD68+ cells are surrounded by small DAPI+ cells suggestive of docking residual leukemic B cells. Presented images are 20X and inserts 40X. Numbers in parenthesis refer to patients Id.

Supplemental Figure 5

Determination of inhibitory effect of CLL-monocytes and Nurse-like cells on T cell proliferation by CFSE staining.

A) Graded concentration of monocytes from normal or CLL donors were used to evaluate inhibition of T cell proliferation. Histograms depict one representative case of each cell type (n=8 cases of CLL patients and n=8 healthy donors studied). Values indicate the percentage of cells with low CFSE expression: the dye fluorescence becomes distributed in peaks of lower intensity with progressive cell divisions. These percentages are lower for mono-CLL than for healthy monocytes for all the graded concentration used. Assays were performed after 5 days of culturing **B)** Evaluation of T cell proliferation inhibition by the presence of NLCs (two depicted cases of n=5

studied) in comparison with normal monocytes by CFSE staining. **C)** Partial recovery of the inhibition of T cell proliferation by CLL-monocytes upon the addition of a mix of neutralizing anti-TGF β , anti-IL10 moAbs or IDO inhibitors as assayed by CFSE staining after 4 days of culturing. One representative case of 5 studied.

Supplemental Figure 6

Representation of putative effects induced by HGF on surrounding cells of the microenvironment. HGF produced by stromal cells induces CLL cells survival (Panel **1**) (Giannoni et al. *Haematologica* 2011, 96:1015). However both NLCs and CLL cells start to produce HGF when in close contact such as in privileged sites (i.e. proliferation centres) thus highlighting a potential autocrine mechanisms of expansion of the leukemic clone (Panel **2**). In turn HGF enhances c-MET and IDO expression on monocytes/macrophages which are further skewed toward a TGF β /IL-10-producing M2 phenotype. Ultimately these changes may drive differentiation of T regulatory cells (Treg) altogether contributing to the immunosuppressive status of CLL patients (Panel **3**).

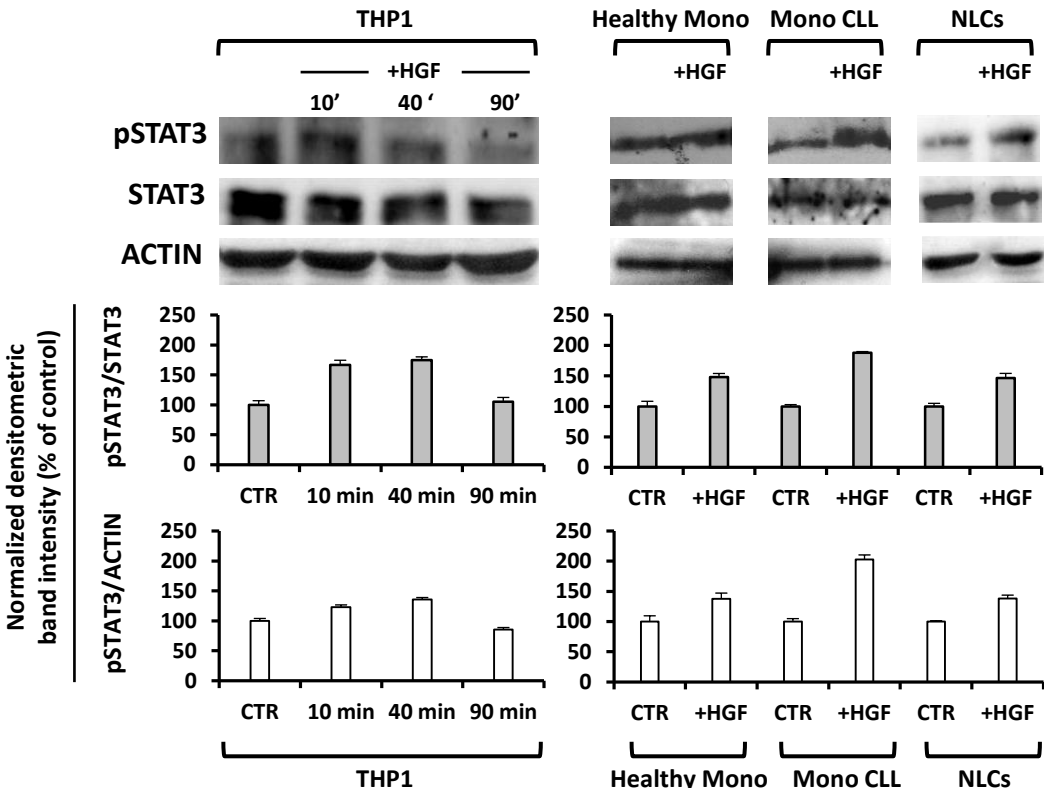
Supplemental Table I.

Patients characteristics.

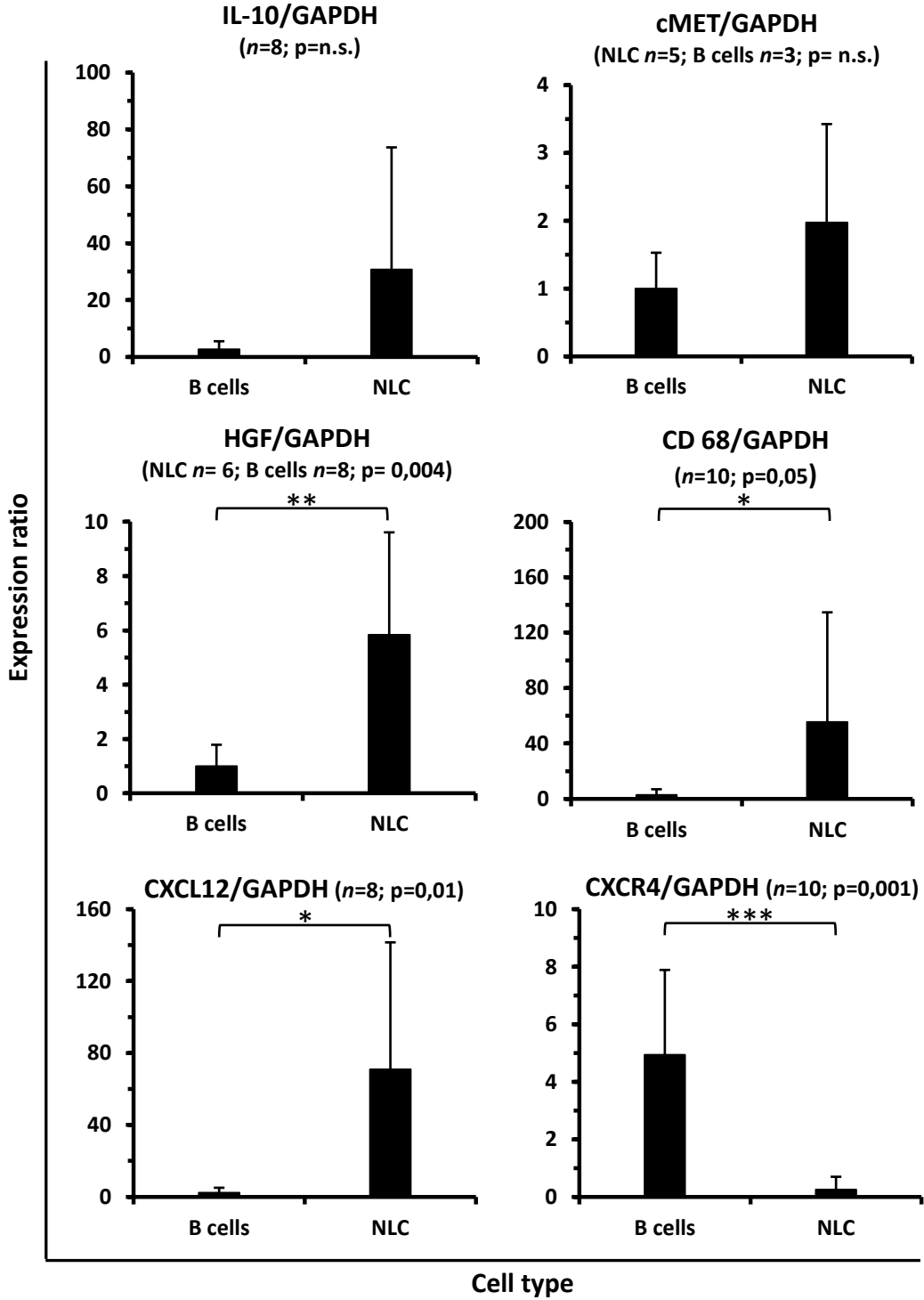
Case Id #	Gender	Clinical stage (RAI/Binet)	Markers			Cytogenetic data	Experimental use
			CD38	Zap70	IgVH		
1	M	2/B	+	+	u	del 13q	C, G, I
2	M	1/A	-	+	m	del 13q	A, C, G, I
3	M	2/B	+	+	m	neg	A, C, G, I
4	M	0/A	-	-	m	N.D.	C, G
5	F	4/C	-	-	m	neg	G, I
6	F	4/C	+	+	u	+12, del 17p	G, I
7	F	0/A	+	N.D.	N.D.	del 13q	A, I, W
8	M	0/A	-	-	N.D.	neg	C, G, I
9	M	1/A	-	-	N.D.	del 13q	C, G, I
10	M	0/A	-	-	m	del 13q	C, I, W
11	F	1/B	+	+	u	N.D.	C, G, I
12	M	1/A	-	N.D.	m	N.D.	C
13	M	1/A	N.D.	N.D.	N.D.	+ 12	C, G
14	F	1/A	-	-	m	del 13q	C, G, I
15	F	2/C	+	-	N.D.	del 13q	A, I, W
16	M	1/A	-	-	m	N.D.	C
17	M	3/C	-	+	u	del 13q	C, G, I
18	M	3/B	+	+	u	+12, del 13q	C, G, I
19	F	3/C	-	-	m	del 13q	C, G
20	F	1/B	+	+	u	+12, del 11q	C, G
21	M	0/A	+	-	m	neg	I, W
22	F	1/A	-	N.D.	m	N.D.	A
23	F	0/A	-	-	m	del 13q	C, G
24	M	0/A	+	+	u	neg	C
25	M	3/C	-	-	u	+12	C
26	F	0/A	-	-	m	del 13q	A, G, I, W
27	F	1/B	+	+	u	N.D.	A, G, I
28	M	0/A	+	+	u	del 13q	A, C
29	M	0/A	-	-	m	N.D.	A, C
30	F	2/B	+	-	u	neg	A, C
31	M	0/A	-	-	m	N.D.	A
32	F	0/A	-	-	m	neg	A, G, W
33	M	3/B	+	+	N.D.	neg	A, C
34	M	0/A	+	-	m	N.D.	A, C
35	M	0/A	-	-	m	del 13q	A, C, G, W
36	M	0/A	+	+	u	N.D.	A, G
37	F	0/A	+	-	m	N.D.	A, C, I
38	F	0/A	-	-	m	N.D.	G
39	M	0/A	-	+	m	del 13q	I
40	M	1/B	-	-	m	N.D.	C, G
41	M	2/A	N.D.	N.D.	N.D.	N.D.	A, C
42	F	2/B	+	+	u	N.D.	A, G
43	M	0/A	-	-	m	N.D.	A
44	M	2/B	-	-	N.D.	del 13q	A, G, W
45	M	4/C	+	+	m	neg	A
46	F	1/C	-	N.D.	u	N.D.	H, I
47	M	1/A	-	N.D.	u	N.D.	H, I
48	M	3/B	-	N.D.	u	N.D.	H, I
49	M	2/A	-	N.D.	m	N.D.	H, I
50	F	2/B	-	N.D.	N.D.	N.D.	H, I
51	F	2/A	-	N.D.	N.D.	N.D.	H, I
52	M	3/C	+	N.D.	N.D.	N.D.	H, I
53	F	1/B	-	N.D.	N.D.	N.D.	H, I
54	M	0/A	+	+	u	N.D.	A, C, W
55	M	2/B	-	+	u	neg	C, W

M: male; **F:** female; **u:** non mutated; **m:** mutated; **del:** deletion; **neg:** negative; **A:** functional assays; **C:** cytofluorimetry; **G:** gene expression; **H:** histochemistry; **I:** immunofluorescence; **W:** western blot; **N.D.:** not determined.

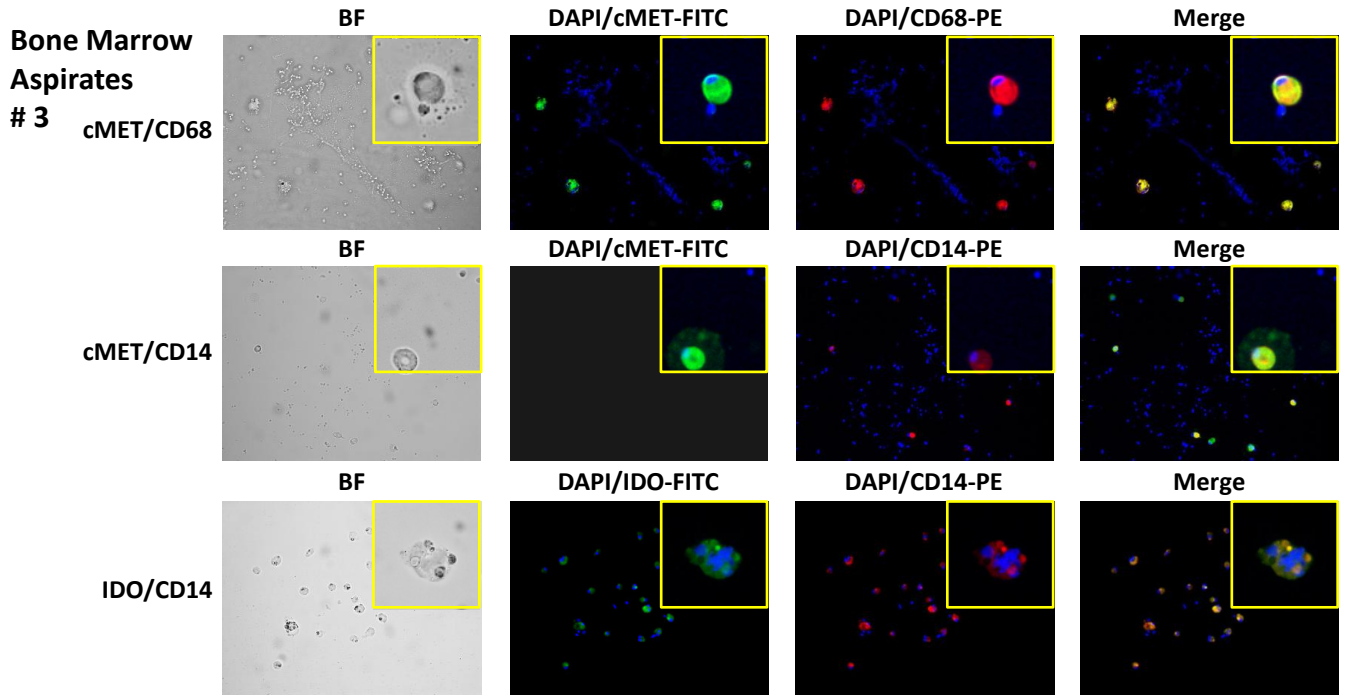
Supplemental Figure 1



Supplemental Figure 2

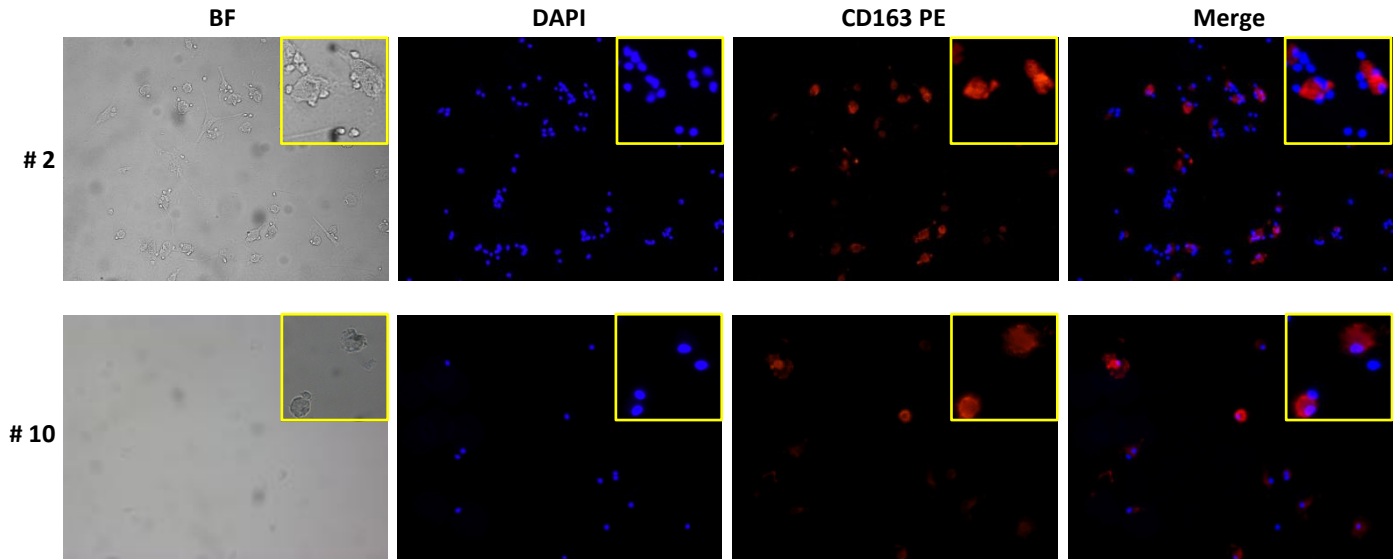


Supplemental Figure 3

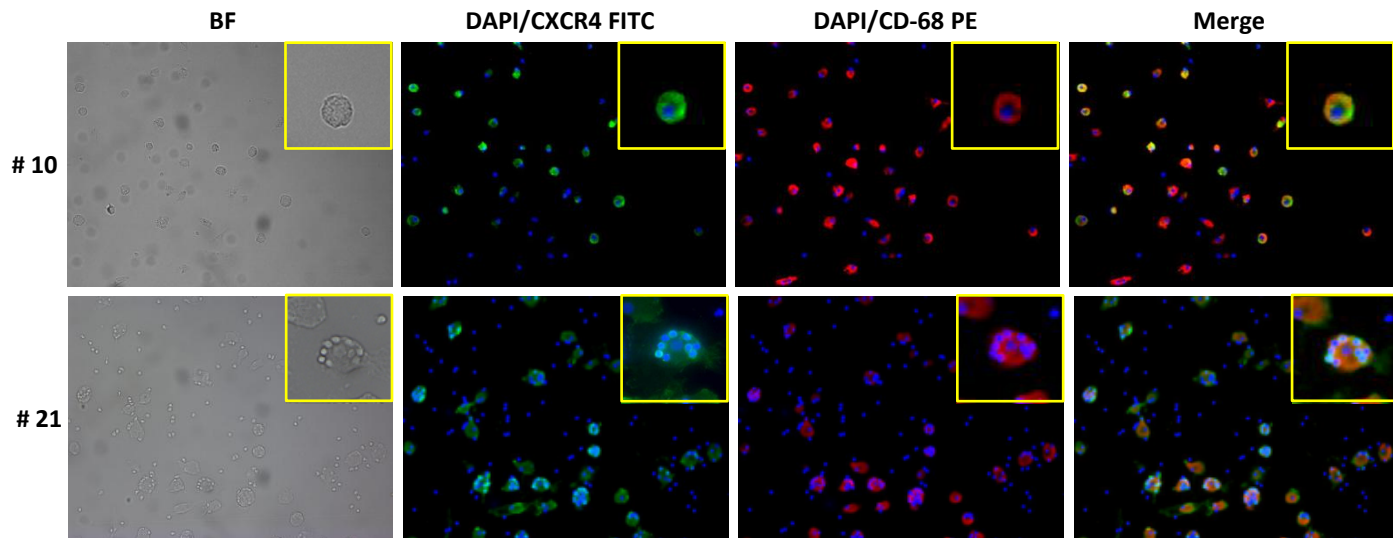


Supplemental Figure 4

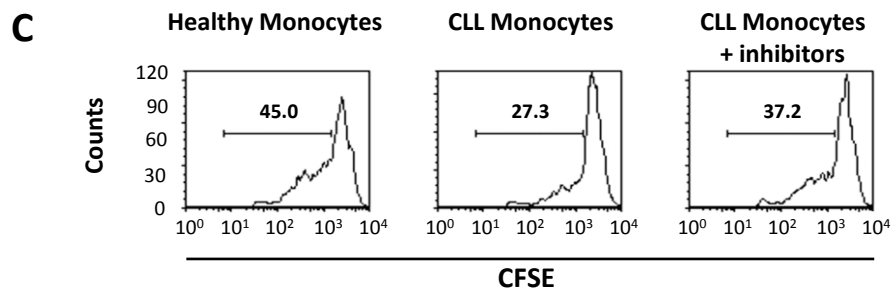
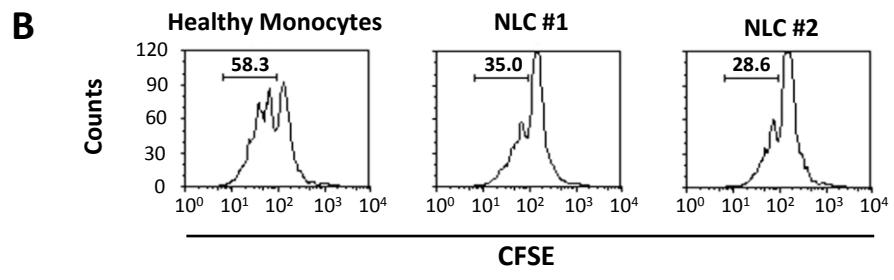
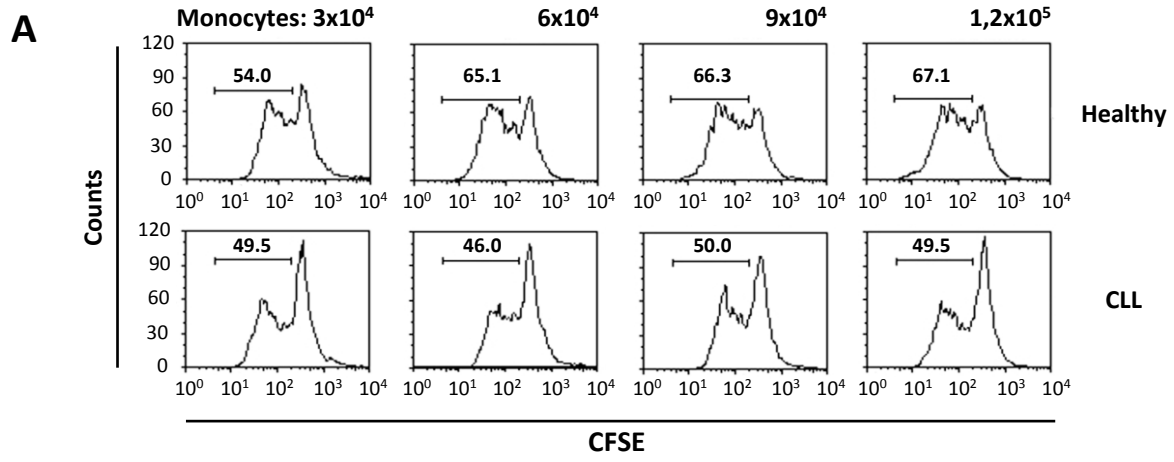
CD163



CXCR4/CD68



Supplemental Figure 5



Supplemental Figure 6

Schematic representation of the HGF effector cascade that may contribute to the expansion of leukemic cells within the CLL microenvironment

