Chronic Lymphocytic Leukemia cells increase neutrophils survival and promote their differentiation into CD16^{high} CD62L^{dim} immunosuppressive subset.

Corresponding author: Podaza Enrique, phD

Institution: Instituto de Medicina Experimental- Academia Nacional de Medicina.

Address: José Andrés Pacheco de Melo 3081. Ciudad de Buenos Aires.

Email: epodaza@gmail.com

Key words: Tumor Associated Neutrophil, Apoptosis, Phenotype Reprogramming, Leukemia.

Abbreviatures: HD: Healthy donor

CLL: Chronic Lymphocytic Leukemia

TAN: Tumor Associated Neutrophils

Category: Tumor Immunology and Microenvironment.

Novelty and Impact.

Reprogramming of neutrophils into an immunosuppressive phenotype has been reported in solid tumor microenvironment. Here we describe for the first time an increase percentage of circulating CD16^{high}CD62L^{dim} immunosuppressive neutrophils in chronic lymphocytic leukemia (CLL) patients and show that leukemic B cells are responsible for inducing neutrophil reprogramming into this subset. Our findings shed light on the potential contribution of this subset to the impaired immune response exhibited by CLL patients and encourage their assessment as complementary therapeutic targets to improve patient's outcome.

Abstract.

Reprogramming of neutrophils by malignant cells is well-described for many types of solid tumors, but data remain scarce for hematological diseases. Chronic lymphocytic leukemia (CLL) is characterized for a deep immune dysregulation mediated by leukemic cells that compromises patient's outcome. Murine models of CLL highlight the relevance of myeloid cells as tumor-driven reprogramming targets. In this study, we evaluated neutrophil reprogramming by CLL cells. We first show that the proportion of the CD16^{high}CD62L^{dim} neutrophil subset in peripheral blood of CLL patients is increased compared to age-matched healthy donors (HD). In vitro, neutrophils from HD cultured in the presence of CLL cells or conditioned media (CM) from CLL cells exhibited a longer lifespan. Depletion of G- and GM-CSF from CM partially reversed the protective effect. In addition, the proportion of viable neutrophils that displayed a CD16^{high}CD62L^{dim} phenotype was increased in the presence of CM from CLL cells, being TGF-β/IL-10 responsible for this effect. Altogether our results describe a novel mechanism through which CLL cells can manipulate neutrophils.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/ijc.31762

Chronic Lymphocytic Leukemia cells increase neutrophils survival and promote their differentiation into $CD16^{high}$ $CD62L^{dim}$ immunosuppressive subset.

Enrique Podaza; Denise Risnik; Ana Colado; Esteban Elías; María Belén Almejún; Horacio Fernandez Grecco; Raimundo Fernando Bezares; Mercedes Borge; Romina Gamberale; Mirta Giordano

Introduction

The immune system, whose basic function is to protect the body from infection, undergoes a change towards immunosuppression in the context of cancer. In this process, tumor cells polarize different infiltrating leukocyte populations into a tumor growth promoting phenotype (1). Neutrophils constitute a significant part of the inflammatory infiltrate in many types of cancer (2-4) and high levels of intratumoral neutrophils have been associated with poor prognosis (5), as they would promote metastasis (6, 7) and angiogenesis (8). However, other studies have reported antitumor effects mediated by neutrophils, demonstrating that they are able to exhibit direct cytotoxic effects toward tumor cells (9) and to prevent metastasis (10).

Reprogramming of neutrophils into tumor associated neutrophils (TANs) is well-described in the setting of solid tumors (3, 4, 11-13), but there are few reports related with TANs in the context of leukemia.

Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of clonal B lymphocytes in blood, lymph nodes, spleen and bone marrow (14). Infections persist as the leading cause of morbidity and mortality in CLL patients, being bacterial infections the most frequent. The major cause of the high incidence of infections is dysregulation of the immune system, which is one of the main characteristics of CLL from early stages, worsening with the progression of the disease (15). Previous reports indicate that CLL cells were capable of modulating immune cell functions turning them into an immunosuppressive/tumor-supportive phenotype. Although almost every cell type in lymphoid tissues can be modified by the leukemic clone, myeloid cells are crucial to promote its progression as shown in the Eμ-TCL1 mouse model of CLL(16). In this model, it was shown that spleen marginal zone neutrophils in contact with CLL cells had a longer lifespan and acquired a tumor-supportive phenotype (16). Recently, Manukyan et al reported that neutrophils from CLL patients exhibit a particular phenotype with reduced expression of TLR2 and LPS-induced release of proinflammatory cytokines(17). However the participation of leukemic cells in these neutrophil dysfunctions remains largely unknown. Herein we show that circulating CLL cells are able to induce peripheral blood neutrophils reprogramming into an immunosuppressive/tumor supportive phenotype.

Methods.

CLL patients and Healthy Donors (HD) samples.

Peripheral blood samples were obtained from CLL patients (age range 50–75), and HD (age range 50-73) after informed consent in accordance with the Declaration of Helsinki (Clinical data from CLL-patients is depicted in supplementary table 1). These studies were approved by the Institutional Review Board of the Institutes of the National Academy of Medicine, Buenos Aires. At the time of the analysis patients were free from clinically relevant infectious complications and were either untreated or had not received antineoplastic treatment for ≥3 months before the study began.

Cell separation procedures and culture.

Leukemic cells and neutrophils were isolated as we previously described (18).

Neutrophils (2 x 10^6/ml) were co-cultured with CLL cells (2 x 10^6/ml) in RPMI medium supplemented with 10% fetal calf serum and antibiotics (Gibco-Thermo Fisher Scientific), during 72 h at 37°C. At 24, 48 and 72 h, samples were collected and stained with Annexin-V-FITC (Biolegend) to assess neutrophil apoptosis by flow cytometry. HD-neutrophil samples used in co-cultures had <1% of lymphocytes and <0,5% of monocytes ($CD14^+$). CLL-samples used in co-cultures were purified using a negative selection kit for CD19 cells (Miltenyi Biotec). CLL-Conditioned media (CM) were obtained culturing CLL cells (2 x 10^6 cells/ml) during 48h. To reduce the levels of IL-10, G-CSF, GM-CSF or TGF-β in CM, it was incubated on plates coated with specific antibodies (anti-IL-10, anti-G-CSF and anti-GM-CSF purchased from Biolegend; anti-TGF-β from Thermo-Fisher*)* during 1h at room temperature. Cytokine depletion was confirmed by ELISA.

To assess neutrophils immunosuppressive function, peripheral blood mononuclear cell from HD were activated with PHA (3µg/ml) for 24 h with or without HD-neutrophils or CLL-neutrophils (1:3 ratio). The expression of the activation marker CD69 was measured on T-cells by flow cytometry and the amount of INFγ released was quantified in culture supernatants by ELISA (Biolengend).

Western blot.

Whole-cell lysates were obtained from 10 × 10^6 neutrophils, cultured or not in CM for 18h, using 120 µL of loading buffer 1× 5% β-mercaptoethanol with 2ul of Halt protease inhibitor cocktail (Thermo Scientific). Lysates were boiled at 99 °C for 5 min, and 50 µL of the protein extracts were separated on a standard 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then blotted with antibodies against Bfl-1 and followed by HRP-conjugated anti-rabbit IgG. Specific bands were developed by enhanced chemiluminescence (ECL) method. The same membrane was blotted with mAb anti-β-actin

followed by HRP-conjugated anti-mouse IgG to compare the total amount of protein in each sample.

Flow cytometry.

Intracellular staining of Arginase I (PE-anti-ArgI; R&D, polyclonal IgG) was performed in whole blood samples fixed with PFA 1% for 15 minutes and permeabilized with PBS 0,1% saponin for 30 min. For surface staining FITC-Annexin-V, PerCP-Cy5.5-anti-CD16 (3G8), FITC-anti-CD16 (NPK15), PE-anti-CD62L (DREG-56), PerCP-Cy5.5-anti-CD62L (MEL-14) and PE-CD11b (ICRF44) were used, all of them purchased from BD-Biosciences.

RNA Preparation, cDNA Synthesis and qRT-PCR.

Total RNA Preparation, cDNA Synthesis and qRT-PCR Total RNA was extracted from 1 x 10^6 purified neutrophils using TRIzol reagent, and cDNA was generated by reverse transcription with MMLV RT according to the manufacturer´s instructions. qRT-PCR was performed using SYBR Green PCR Master Mix in 20 µl reactions. Primers were designed using Primer3 software and purchased from Ruralex-Fagos: GAPDH Fw 5´GAGTCAACGGATTTGGTCGT 3´, GAPDH Rv 5´-TTGATTTTGGAGGGATCTCG-3´, APRIL Fw 5´-CCTGGGACTTTGATTTTACGG-3´, APRIL Rv 5´-TGGTGGTGCTGTTCTGGATG-3´, BAFF Fw 5´-TGGTGACTTTGTTTCGATGTATTC-3´, BAFF Rv 5´-GTTCATCTCCTTCTTCCAGTTTTG-3´ and were used at a concentration of 250 nM. Reactions were carried out in CFX Connect™ real-time PCR detection system (Biorad). The cycling program used was 50 ˚C for 2 minutes, 95˚C for 1 minute, followed by 40 cycles of 95˚C for 15 seconds, 60˚C for 60 seconds. Data were analyzed using GAPDH as a reference gene.

Results and discussion

Although most reports describe TANs differentiation at solid tumor microenvironment, recently a new subset of circulating immunosuppressive neutrophils ($CD16^{high}CD62L^{dim}$) has been identified in patients with head and neck squamous cells carcinoma (HNSCC) (19). To determine if this neutrophil subset is also expanded in CLL patients, aliquots of fresh peripheral blood samples from age-matched HD and CLL patients were stained with anti-CD16 and anti-CD62L antibodies and fixed immediately before erythrocytes lysis. This approach has been reported to prevent CD62L downregulation due to neutrophil manipulation (20). Clinical features of CLL patients evaluated are depicted in Supplementary Table 1. We found that CLL samples exhibit a significantly higher percentage of the $CD16^{high}CD62L^{dim}$ neutrophil subset compared to age-matched HD samples (Fig 1 A-B). Although there was no statistically significant association between the percentages of $CD16^{high}CD62L^{dim}$ neutrophils and any of the clinical parameters recorded, CLL patients with intermediate or advanced disease (Binet B and

C stages) showed a strong trend towards higher proportion of TANs (Supplementary Fig 1). While future studies with a larger cohort of patients are necessary to corroborate these findings, they are in line with recent data from Manukyan et al who showed that active disease was associated with downregulation of CD62L expression in CLL neutrophils (17). As previously reported (21), $CD16^{high}CD62L^{dim}$ neutrophils expressed higher levels of CD11b (Fig 1.C). By contrast, we did not find differences in arginase 1 expression (Supplementary Fig 2), a known marker of TANs, nor in BAFF and APRIL mRNA levels between HD and CLL neutrophils (Fig 1D).

Given that $CD16^{high}CD62L^{dim}$ neutrophils were reported to exert immunosuppressive activity on T cells (21), we next compared the capacity of neutrophils from HD and CLL patients to impair T cell activation. To that aim T lymphocytes from HD samples were stimulated with PHA in the presence of HD-neutrophils or CLL-neutrophils (1:3 T cell: neutrophil ratio). Twenty-four hours later, the expression of the activation marker CD69 on T cells and the release of interferon γ to the supernatant were recorded. Neutrophils from both sources were able to reduce PHA-induced activation of T cells; however the inhibition was significantly higher in the presence of CLL neutrophils compared to HD neutrophils (Fig. 1 E-F). These results suggest that, as reported in gastric cancer (22), neutrophils from CLL patients can impair T cell responses.

Because increased lifespan is crucial for neutrophils to acquire a TAN phenotype, we compared *in vitro* survival of neutrophils from HD and CLL patients. At 24 h, CLL neutrophils displayed a significantly higher survival that was not evident at longer times (Fig. 1 G).

We next determined if CLL cells were responsible for the increased neutrophils survival by co-culturing purified HD-neutrophils with or without CLL cells. Neutrophil viability was evaluated at 24, 48 and 72 h using Annexin V-FITC and flow cytometry analysis. Representative results are depicted in Figure 2 A-B. As expected when cultured alone, less than 40% of neutrophils (Annexin V^-) were viable at 24 h. However, in the presence of CLL cells, a significant proportion of neutrophils remained viable even after 72 h in culture. To assess if this delay of neutrophil apoptosis could be attributed to cell-to-cell signals and/or to soluble factors, we performed co-cultures of neutrophils and CLL cells in transwell chambers. There were no significant differences in neutrophil viability between those separated from CLL cells by transwell membrane or mixed together, suggesting that soluble factors released by CLL cells might be responsible for neutrophil protection (Fig. 2C). Of note, B lymphocytes purified from age-matched- HD (>97% CD19+) were not able to rescue neutrophils from apoptosis suggesting that it is a feature of CLL cells (Fig. 2D). Since soluble factors released from CLL cells were responsible for the protective effects on neutrophils, we decided to use conditioned media (CM) from purified CLL cells in subsequent experiments. We first evaluated the involvement of GM-

CSF and G-CSF, two well-known factors released by CLL cells that are crucial to increase neutrophil lifespan (20). To that aim we cultured HD-neutrophils for 24 h with whole CM or CM depleted from GM-CSF and/or G-CSF before evaluating apoptosis. As shown in Fig 2E, depletion of both cytokines significantly though not completely counteracted the anti-apoptotic effect of CM suggesting that G- and GM-CSF released by leukemic cells were involved in increasing neutrophil lifespan. Additionally, since that IL-10 and TGF-β had been implicated in TANs reprogramming (23) we assessed their participation as antiapoptotic agents in CM. Depletion of both anti-inflammatory cytokines did not impair CM antiapoptotic effect (Fig 2F). Given that longer lifespan of TANs has been associated with increased expression of Bfl-1 (16, 23), an anti-apoptotic member of the Bcl-2 family, we evaluated Bfl-1 expression on HD-neutrophils exposed to CM. By western blot we found that Bfl-1 levels were significantly up-regulated in HD-neutrophils incubated for 18 h in CM compared to those incubated in control medium (Fig. 2G).

Finally, we evaluated the expression of CD16 and CD62L in viable HD-neutrophils cultured in the presence of CM to determine if soluble factors released by malignant B cells were able to induce neutrophil reprogramming into TANs. Exposure of HD-neutrophils to CM for 24 h resulted in a significant increase of the $CD16^{high}CD62L^{dim}$ subset. Even though IL-10 and TGF-β were not involved in antiapoptotic effect of CM we assessed their participation in the downregulation of CD62L by depleting each or both cytokines from CM. As shown in Fig 3C, single depletions of IL-10 or TGF-β did not modify CM capacity to increase the percentage of $CD62L^{dim}$ neutrophils. However, depletion of both cytokines significantly reduced reprogramming activity of CM suggesting a cooperative effect of IL-10 and TGF-β.

Millrud et al. (19) showed that a higher percentage of $CD16^{high}CD62L^{dim}$ neutrophils in HNSCC correlates with better clinical outcome due to the antitumor activity exerted by these cells through the release of NETs. By contrast, we previously reported in CLL that NETs promote leukemic cells survival and activation (18), suggesting that an increase percentage of the $CD16^{high}CD62L^{dim}$ subset could be detrimental instead of beneficial to CLL patients. Neutrophils displaying a B-helper phenotype have been previously described (24) at the marginal zone of the spleen where IL-10 released by activated endothelial cells enable neutrophils to produce BAFF, APRIL and NET-like structures that promote B-cell activation. Although we did not observe a BAFF or APRIL differential expression between circulating neutrophils from HD and CLL patients, it is conceivable that, once in the lymphoid tissues, reprogrammed $CD16^{high}CD62L^{dim}$ cells receive additional signals from the microenvironment to promote leukemic B cell survival. This hypothesis is supported by a previous report in the Eμ-TCL1 murine model of CLL that showed an expanded neutrophil population with B-helper properties in the spleen of leukemic mice which does not exist in the bone marrow (16),

suggesting that the local environment is critical for shaping the gene expression program of B helper neutrophils.

Here we described the capacity of CLL cells to reprogram circulating neutrophils into a tumor-supportive/immunosuppressive phenotype. The $CD16^{high}CD62L^{dim}$ subset may influence CLL patient outcome not only by favoring leukemic cell survival through NETs release, but also by impairing T-cell responses with increased risk of infectious complications.

References:

1. Granot Z, Fridlender ZG. Plasticity beyond cancer cells and the "immunosuppressive switch". Cancer research. 2015;75(21):4441-5.
2. Wislez M, Rabbe N, Marchal J, Milleron B, Crestani B, Mayaud C, et al. Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: role in tumor progression and death. Cancer research. 2003;63(6):1405-12.
3. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, von der Maase H. Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2009;27(28):4709-17.
4. Trellakis S, Bruderek K, Dumitru CA, Gholaman H, Gu X, Bankfalvi A, et al. Polymorphonuclear granulocytes in human head and neck cancer: enhanced inflammatory activity, modulation by cancer cells and expansion in advanced disease. International journal of cancer. 2011;129(9):2183-93.
5. Shen M, Hu P, Donskov F, Wang G, Liu Q, Du J. Tumor-associated neutrophils as a new prognostic factor in cancer: a systematic review and meta-analysis. PloS one. 2014;9(6):e98259.
6. Tabaries S, Ouellet V, Hsu BE, Annis MG, Rose AA, Meunier L, et al. Granulocytic immune infiltrates are essential for the efficient formation of breast cancer liver metastases. Breast cancer research : BCR. 2015;17:45.
7. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. Nature. 2015;528(7582):413-7.
8. Tazzyman S, Lewis CE, Murdoch C. Neutrophils: key mediators of tumour angiogenesis. International journal of experimental pathology. 2009;90(3):222-31.
9. Ishihara Y, Fujii T, Iijima H, Saito K, Matsunaga K. The role of neutrophils as cytotoxic cells in lung metastasis: suppression of tumor cell metastasis by a biological response modifier (PSK). In vivo. 1998;12(2):175-82.
10. Granot Z, Henke E, Comen EA, King TA, Norton L, Benezra R. Tumor entrained neutrophils inhibit seeding in the premetastatic lung. Cancer cell. 2011;20(3):300-14.
11. Kuang DM, Zhao Q, Wu Y, Peng C, Wang J, Xu Z, et al. Peritumoral neutrophils link inflammatory response to disease progression by fostering angiogenesis in hepatocellular carcinoma. Journal of hepatology. 2011;54(5):948-55.
12. Ilie M, Hofman V, Ortholan C, Bonnetaud C, Coelle C, Mouroux J, et al. Predictive clinical outcome of the intratumoral CD66b-positive neutrophil-to-CD8-positive T-cell ratio in patients with resectable nonsmall cell lung cancer. Cancer. 2012;118(6):1726-37.
13. Fossati G, Ricevuti G, Edwards SW, Walker C, Dalton A, Rossi ML. Neutrophil infiltration into human gliomas. Acta neuropathologica. 1999;98(4):349-54.
14. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. The New England journal of medicine. 2005;352(8):804-15.
15. Morrison VA. Infectious complications in patients with chronic lymphocytic leukemia: pathogenesis, spectrum of infection, and approaches to prophylaxis. Clinical lymphoma & myeloma. 2009;9(5):365-70.

16. Gatjen M, Brand F, Grau M, Gerlach K, Kettritz R, Westermann J, et al. Splenic Marginal Zone Granulocytes Acquire an Accentuated Neutrophil B-Cell Helper Phenotype in Chronic Lymphocytic Leukemia. Cancer research. 2016;76(18):5253-65.
17. Manukyan G, Papajik T, Gajdos P, Mikulkova Z, Urbanova R, Gabcova G, et al. Neutrophils in chronic lymphocytic leukemia are permanently activated and have functional defects. Oncotarget. 2017;8(49):84889-901.
18. Podaza E, Sabbione F, Risnik D, Borge M, Almejun MB, Colado A, et al. Neutrophils from chronic lymphocytic leukemia patients exhibit an increased capacity to release extracellular traps (NETs). Cancer Immunol Immunother. 2017;66(1):77-89.
19. Millrud CR, Kagedal A, Kumlien Georen S, Winqvist O, Uddman R, Razavi R, et al. NET-producing CD16high CD62Ldim neutrophils migrate to tumor sites and predict improved survival in patients with HNSCC. International journal of cancer. 2017;140(11):2557-67.
20. Lizcano A, Secundino I, Dohrmann S, Corriden R, Rohena C, Diaz S, et al. Erythrocyte sialoglycoproteins engage Siglec-9 on neutrophils to suppress activation. Blood. 2017;129(23):3100-10.
21. Pillay J, Kamp VM, van Hoffen E, Visser T, Tak T, Lammers JW, et al. A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1. The Journal of clinical investigation. 2012;122(1):327-36.
22. Wang TT, Zhao YL, Peng LS, Chen N, Chen W, Lv YP, et al. Tumour-activated neutrophils in gastric cancer foster immune suppression and disease progression through GM-CSF-PD-L1 pathway. Gut. 2017;66(11):1900-11.
23. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer cell. 2009;16(3):183-94.
24. Puga I, Cols M, Barra CM, He B, Cassis L, Gentile M, et al. B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. Nature immunology. 2011;13(2):170-80.

Figure 1.

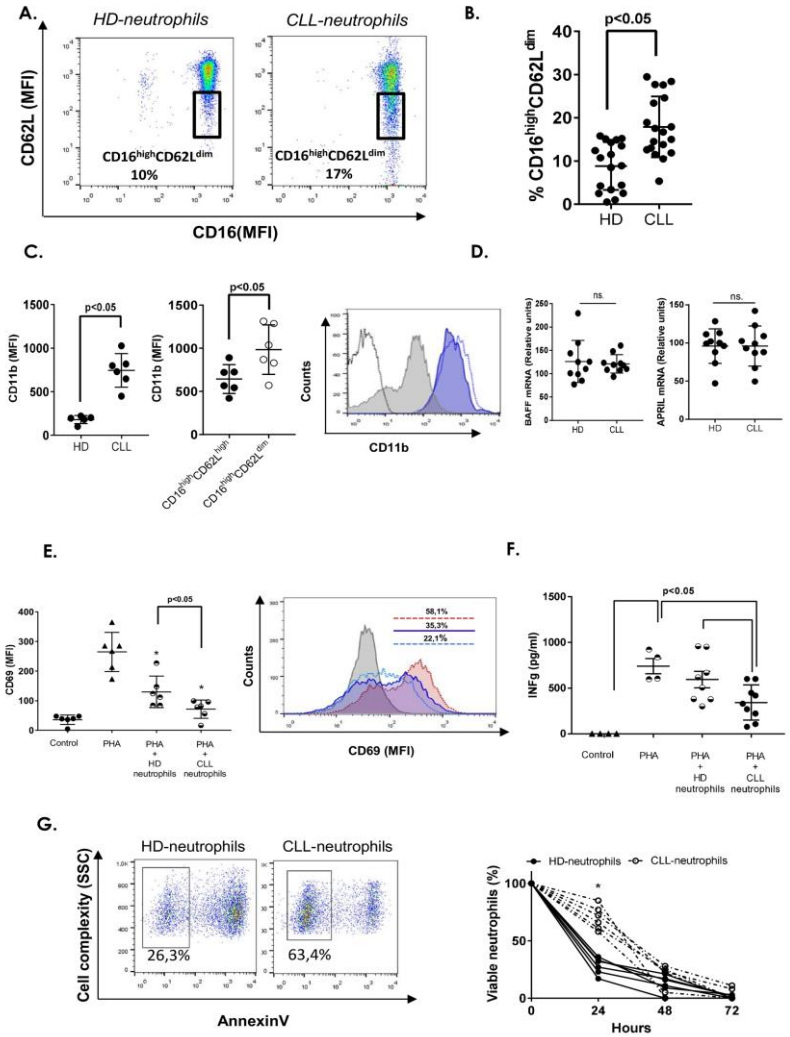


Figure 2

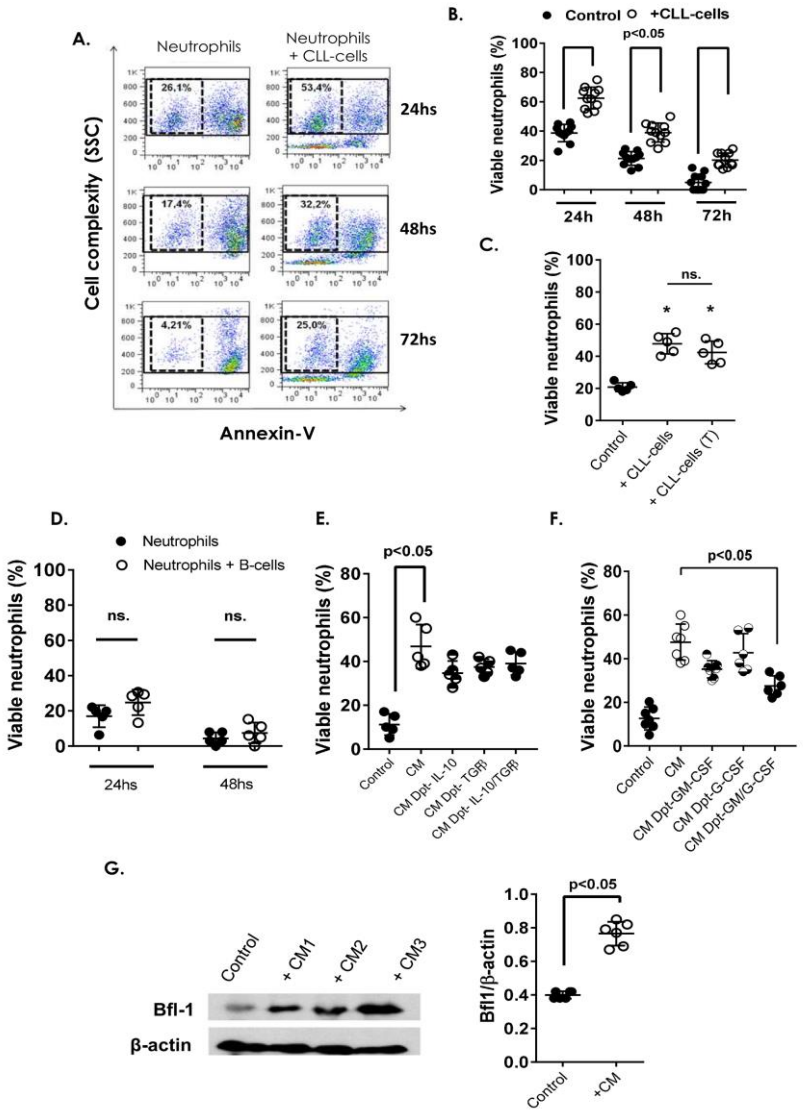
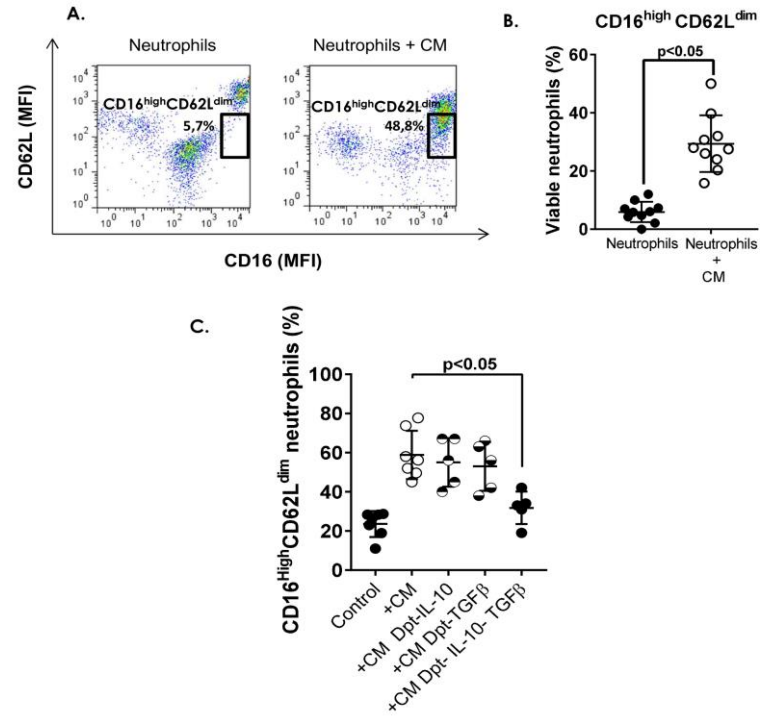


Figure 3



Novelty & Impact Statement:

The reprogramming of neutrophils into an immunosuppressive phenotype is known to occur in the solid tumor microenvironment. The present study shows that this phenomenon also exists in a hematological disease, wherein an increased percentage of circulating immunosuppressive neutrophils, namely the $CD16^{high}CD62L^{dim}$ subset, was identified in chronic lymphocytic leukemia (CLL) patients. Cooperative effects of the soluble factors IL-10 and TGFβ, released from leukemic B cells, appear to play a role in neutrophil reprogramming into the $CD16^{high}CD62L^{dim}$ subset. The findings shed light on the potential contribution of neutrophil reprogramming to the impaired immune response exhibited by CLL patients.