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Haematologica 2017 [Epub ahead of print]

doi:10.3324/haematol.2016.148965

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Lymphocyte activation gene 3- A novel therapeutic target in chronic lymphocytic leukemia

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Key words: CLL, LAG3, therapeutic target
Running title: LAG-3 A novel therapeutic target in CLL

Abstract: 221 words
Main Text: 3154 words
No. of figures: 5
Supplemental files: 1
References: 31
Abstract

A novel therapeutic approach in cancer, attempting to stimulate host anti-tumor immunity, involves blocking of immune checkpoints. LAG3 is an immune checkpoint receptor expressed on activated/exhausted T-cells. When engaged by MHC class II molecules, LAG3 negatively regulates T-cell function thereby contributing to tumor escape. Intriguingly, a soluble LAG3 variant activates both immune and malignant MHC class II-presenting cells. In this study, we examined the role of LAG3 in the pathogenesis of chronic lymphocytic leukemia, an MHC class II-presenting malignancy, and show that chronic lymphocytic leukemia cells express and secrete LAG3. High levels of surface and soluble LAG3 were associated with the unmutated immunoglobulin variable heavy chain leukemic subtype and a shorter median time from diagnosis to first treatment. Utilizing a mechanism mediated through MHC class II engagement, recombinant soluble LAG3-Ig fusion protein, LAG3-Fc, activated chronic lymphocytic leukemia cells, induced anti-apoptotic pathways and protected the cells from spontaneous apoptosis, effects mediated by SYK, BTK and MAPK signaling. Moreover, LAG3 blocking antibody enhanced in vitro T cell activation. Our data suggest that soluble LAG3, promotes leukemia cell activation and anti-apoptotic effects, through its engagement with MHC class II. Furthermore, MHC class II-presenting chronic lymphocytic leukemia cells may affect LAG3-presenting T cells and impose immune exhaustion on their microenvironment hence; blocking LAG3-MHC class II interactions is a potential therapeutic target in chronic lymphocytic leukemia.
**Introduction**

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder (LPD) characterized by the progressive accumulation of small CD5+ mature-looking B-cells in the peripheral blood, bone marrow (BM) and secondary lymphoid organs(1). Despite recent advances in understanding the pathophysiology of CLL, it is still mostly regarded as an incurable disorder, despite the long term remissions observed in some of the patients treated with Fludarabine-Cyclophosphamide-Rituximab (FCR) regimen or patients who underwent allogeneic stem cell transplantation (2, 3). There are two main subgroups of CLL based on the presence or absence of somatic mutations in the immunoglobulin heavy chain variable domain (IGHV)(1). The presence of a mutated IGHV (M-IGHV) identifies a leukemic subtype that has a stable or slowly progressive course, while the expression of an unmutated IGHV (UM-IGHV) gene is associated with a more aggressive disease and an inferior survival(4-6).

The inability of the immune system to eradicate malignancy is one of the fundamental hallmarks of cancer. Due to chronic antigen stimulation induced by cancer cells, effector T-cells may gradually lose their effector activities, a process termed “exhaustion”(7). In this respect, expression of immune checkpoint receptors is regarded as a hallmark of "exhaustion". Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1) are particularly important immune checkpoint receptors(8-10).

The CD4 homologue Lymphocyte-activation gene 3 (LAG3;CD223) is an immune checkpoint receptor. Among others, LAG3 is expressed on exhausted T-cells as well as on tumor infiltrating lymphocytes (TILs)(11, 12). LAG3 binds to MHC Class II (MHCII) molecules on antigen presenting cells (APC), but with higher affinity than CD4, an interaction that negatively regulates CD3-T cell receptor (TCR) complex signaling, thus affecting T cell proliferation, function and homeostasis(11).

In humans, a 52kDa soluble LAG3 protein variant (LAG-3V3, sLAG3) is formed by an alternatively spliced RNA(13, 14) (Supplement 1). sLAG3 has also been shown to bind MHCII, yet, this variant was reported to activate APCs and enhance tumor-specific cytotoxic T-cells(15). However, in melanoma cells that express MHCII, the interaction with sLAG3 activates MAPK/ERK and PI3K/AKT pathways, thus contributing to resistance of the malignant cells to apoptosis(15). Interestingly, LAG3 expression was recently suggested as a prognostic marker in patients with CLL, as gene expression profiling of CLL cells detected increased
LAG3 expression levels that were in correlation with *UM-IGHV* and with reduced treatment free survival (16).

We hypothesized that LAG3-MHCII interaction may play an important role in the pathogenesis of CLL and contribute to leukemic cell’s resistance to apoptosis and its ability to evade anti-cancer immunity. For that, we analyzed the expression of LAG3 and its soluble variant, sLAG3, in patients with CLL, and explored the effects of LAG3-MHCII interaction on CLL cells’ activation, survival and downstream signaling pathways that mediate these effects.

**Methods**

*Patients and samples*

After obtaining informed consent in accordance with the Declaration of Helsinki and approval from the institutional ethics committee, peripheral blood samples were collected from CLL patients (17) and healthy controls. Lymph nodes and spleen samples were also collected from CLL patients. Handling protocol is available in supplement 2.

*Reagents and antibodies*

Detailed in Supplement 2.

*Enrichment of CLL cells*

Peripheral blood mononuclear cells were magnetically labeled either using CD19 microbeads for positive selection or by B-CLL Cell Isolation kit for negative selection and then separated on a magnetic cell separation column (MACS), all from Miltenyi Biotec Inc, Auburn, CA.

*RNA extraction and cDNA synthesis*

RNA was extracted using RNeasy kit (Qiagen, CA USA). Reverse transcription was performed using oligo(dT) priming and Verso cDNA kit (Thermo Fisher Scientific/ABgene, Epsom, U.K.) according to the manufacturer’s instructions.

*IGHV gene analysis*

Analysis of *IGHV* gene status was performed as described in Wiestner et. al., (18) and detailed in Supplement 2.
**Cell stimulation, apoptosis and LAG3-Fc binding assay**

*Stimulation:* CLL cells were incubated with FcR Blocking Reagent, before stimulated by either a recombinant soluble human LAG3-Ig fusion protein (LAG3-Fc) (1µg/ml) or control Ig-Fc (1µg/ml), for the indicated time followed by flow cytometry analysis.

*Analysis of cell viability and apoptosis:* After incubation with LAG3-Fc or Ig-Fc for the indicated time, cells were either harvested for Western blot assays or stained with the AnnexinV/propidium Iodide MEBCYTO® Apoptosis Kit (MBL, Nagoya, Japan), according to the manufacturer’s instructions. For inhibition assays, CLL cells were pre-incubated with Wortmannin (50nM), PD98059 (100µM), Idelalisib (10µM), R406 (100µM) or Ibrutinib (0.5µM) for 1 hour, prior to stimulation, then, cultured for 48 hours and analyzed by flow cytometry.

*LAG3-Fc binding:* CLL cells were incubated for 15 min with either LAG3-Fc or Ig-Fc and stained for CD19 and anti-human IgG (Fc gamma-specific), for flow cytometry analysis. For *Inhibition assays:* LAG3-Fc (1µg/ml) was incubated with anti-LAG3 [aimed at the MHCII molecules binding site (clone 17B4), 10µg/ml] for 30 min before added to the cultured CLL cells for one hour incubation. Then, cells were washed and incubated for 72 hours.

*T cells activation and blocking antibody treatment*

Cells were incubated for 48 hours in the presence of i) anti LAG3 (17B4) (20µg/mL), ii) anti PD-1 (J116) with anti PD-L1 (M1H1) (10 µg/mL each), iii) the combined three antibodies or iv) IgG1 isotype control, then activated by CD3/CD28 Dynabeads for 6 hours, followed by flow cytometry analysis.

**Western blotting and Flow Cytometry**

Detailed in Supplement 2.

**Quantitative PCR**

Real Time PCR was performed using LightCycler® 480 SYBR Green I Master and analyzed on a LightCycler 480 II (Roche, Basel, Switzerland). Primers are presented in Supplement 2.
**Enzyme-linked immunosorbent assay (ELISA)**

Soluble (s)LAG3 serum concentrations were determined using RayBio Human LAG3 Elisa kit (RayBiotech, GA, USA) following the manufacturer's instruction, using SpectraMax M2 ELISA reader (Molecular Devices, CA, USA).

**Statistical analysis**

We used unpaired and paired t-tests or one-way ANOVA to assess differences in the means of two groups or three groups respectively. A p-value<0.05 was considered significant.

**Results**

**LAG3 expression in CLL cells and disease course**

Based on previously reported gene expression profiles that have shown overexpression of LAG3 in *UM-IGHV* CLL(16), we first evaluated the expression of full length LAG3 mRNA in CLL cells from patients with *M-IGHV* and *UM-IGHV* CLL as well as in B cells from normal controls. Patient characteristics are presented in supplement 3. Peripheral blood CLL and normal B cells were purified using positive selection to obtain B cells purity (>96%) and LAG3 expression was analyzed by RT-PCR. Full-length LAG3 mRNA expression levels were increased in CLL cells compared to normal B cells (p=0.0028, Fig 1A). When evaluated among patients with CLL, LAG3 mRNA levels were significantly increased in *UM-IGHV* CLL cells compared to cells with *M-IGHV* gene (p=0.026, Fig. 1B). Moreover, patients with higher levels of full-length LAG3 mRNA (defined as above the median LAG3 mRNA level) had a shorter median time from diagnosis to first treatment (Fig. 1C).

At protein level, LAG3 was detected by Western blot in CD19+ purified CLL cells in all analyzed patients. However, no differences were detected in LAG3 levels between *M-IGHV* and *UM-IGHV* CLL cells (Fig. 1D,E). Using flow cytometry, we evaluated LAG3 cellular localization in CLL cells. LAG3 was detected at very low levels on the surface of CLL cells and only a small fraction of the cells expressed substantial levels of surface LAG3 (Fig. 1F). Most CLL cells though, expressed high levels of intracellular LAG3 (6.4%±5.4 expressed surface LAG3 while 60.9%±24.8% expressed intracellular LAG3, Fig 1F). The intensity of surface LAG3 expression was further evaluated in peripheral blood lymphocytes; mean fluorescence intensity (MFI)
of surface LAG3 was increased in CLL cells compared to normal B cells (p<0.001, Fig 1G). Surface LAG3 MFI was also increased in UM-IGHV compared to M-IGHV CLL cells (9.2±7.1 Vs 3.9±1.9, respectively; p=0.026, Fig 1H). In patients with CLL, surface LAG3 MIF was elevated in CLL cells compared to CD4+ and CD8+ lymphocytes [6.5±5.8 Vs 2.6±2.0 (p=0.005), Vs 2.3±1.7 (p=0.002)], respectively, (Fig.1I). No statistically significant differences were detected in the intensity of intracellular LAG3 expression between CLL and normal B cells (Supplement 4A).

*Increased expression of soluble (s)LAG3 is associated with both UM-IGHV status and shorter time to treatment*

The levels of LAG3V3, the soluble, shorter LAG3 isoform, encoded by alternatively spliced RNA, were determined in patients with CLL and in normal controls. In this analysis, IGHV mutational status data were available for 32 patients. Thirteen out of 17 patients with UM-IGHV, but only 3 out of 15 with M-IGHV, had progressive disease (Supplement 3). Increased levels of LAG3V3 mRNA were evident in UM-IGHV CLL cells compared to both M-IGHV CLL cells (p=0.039) and normal B cells (p=0.03, Fig. 2A). Elevated levels of LAG3V3 mRNA (defined as levels higher than the median value) were significantly associated with a shorter time to first treatment (Fig.2B).

Soluble (s)LAG3 protein levels were determined in the serum of patients with CLL and healthy controls and were found to be higher in patients with UM-IGHV CLL, compared to patients with M-IGHV gene and healthy controls (Fig.2C). The median serum sLAG3 levels were 2.5ng/ml (0.11-6.67), 0.2ng/ml (0.03-13.0) and 0.15 ng/ml (0.09-1.79) in patients with UM-IGHV, M-IGHV CLL and healthy controls, respectively (Fig.2C). High sLAG3 levels were also detected in patients whose disease progressed compared to patients with stable CLL [median levels of 2.9 ng/ml (0.15-13.0) and 0.06 ng/ml (0.03-1.48), respectively, p<0.001; (Fig. 2D)]

Next, we explored whether CLL cells secrete sLAG3. sLAG3 levels progressively increased in the culture medium of negatively selected CLL cells, and the highest levels were detected at the 72 hours’ time point (Fig. 2E). Overall, our data suggest that CLL cells express and secrete sLAG3.
**LAG3 binds MHC class II molecules on CLL cells**

As CLL cells express MHCII molecules on their cell surface (19), we further determined the specific binding of LAG3 to CLL cells. As shown in Fig. 3A, B, LAG3-Fc (a fusion protein that consists of an extracellular portion of LAG3 fused to the Fc fraction of human IgG1 that binds to MHCII) was found to bind a large proportion of CD19+ CLL cells, as opposed to Ig-Fc control. Mean fluorescence intensity (MIF) representing LAG3-Fc binding to CLL cells was 226 as compared to 51 in cells incubated with the Ig-Fc control. The addition of anti-LAG3 antibody, directed to the extra loop of the Ig-like domain 1 of LAG-3 that binds MCHII molecules (20, 21), completely abolished soluble LAG3 ligation to CLL cells. Therefore, our results suggest that sLAG3 binds to CLL cells through interaction with MHCII molecules.

**Soluble LAG3 activates CLL cells and exerts an anti-apoptotic effect**

We further studied the biological effects of sLAG3 on CLL cells. For this, peripheral blood CLL cells were incubated with LAG3-Fc and its effect on CLL cell activation was studied by evaluating cell surface CD86 expression. Expression of the costimulatory B7 molecules, CD80 and CD86, is low in CLL cells but it is upregulated upon cell activation (22). Activation of B cells via MHCII engagement was reported to induce B7 co-stimulatory molecules (23). As LAG3 interacts with CLL cells via MHCII, we used CD86 expression as a marker of LAG3-induced CLL cell activation. After 24 hours incubation with LAG3-Fc the expression of CD86+ CLL cells increased significantly compared to controls (Fig. 3C). CD86 upregulation in response to sLAG3 activation was completely blocked by pre-incubation with anti-LAG3 antibody (Fig. 3C). Incubation with LAG3-Fc also induced a mild, though statistically significant, increase in the expression of another marker of CLL cell activation, CD69 (Supplement 4B).

We next investigated the effect of LAG3-Fc on the PI3K/AKT and MAPK/ERK pathways, which have been reported to be activated following MHCII engagement (15). Stimulation of CLL cells with LAG3-Fc induced AKT and ERK1/2 phosphorylation, an effect that peaked 15 minutes after activation (Fig. 3D).

To explore possible effects of soluble LAG3 on CLL cell survival, CLL cells were incubated with LAG3-Fc and cells' viability was evaluated after 24, 48, 72 and 96 hours. The percentage of live cells increased significantly after incubation with
LAG3-Fc compared to unstimulated CLL cells. Maximal effect was detected after 48 and 72 hours' incubation (Fig. 4A-C and Supplement 4C). The effect of LAG3-Fc on CLL cell survival was abolished by PD98059 (MEK1/2 inhibitor), Ibrutinib (Bruton’s tyrosine kinase inhibitor) and R406 (SYK inhibitor and the active metabolite of Fostamatinib) (Fig. 4D), as well as by anti-LAG3 blocking antibody (Fig. 4A,E). However, LAG3-Fc anti apoptotic effect was not affected by pre-incubating with either wortmannin [phosphatidylinositol 3-kinase (PI3K) inhibitor] or Idelalisib (a specific PI3Kδ inhibitor), Fig 4D.

Incubation with LAG3-Fc was also associated with a prominent decrease in cleaved PARP and a robust increase in Mcl-1 levels. (Fig. 4F,G and Supplement 4D,E). The levels of Bcl-XL and Bcl-2 increased slightly and inconsistently after 48 and 72 hour incubation with LAG3-Fc (Fig. 4F,G and Supplement 4D,E). Interestingly, incubating CLL cells with anti-LAG3 antibody resulted in increased levels of apoptotic cells compared to control (Fig. 4H), suggesting that blocking sLAG3-MHCII interaction prevented autocrine effects of sLAG3, excreted by the cultured CLL cells.

**T-cells in CLL microenvironment express both LAG3 and PD1**

We also studied the expression of LAG3 on tumor infiltrating T lymphocytes in secondary lymphoid tissues (lymph nodes and spleens) obtained from patients with CLL and compared it to LAG3 expression on concurrently collected circulating peripheral blood T-cells. There was no statistically significant difference between LAG3 expression on CD4+ T-cells in peripheral blood and secondary lymphoid organs. However, we found that the percentage of CD8+ T-cells expressing LAG3 was significantly higher in secondary lymphoid tissues compared to paired peripheral blood CD8+ lymphocytes isolated from the same patient (5.7%±5.4 vs. 1.2±2.2% of CD8+ T-cells in secondary lymphoid tissues and peripheral blood, respectively, p=0.026, Fig. 5A). CD8+ cells, obtained from secondary lymphoid organs of patients with CLL, were analyzed further, and PD1 expression on these cells was evaluated. We found that LAG3 expression was confined to PD1 expressing CD8+ lymphocytes (Fig. 5B).

Next, we evaluated possible combined effects of LAG3 and PD1 blockade on T cell activation, in patients with CLL. For that, we determined the expression of
CD69 (as a marker of T cell activation) on T cells from peripheral blood of CLL patients, that were activated in vitro (using anti-CD3/CD28 beads), after pre-incubation with either anti-LAG3 antibody, anti-PD1 combined with anti-PD-L1 antibodies (to fully block PD-1 pathway) or both (Fig. 5C). We found that T cell activation was increased in the presence of anti-LAG3 antibody but was unaffected by PD-1 pathway blockade. Combining anti-LAG3 with anti-PD1/anti-PD-L1 antibodies abolished the positive effect induced by anti-LAG3 abs on both CD4+ and CD8+ T cell activation (Fig. 5C).

Discussion
Here we examined the role of the immune checkpoint receptor LAG3 and the interactions with its ligand, MHCII, in the pathogenesis of CLL. We show that CLL cells express LAG3 and excrete its soluble isoform, LAG3V3. Soluble LAG3 activated CLL cells and prevented them from undergoing spontaneous apoptosis, both effects mediated by its binding to MHCII molecules present on their surface.

LAG3 mRNA was detected in CLL cells at higher levels than in normal B cells. Full length LAG3 mRNA levels were also significantly higher in patients with the prognostically unfavorable UM-IGHV compared to those with M-IGHV gene. The latter observation is similar to gene expression profiles results reported earlier by Kostaskova et al (16). LAG3 was detected intracellularly in CLL cells, while only a small proportion of cells presented surface LAG3. However, in cells expressing surface LAG3, the levels were significantly higher in UM-IGHV cells, perhaps implying a role for LAG3 in the unfavorable prognosis of patients with UM-IGHV CLL. mRNA LAG3V3 and serum levels of sLAG3 were elevated in the UM-IGHV sub-group of patients compared to the subgroup of patients with M-IGHV gene. Increased levels of full length LAG3 mRNA, LAG3V3 mRNA and serum sLAG3 were all associated with a more aggressive clinical course and a shorter median time to first treatment. Thus, we can conclude that higher levels of LAG3 are associated with poor prognostic features and an aggressive course of disease in patients with CLL.

Previous studies have reported that increased levels of sLAG3 were associated with favorable outcome in patients with breast cancer (24). In these cases, sLAG3 binds MHCII molecules on APCs, increase the capacity of MHCII positive immune cells to induce T-cell response and enhance tumor-specific cytotoxic T-cells (15). However,
in malignant melanoma cells that express MHCII, sLAG3 binding appears to up-regulate anti-apoptotic pathways (15).

Similarly, we found that sLAG3 binds to MHCII on CLL cells, and induces CLL cell activation and stimulation of the PI3K/AKT and MAPK/ERK pathways, as well as promotes anti-apoptotic effects. Incubating CLL cells with sLAG3 resulted in an increase in the number of live cells, an effect abrogated through inhibition of BTK, SYK and LAG3-MHCII interaction but not through inhibition of PI3K pathway. Our findings are compatible with previous reports showing that ligation of MHCII generates downstream signals which is mediated through SYK, AKT and ERK (15, 25). Activation of CLL cells via sLAG3 also resulted in decreased degradation of PARP and increased expression of anti-apoptotic proteins, which was substantial for Mcl-1 and more subtle for Bcl-XL and Bcl-2. Constitutive expression of anti-apoptotic proteins and resistance to apoptosis are major hallmarks of CLL. Our data suggest a role for LAG3 in the pathogenesis of CLL, not only as an immune modulator but also in the regulation of anti-apoptotic pathways in CLL cells.

We show here that in CLL patients, LAG3 is expressed both by tumor cells as well as in the tumor microenvironment: We found that LAG3 expression on CD8+ T-cells was increased in secondary lymphoid tissues obtained from CLL patients, compared to peripheral blood lymphocytes. This is in agreement with previous studies that reported increased expression of LAG3 on CD8+ T-cells infiltrating some solid tumors as well as in a murine model of CLL (12, 26, 27). We also show that LAG3 expression was detected almost exclusively on PD1 presenting CD8+ lymphocytes. Co-expression of LAG3 together with PD1 on TILs identifies a highly exhausted T cell population, and the synergy between these inhibitory receptors appears to impose tumor-induced immune tolerance in solid tumors (8, 11, 28-30).

Blocking LAG3 enhanced both CD4+ and CD8+ T cell activation, while blocking PD-1/PD-L1 pathway did not affect T cell activation. This is perhaps in agreement with recently published data showing only a modest effect for anti PD1 Pembrolizumab in patients with CLL (31). When expressed on immune cells present in the microenvironment, LAG3 may induce immune tolerance and exhaustion of LAG3-expressing cells through its interaction with the MHCII-presenting CLL cells. Hence, it is feasible that LAG3 could be targeted in an attempt to enhance anti-tumor immunogenicity.
In this study, we show that CLL cells not only express, but also secrete sLAG3. Additionally, the mere addition of anti-LAG3 antibodies to CLL cells increased spontaneous apoptosis. This may be indicative of the existence of a vicious cycle in which LAG3 (either secreted by CLL or T cells, or presented on immune cells) and its interaction with MHCII on CLL cell surface promote CLL cell activation and enhance their survival. Our data suggests that targeting sLAG3-MHCII engagement could be considered as a potentially novel form of anti-CLL immunotherapy.

Acknowledgments: The study was supported by The Varda and Boaz Dotan Research Center in Hemato-Oncology affiliated with the CBRC at Tel-Aviv University.

The authors have no conflict of interest to declare.

Author's contributions: MS-performed the experiments and wrote the paper, YH-initiated the study, supervised the research, analyzed the data and wrote the paper, BZK-analyzed the data, ND- performed the experiments, CS- performed the experiments, SK- performed the experiments, IA-provided essential reagents, AP-critical reading of the paper, AW- critical reading of the paper, CP-supervised the research and wrote the paper.
References


**Figure Legend**

**Figure 1. Full length LAG3 expression in CLL patients**
(A-C) Full length LAG3 mRNA levels in CD19⁺ selected normal B-cells and CLL cells quantitated by qPCR, and normalized to GAPDH. (A) LAG3 mRNA expression in normal B-cells (n=7) vs. CLL cells (n=28) (B) LAG3 mRNA expression in M-IGHV cells (n=15) compared to UM-IGHV CLL cells (n=13). (C) Kaplan-Meier analysis of time from diagnosis to first treatment in CLL patients with “low” and “high” LAG3 mRNA levels (n=28), using the median value as cut-off level. (D-E) Detection of LAG3 protein levels by Western blot assay in CD19⁺ purified CLL cells, M-IGHV cells compared to UM-IGHV CLL cells. (D) Representative blot analyzing CLL cells from 8 patients, 4 M-IGHV and 4 UM-IGHV CLL (patients’ characteristics are presented in Supplement 3, according to the designated numbers). Actin was used to verify equal loading. (E) Statistic analysis of CLL cells from 16 patients with CLL, 8 M-IGHV compared to 8 UM-IGHV CLL cells. (F-I) LAG3 expression in peripheral blood samples, evaluated by flow cytometry (F) Representative dot plots showing surface and intracellular expression of LAG3 in CLL cells. (G) Summary of surface LAG3 mean fluorescence intensity normalized to isotype control (MFIR), in normal B-cells (n=8) compared to CLL cells (n=22) isolated from peripheral blood of healthy controls and patients with CLL, respectively (H) Surface LAG3 MFI normalized to isotype control (MFIR) as detected in peripheral blood of M-IGHV (n=11) and UM-IGHV (n=11) CLL cells. (I) Summary of surface LAG3 MFIR in CLL, CD4⁺ and CD8⁺ cells isolated from peripheral blood of patients with CLL (n=22).

**Figure 2. soluble (s)LAG3 is associated with UM-IGHV status and progressive disease**
(A-B) LAG3V3 mRNA levels encoding soluble LAG3 (sLAG3) were quantitated in CD19⁺ selected normal B and CLL cells by qPCR, and normalized to GAPDH. (A) LAG3V3 mRNA levels in normal B-cells (n=7), M-IGHV (n=12) and UM-IGHV (n=11) CLL cells. (B) Kaplan-Meier analysis of time from diagnosis to first treatment in patients with CLL (n=23), expressing “low” and “high” LAG3V3 mRNA levels, using median value as cut-off level. (C-D) Serum sLAG3 levels in CLL patients and healthy individuals, determined by ELISA (C) Serum sLAG3 levels in healthy controls (n=8) and patients with M-IGHV (n=16) and UM-IGHV (n=17) CLL. (D)
Comparison between serum sLAG3 levels in CLL patients with either stable (n=17) or progressive (n=18) disease. (E) Measurement of sLAG3 levels in the medium of cultured CLL cells; negatively selected CLL cells were cultured and medium sLAG3 levels in the culture medium were determined by ELISA after 24, 48 and 72 hours (n=5).

**Figure 3. soluble (s)LAG3 binds and activates MHC class II molecules on CLL cells**

(A-B) Detection of sLAG binding to CLL cells: Peripheral blood CLL cells were incubated for 15 min with either LAG3-Fc, LAG3-Fc after pre-incubation with anti-LAG3 blocking antibody, or Ig-Fc that served as control. LAG3 binding to CLL cells was detected by flow cytometry, using a fluorophore-conjugated secondary antibody (anti-human Fc). (A) Representative dot plot analysis showing that LAG3-Fc binding to CLL cells (middle box) was completely abolished by anti-LAG3 blocking antibody (right box) (B) The mean fluorescence intensity of LAG3-binding CLL cells after incubation with LAG3-Fc (middle bar) decreases to control levels after pre-incubation with anti-LAG3 Abs (right bar); cumulative results of 11 experiments. (C) Measurement of CD86 surface expression on CLL cells in response to sLAG3 activation: CLL cells were incubated with either LAG3-Fc or Ig-Fc that served as control, for 24 hours and surface CD86 expression was analyzed on CD5+/CD23+ gated CLL cells, by flow cytometry. C-Left: representative dot plots showing an increase in surface CD86 expression on CLL cells in the presence of LAG3-Fc. C-middle: surface CD86 mean fluorescence intensity (MFI) levels on LAG3-Fc-activated CLL cells are presented as fold-change increase over control (Ig-Fc) levels, n=11. C-Right: comparison of CD86 MFI expression on CLL cells incubated with either Ig-Fc (control, left bar), LAG3-Fc (middle bar) or LAG3-Fc pre-incubated with anti-LAG3 blocking antibody [right bar, (n=7)]. (D) Changes in the mean fluorescence levels (normalized to baseline) of pERK and pAKT in CLL cells, measured by flow cytometry at 0, 5, 15, 45, 60 and 120 min after activation by LAG3-Fc (n=5).

**Figure 4. sLAG3 protects CLL cells from spontaneous apoptosis**
Peripheral blood CLL cells were incubated for 48 or 72 hours with either Ig-Fc (control), LAG3-Fc, or with LAG3-Fc pre-incubated with anti-LAG3 blocking antibody aimed at the MHCII molecules binding site. Cell viability was determined by flow cytometry, using Annexin V/PI apoptosis detection kit. The levels of anti-apoptotic proteins and cleaved PARP (cPARP) were determined by Western blot analysis and quantified: (A) Representative dot plots showing the percentage of apoptotic cells in the presence of Ig-Fc (left), LAG3-Fc (middle) and LAG3-Fc with anti-LAG3 blocking antibody (right). (B-C) Percentage of live CLL cells in the presence of either Ig-Fc (control) or LAG3-Fc, as seen in 10 independent experiments after 48 (B) and 72 hours (C) incubation. (D) The percentage of live CLL cells in the presence of Ig-Fc control [marked as (-)], or LAG3-Fc [marked as (+)], with or without one hour pre-incubation with PD98059, Wortmannin (left graph), Ibrutinib, R406 or Idelalisib (right graph). After cultured for 48 hours, cell viability was determined by flow cytometry, using Annexin V/PI apoptosis detection kit (n=7). (E) The percentage of live CLL cells cultured with either Ig-Fc (Left bar), LAG3-Fc (middle bar), or LAG3-Fc pre-incubated with anti-LAG3 (right bar), for 1 hour, washed and incubated for an additional 72 hours before analyzed by flow cytometry (n=6). (F) Representative Western blot analysis showing the levels of cleaved PARP, MCL-1, Bcl-XL and Bcl-2 in CLL cells after 72 hours incubation with Ig-Fc as a control [marked (-)] or LAG3-Fc [marked (+)]. Actin was used to verify equal loading. (G) Cumulative results from 8 independent experiments, performed as described in Fig 4F. Shown are quantified levels of cleaved PARP, MCL-1, Bcl-XL and Bcl-2 in LAG3-Fc activated CLL cells, normalized to control (incubation with Ig-Fc). (H) The percentage of apoptotic CLL cells increases following LAG3 blockade. The levels of apoptotic CLL cells were determined after 72 hours incubation with anti-LAG3 blocking antibody and normalized to control levels, in 15 independent experiments.

Figure 5. Increased surface LAG3 expression on CD8+ tumor infiltrating lymphocytes and blocking LAG3 enhance in-vitro T cell activation. (A) The expression of surface LAG3 on CD4+ and CD8+ cells (analyzed by flow cytometry), in 10 paired samples of peripheral blood and secondary lymphoid tissues [lymph node (n=3) or spleen cells (n=7)] from patients with CLL. B. A representative dot plot analysis, showing co-expression of surface LAG3 and PD1 on...
lymph node-derived CD8⁺ lymphocytes isolated from a patient with CLL (n=5). C. CD69 expression on activated T-cells, with or without LAG3 and PD1 blockade. Cells from 7 patients with CLL were incubated for 48 hours in the presence or absence of the indicated blocking antibodies or with IgG1 isotype control. T-cells were then activated by CD3/CD28 beads for 6 hours and CD69 expression was analyzed in CD4⁺ (left) and CD8⁺ cells (right) by flow cytometry.
Supplement 1. Illustration of the membrane-expressed and secreted LAG-3 molecules.
Supplement 2

Material and methods

Patients and samples

Mononuclear cells from patients with CLL and healthy controls were isolated by density-gradient centrifugation over Ficoll-Paque PLUS (GE healthcare, NJ, USA). Viably frozen cells were kept in fetal calf serum (FCS) containing 10% dimethyl sulfoxide and stored in liquid nitrogen. Before use, frozen cells were thawed and cultured at 37°C, 5% CO₂, in RPMI medium 1640 (Gibco, Life Technologies, CA, USA) supplemented with 10% FCS, penicillin, streptomycin, and L-glutamine all purchased from Biological Industries (BI, Kibbutz Beit Haemek, Israel). Serum samples from patients with CLL and healthy controls were also stored in -80°C for ELISA analysis.

Reagents and antibodies

Monoclonal antibodies (MoAb) anti-human CD19-APC (SJ25C1), CD86-PE (IT2.2), CD69-PE (L78), CD4-APC (RPA-T4), CD8-BV421 (RPA-T8), CD5-APC (L17F12), CD23-FITC (M-L233), AKT (pS473)-PE (M89-61), ERK1/2 (pT202/pY204)-PE (20A), cytofix fixation buffer, and Phosflow perm/wash buffer I were purchased from BD Biosciences (San Jose, CA, USA), Mouse IgG1 κ isotype control PerCP-eFluor® 710 (P3.6.2.8.1), anti-Human CD223 (LAG3) PerCP-eFluor® 710 (3DS223H), CD8a-PE (HIT8a), CD4 FITC (RPA-T4), Anti-Human CD279 (PD-1) PE-Cyanine7 (J105) Mouse IgG1 κ isotype control PE- Cyanine7 (P3.6.2.8.1), anti-Human CD274 (PD-L1, B7-H1) (M1H1), anti-human CD279 (PD-1) (J116) and mouse IgG1 K Isotype control purified (P3.6.2.8.1) and polyclonal anti-Human IgG (Fc gamma-specific) PE, were purchased from eBioscience (San Diego, CA, USA). PE Mouse IgG1, κ Isotype control antibody was from BioLegend (San Diego, CA, USA). Anti-human Bcl-2 (50E3), Bcl-xL (54HS), Cleaved PARP (D64E10) and Polyclonal Mcl-1 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-LAG3 (17B4) was purchased from Abcam (Cambridge, UK). Purified anti-human Actin was from MP Biomedicals (Illkirch, France). Goat anti-Rabbit IgG (H+L)-HRP conjugate and Goat anti-Mouse IgG (H+L)-HRP conjugate were from Bio-Rad Laboratories (Richmond, CA). Recombinant soluble human LAG3-Ig fusion protein (LAG3-Fc) and human IgG1-Fc control were purchased from Enzo Life Sciences. Anti-LAG3 (blocking) (17B4)
(PF) was purchased from AdipoGen (Liestal, Switzerland). All antibodies utilized in the study were used in concentrations according to the manufacturer's instructions. Dynabeads Human T-Activator CD3/CD8 was purchased from Invitrogen (Oslo, Norway). PD-98059 and Syk Inhibitor VI, R406 were purchased from Merck Millipore’s Calbiochem (Darmstadt, Germany). Ibrutinib was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA), CAL-101 (Idelalisib, (GS-1101) was from Selleckchem (TX, USA), and Wortmannin was purchased from Sigma-Aldrich (St. Louis, MO, USA). FcR Blocking Reagent was from Miltenyi Biotec Inc. (Auburn, CA, USA).

**IGHV gene analysis**

Analysis of IGHV gene status was performed as described in Wiestner et al. Amplification of the immunoglobulin heavy chain variable (IGHV) gene was performed as described by Hamblin et al. Briefly, cDNA was amplified by polymerase chain reaction (PCR) using a mixture of 5’ oligonucleotides specific for each leader sequence of the VH1 to VH7 IGHV families as forward primers and a 3’ oligonucleotide complementary to either the consensus sequence of the joining region or the constant region of the IgM locus as reverse primer. PCR was performed in 25 μL reactions with FastStart Taq (Roche) and 20 pmol of each primer. Products were purified (QIAquick Kit; Qiagen, Valencia, CA) and sequenced directly with the appropriate 3’ and 5' oligonucleotides by HyLab Sequencing Service (Rehovot, Israel). Nucleotide sequences were aligned to the VBase sequence directory ([http://www.ncbi.nlm.nih.gov/igblast/](http://www.ncbi.nlm.nih.gov/igblast/)). Sequences with 2% or less deviation from any germline IGHV sequence were considered unmutated.

**Western blotting**

Purified CLL cells were lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing Phosphatase Inhibitor Cocktail 2 and protease inhibitor Cocktail (Sigma-Aldrich, St. Louis, Missouri, USA). Extracts from cell lysates were separated on 10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose paper. The nitrocellulose paper was incubated with the designated antibodies and HRP-conjugated secondary antibodies according to the manufacturer's instructions, detected using myECL™ Imager and quantified using MyImageAnalysis Software (both from Thermo Fisher Scientific, Waltham, MA USA).
**Flow cytometry**
For surface staining, cells were incubated for 30 min at 4°C with the appropriate antibodies, and intracellular staining was performed using Cytofix fixation buffer and Phosflow Perm/Wash buffer I, according to the manufacturer’s instructions. Samples were acquired by the BD FACSCanto II and analyzed using BD FacsDiva software (Becton Dickinson, San Jose, CA).

**Quantitative PCR primers**
GAPDH forward 5'-ATGGGGAAGGTGAAGGTCG
and reverse 5'-GGGGTCATTGATGGCAACAATA,
LAG3 forward 5'-TCACAGTGACTCCCAAATCCT
and reverse 5'-GCTCCACACAAAGCGTTCTT,
sLAG3 forward 5'-GACCTCCTGGTGACTGGAGA
and reverse 5' TCCCACCTGAGGCTGACC.
All primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA).
## Supplement 3. Patient characteristics

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A. Summary of intracellular LAG3 MFI normalized to Isotype control in B-cells (left, n=8) compared to CLL cells (right, n=22) isolated from peripheral blood of healthy controls and CLL patients. B. Statistical analysis of CD69 surface expression on CLL cells (n=8) after sLAG3 activation: CLL cells were incubated with either Ig-Fc as control or LAG3-Fc for 24 hours and surface CD69 expression was analyzed on CD5⁺/CD23⁺ gated CLL cells, by flow cytometry. C. Differences in percentage of live CLL cells in the presence of LAG3-Fc compared to Ig-Fc (control), after 24, 48, 72 or 96 hours incubation (summary of 6-9 independent experiments). D. Representative Western blot analysis showing the levels of cleaved PARP, MCL-1, Bcl-XL and Bcl-2 in CLL cells after 48 hours incubation with Ig-Fc (control) or with LAG3-Fc. Actin was used to verify equal loading. E. Cumulative results of 7 independent experiments, performed as described in Fig 4, presenting quantified levels of cleaved PARP, MCL-1, Bcl-XL and Bcl-2 in LAG3-Fc activated CLL cells, normalized to control (Ig-Fc), after 48 hours incubation.