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Lymphocyte activation gene 3: a novel therapeutic target in chronic lymphocytic leukemia

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ABSTRACT

A novel therapeutic approach in cancer, attempting to stimulate host anti-tumor immunity, involves blocking of immune checkpoints. Lymphocyte activation gene 3 (LAG3) is an immune checkpoint receptor expressed on activated/exhausted T cells. When engaged by the major histocompatibility complex (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 the study herein, 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 leukemic 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.

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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 the 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 rate of survival.^{4,6}

The inability of the immune system to eradicate malignancy is one of the funda-

mental 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, the 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 homolog 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} (*Online Supplementary Figure S4*). 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 the 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 cells resistance to apoptosis and their ability to evade anti-cancer immunity. For that reason, 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 patients¹⁷ and healthy controls. Lymph nodes and spleen samples were also collected from CLL patients. Handling protocol is available in the *Online Supplementary Material and Methods*.

Reagents and antibodies

These are detailed in the *Online Supplementary Material and Methods*.

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, USA.

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, UK) 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 the *Online Supplementary Material and Methods*.

Cell stimulation, apoptosis and LAG3-Fc binding assay

Stimulation: CLL cells were incubated with FcR blocking reagent, before being stimulated by either a recombinant soluble human LAG3-Ig fusion protein (LAG3-Fc) (1µg/ml) or control Ig-Fc (1µg/ml).

Analysis of cell viability and apoptosis: After incubation with LAG3-Fc or Ig-Fc, cells were either harvested for Western blot assays or stained with the Annexin V/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 γ -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 being added to the cultured CLL cells for 1 hour of incubation. Subsequently cells were washed and incubated for 72 hours.

Activation of T cells 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

These are detailed in the *Online Supplementary Material and Methods*.

Quantitative PCR

Real-time polymerase chain reaction (PCR) was performed using LightCycler® 480 SYBR Green I Master and analyzed on a LightCycler 480 II (Roche, Basel, Switzerland). Primers are presented in the *Online Supplementary Material and Methods*.

Enzyme-linked immunosorbent assay (ELISA)

sLAG3 serum concentrations were determined using RayBio Human LAG3 Elisa kit (RayBiotech, GA, USA) following the manufacturer's instructions, 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*

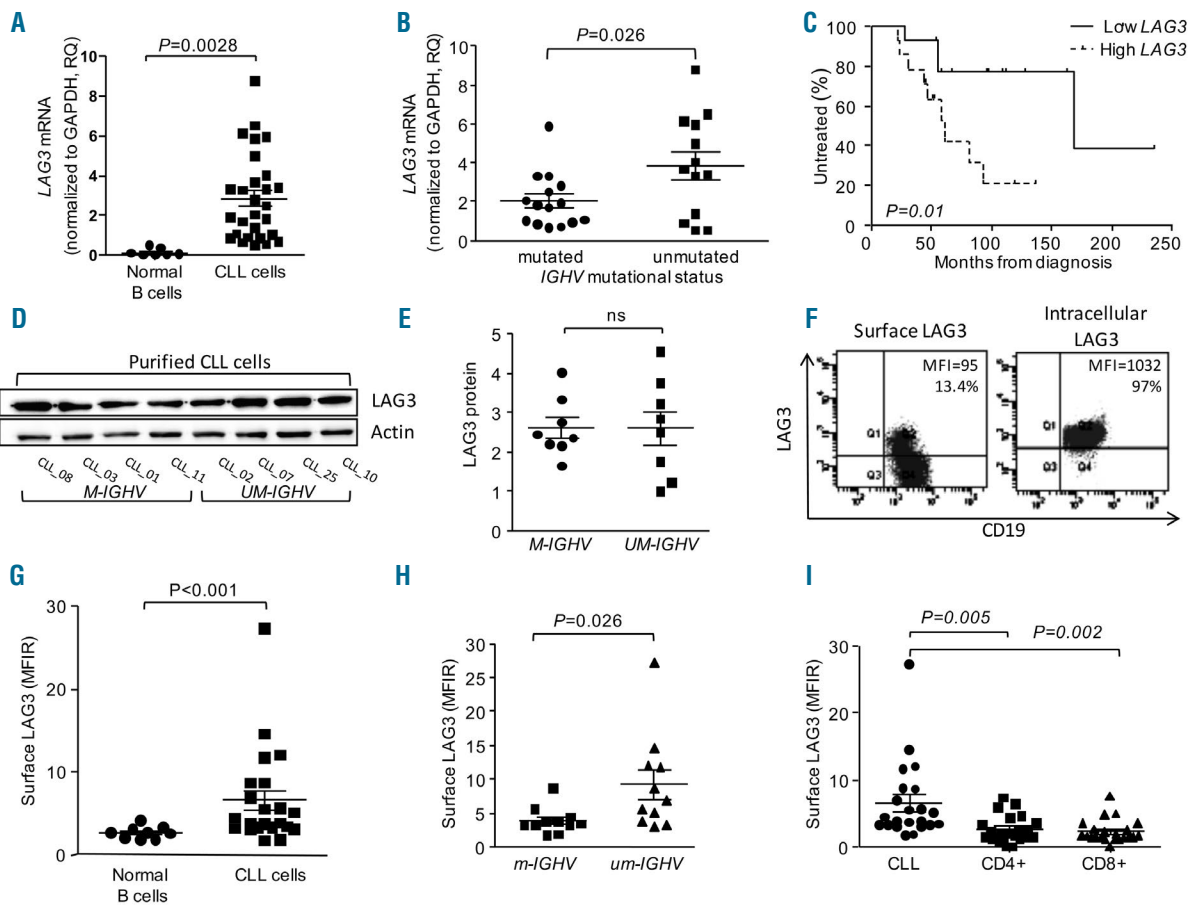


Figure 1. Full-length LAG3 expression in CLL patients. (A-C) Full-length LAG3 mRNA levels in CD19⁺ selected normal B cells and CLL cells quantitated by qPCR, and normalized to GAPDH. (A) LAG3 mRNA expression in normal B cells (n=7) vs. CLL cells (n=28). (B) LAG3 mRNA expression in *M-IGHV* cells (n=15) 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 cutoff level. (D-E) Detection of LAG3 protein levels by Western blot assay in CD19⁺ purified CLL cells, *M-IGHV* cells compared to 8 *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 the *Online Supplementary Table S1*, 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 (MFI) normalized to isotype control, 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). CLL: chronic lymphocytic leukemia; LAG3: lymphocyte activation gene 3; ns: not significant.

CLL,¹⁶ we first evaluated the expression of full-length LAG3 messenger RNA (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 the *Online Supplementary Table S1*. Peripheral blood CLL and normal B cells were purified using positive selection to obtain B cell 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 ($P=0.0028$; Figure 1A). When evaluated among patients with CLL, LAG3 mRNA levels were significantly increased in *UM-IGHV* CLL cells compared to cells with the *M-IGHV* gene ($P=0.026$; Figure 1B). Moreover, patients with higher levels of full-length LAG3 mRNA (defined as being above the median LAG3 mRNA level) had a shorter median time from diagnosis to first treatment (Figure 1C). At the 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 (Figure 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 (Figure 1F). Most CLL cells, however, expressed high levels of intracellular LAG3 (6.4±5.4% expressed surface LAG3 while 60.9±24.8% expressed intracellular LAG3, Figure 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 ($P<0.001$; Figure 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; $P=0.026$; Figure 1H). In patients with CLL, surface LAG3 MFI was elevated in CLL cells compared to CD4⁺ and CD8⁺ lymphocytes (6.55±5.8 vs. 2.6±2.0 ($P=0.005$), vs. 2.3±1.7 ($P=0.002$), respectively, Figure 1I). No statistically significant differences were detected in the intensity of intracellular LAG3 expression between CLL and normal B cells (*Online Supplementary Figure S2A*).

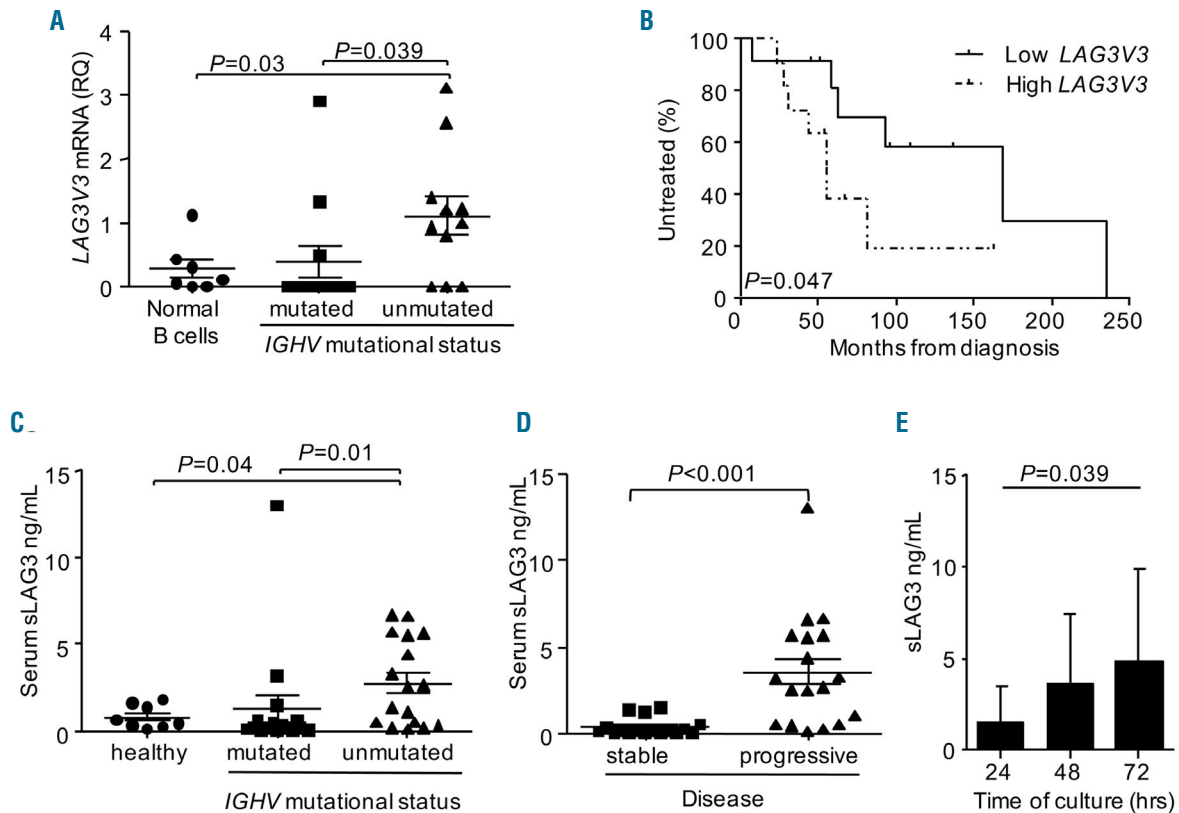


Figure 2. Soluble (s)LAG3 is associated with UM-IGHV status and progressive disease. (A-B) LAG3V3 mRNA levels encoding sLAG3 were quantitated in CD19⁺ selected normal B and CLL cells by qPCR, and normalized to GAPDH. (A) LAG3V3 mRNA levels in normal B cells (n=7), M-IGHV (n=12) and UM-IGHV (n=11) CLL 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 the median value as cutoff 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). mRNA (RQ): messenger RNA (relative quantification).

Increased expression of sLAG3 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 (Online Supplementary Table S1). 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 ($P=0.03$; Figure 2A). Elevated levels of LAG3V3 mRNA (defined as levels higher than the median value) were significantly associated with a shorter time to first treatment (Figure 2B).

sLAG3 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 the M-IGHV gene and healthy controls (Figure 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, (Figure 2C). High sLAG3 levels were also detected in patients whose disease progressed compared to patients with stable CLL (median lev-

els of 2.9 ng/ml (0.15-13.0) and 0.06 ng/ml (0.03-1.48), respectively, $P<0.001$; Figure 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 hour time point (Figure 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 Figure 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. MFI value, 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 LAG3 that binds MHCII 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.

sLAG3 activates CLL cells and exerts an anti-apoptotic effect

We further studied the biological effects of sLAG3 on CLL cells. For this purpose, 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 costimulatory 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 control (Figure 3C). CD86 upregulation in response to sLAG3 activation was completely blocked by pre-incubation with anti-LAG3 antibody (Figure 3C). Incubation with LAG3-Fc also induced a mild, though statistically significant, increase in the expression of another marker of CLL cell activation, CD69 (*Online Supplementary Figure S2B*).

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

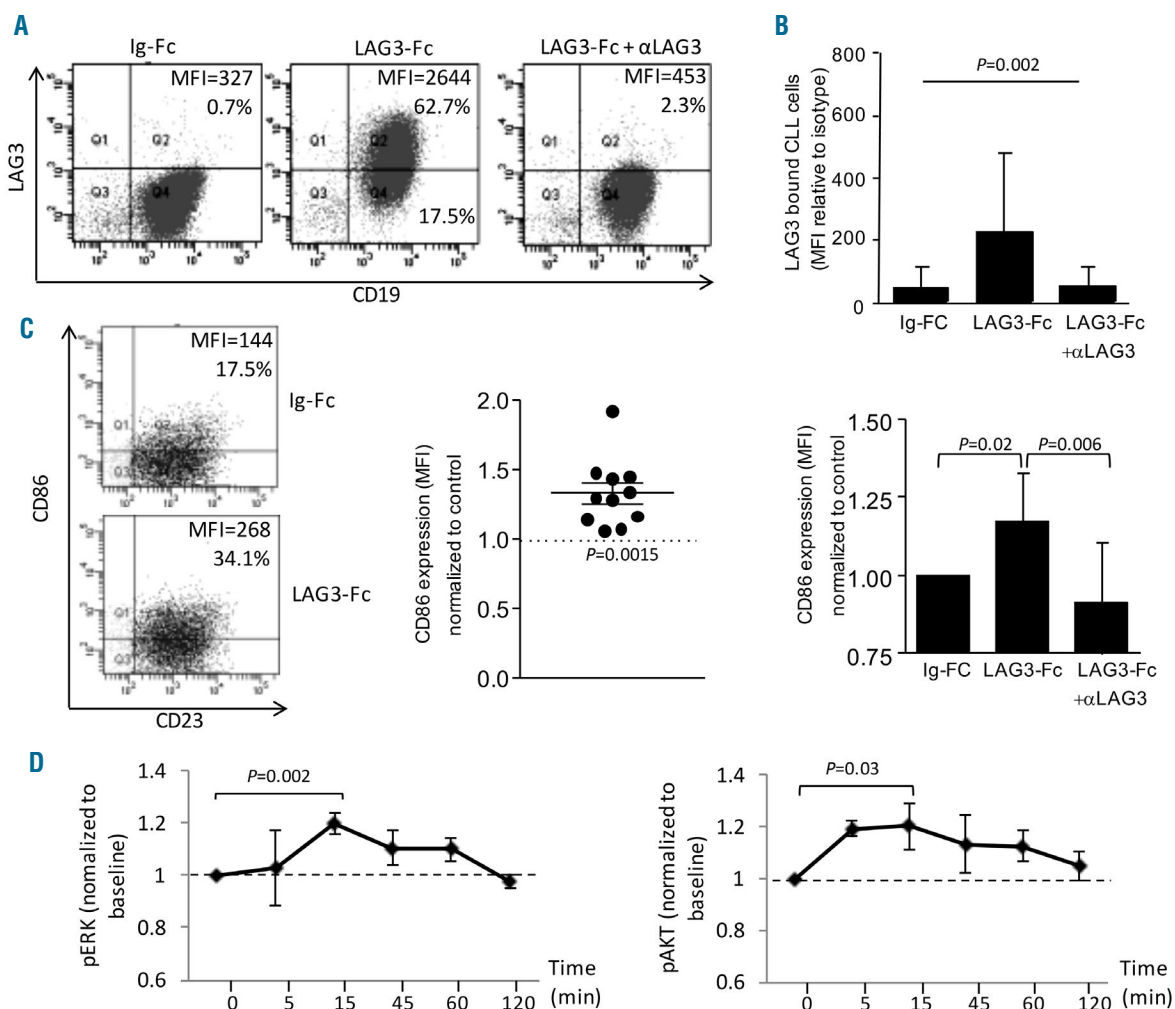


Figure 3. Soluble (s)LAG3 binds and activates MHC class II molecules on CLL cells. (A-B) Detection of sLAG binding to CLL cells: peripheral blood CLL cells were incubated for 15 min with either LAG3-Fc, LAG3-Fc after pre-incubation with anti-LAG3 blocking antibody, or Ig-Fc that served as control. LAG3 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 (MFI) of LAG3-binding CLL cells after incubation with LAG3-Fc (middle bar) decreases to control levels after pre-incubation with anti-LAG3 antibodies (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 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). CLL: chronic lymphocytic leukemia; LAG3: lymphocyte activation gene 3.

ERK1/2 phosphorylation, an effect that peaked 15 min after activation (Figure 3D).

To explore possible effects of soluble LAG3 on CLL cell survival, CLL cells were incubated with LAG3-Fc and their viability was evaluated after 24, 48, 72 and 96 hours. The percentage of live cells increased significantly after incuba-

tion with LAG3-Fc compared to unstimulated CLL cells. Maximal effect was detected after 48 and 72 hours incubation (Figure 4A-C and *Online Supplementary Figure S2C*). 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

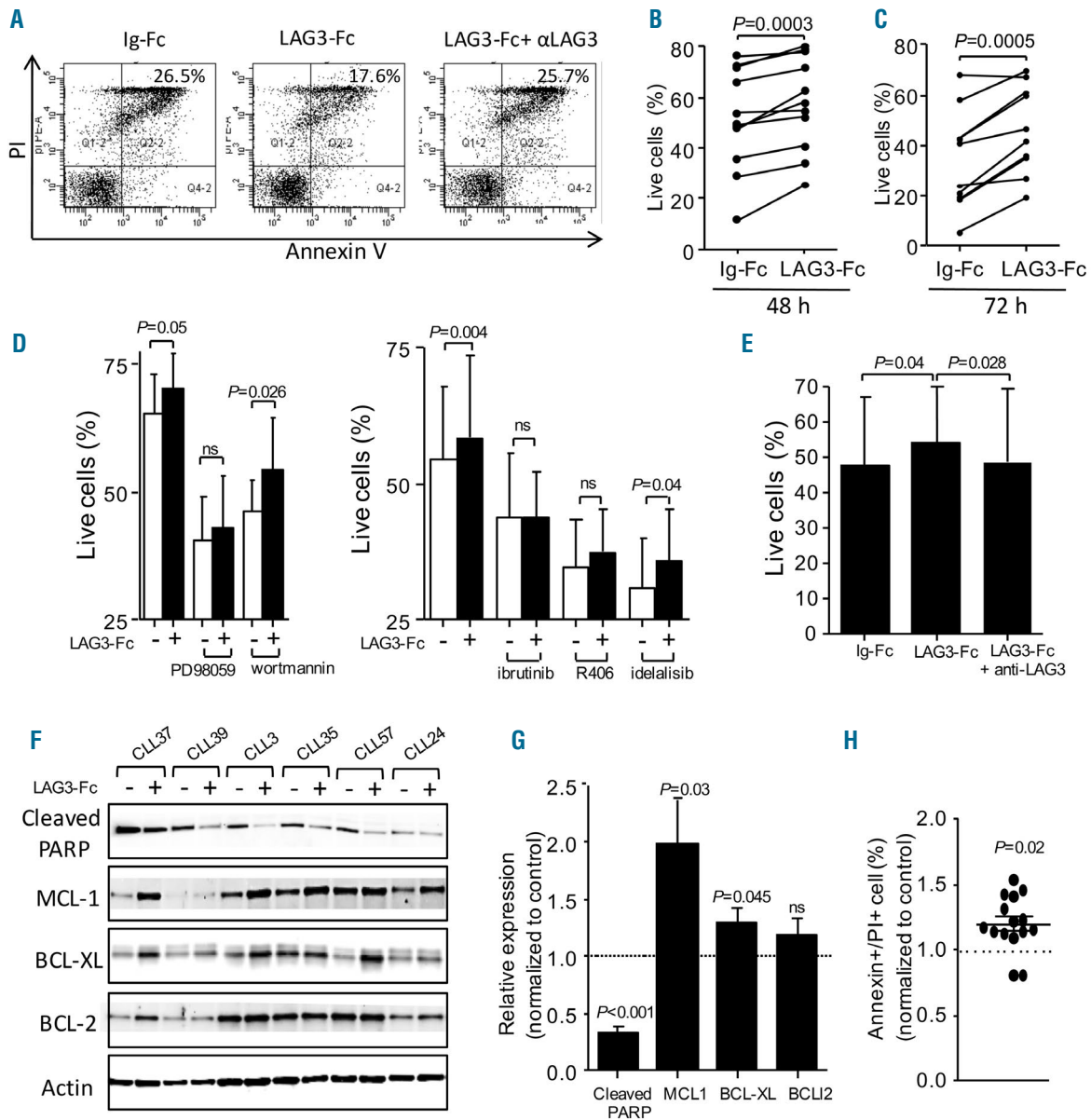


Figure 4. Soluble (s)LAG3 protects CLL cells from spontaneous apoptosis. (A-F) Peripheral blood CLL cells were incubated for 48 or 72 hours with either Ig-Fc (control), LAG3-Fc, or with LAG3-Fc pre-incubated with anti-LAG3 blocking antibody aimed at the MHCII molecules binding site. Cell viability was determined by flow cytometry, using an Annexin V/PI apoptosis detection kit. The levels of anti-apoptotic proteins and cleaved PARP 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 1 hour pre-incubation with PD98059, wortmannin (left graph), ibrutinib, R406 or idelalisib (right graph). After being cultured for 48 hours, cell viability was determined by flow cytometry, using an 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 being 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 Figure 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 increased 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. LAG3: lymphocyte activation gene 3; CLL: chronic lymphocytic leukemia; ns: not significant.

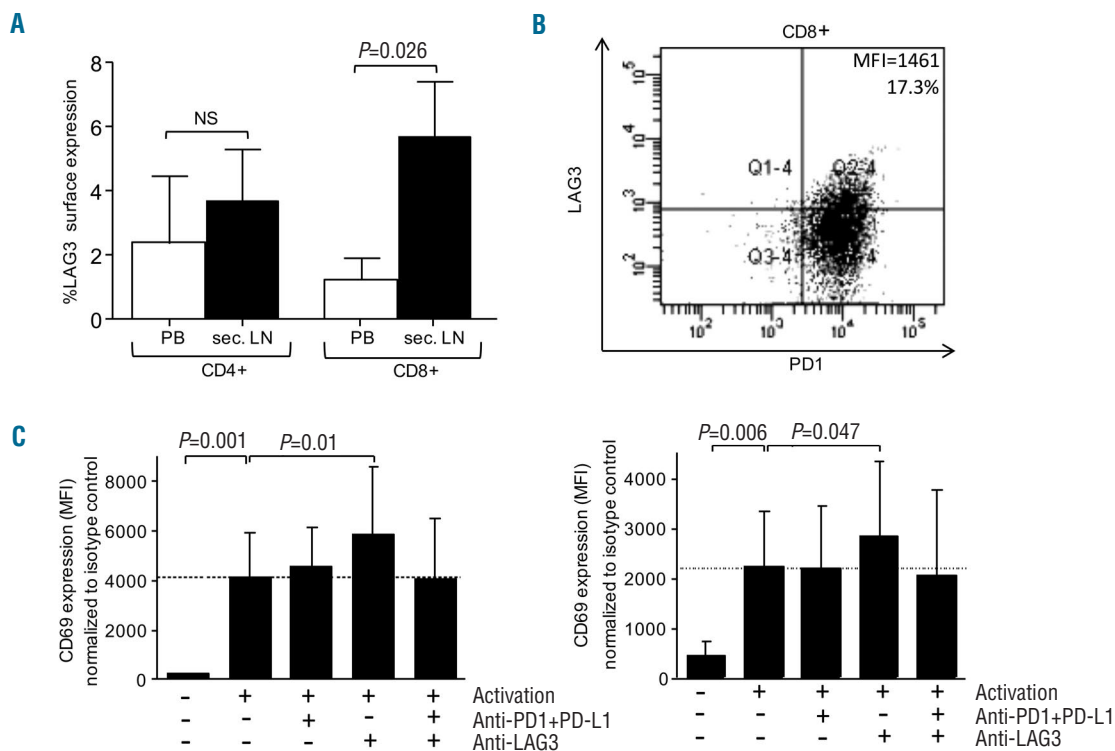


Figure 5. Increased surface LAG3 expression on CD8⁺ tumor infiltrating lymphocytes and blocking LAG3 enhance *in vitro* T-cell activation. (A) The expression of surface LAG3 on CD4⁺ and CD8⁺ cells (analyzed by flow cytometry), in 10 paired samples of peripheral blood (PB) and secondary lymphoid tissues [sec. LN; 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. LAG3: lymphocyte activation gene 3; MFI: mean fluorescence intensity; ns: not significant.

active metabolite of fostamatinib) (Figure 4D) as well as by the anti-LAG3 blocking antibody (Figure 4A and 4E). However, LAG3-Fc anti-apoptotic effect was affected neither by pre-incubating with wortmannin (phosphatidylinositol 3-kinase (PI3K) inhibitor) nor by idelalisib (a specific PI3K δ inhibitor), Figure 4D.

Incubation with LAG3-Fc was also associated with a prominent decrease in cleaved PARP and a robust increase in Mcl-1 levels. (Figure 4F,G and *Online Supplementary Figure S2D,E*). The levels of Bcl-XL and Bcl-2 increased slightly and inconsistently after 48 and 72 hour incubation with LAG3-Fc (Figure 4F,G and *Online Supplementary Figure S2D,E*). Interestingly, incubating CLL cells with anti-LAG3 antibody resulted in increased levels of apoptotic cells compared to control (Figure 4H), suggesting that blocking sLAG3-MHCII interaction prevented autocrine effects of sLAG3, excreted by the cultured CLL cells.

T cells in the 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 \pm 5.4% vs. 1.2 \pm 2.2% of CD8⁺ T cells in secondary lymphoid tissues and peripheral blood, respectively, $P=0.026$; Figure 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 (Figure 5B).

Next, we evaluated the possible combined effects of LAG3 and PD1 blockade on T cell activation in patients with CLL. In order to do so, 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 the PD-1 pathway), or both (Figure 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 antibodies on both CD4⁺ and CD8⁺ T-cell activation (Figure 5C).

Discussion

In the study herein, we examined the role of the immune checkpoint receptor LAG3 and the interactions with its ligand, MHCII, in the pathogenesis of CLL. We showed that CLL cells express LAG3 and excrete its soluble isoform, LAG3V3. sLAG3 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 the *M-IGHV* gene. The latter observation is similar to gene expression profile 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, the short, soluble LAG3 isoform, were elevated in the *UM-IGHV* subgroup of patients compared to patients with the *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 a favorable outcome in patients with breast cancer.²⁴ In these cases, sLAG3 binds MHCII molecules on APCs, increases the capacity of MHCII positive immune cells to induce T-cell response and enhances tumor-specific cytotoxic T cells.¹⁵ However, in malignant melanoma cells that express MHCII, sLAG3 binding appears to upregulate 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 promoting anti-apoptotic effects. Incubating CLL cells with sLAG3 resulted in an increase in the number of live cells, an effect abrogated through the inhibition of BTK, SYK and LAG3-MHCII interaction, but not through the inhibition of the 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} The activation of CLL cells *via* sLAG3 also resulted in a decreased degradation of PARP

and an 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 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.^{9,11,28-30}

Blocking LAG3 enhanced both CD4⁺ and CD8⁺ T-cell activation, while blocking the 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 the study herein, we demonstrated 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 surfaces promotes CLL cell activation and enhances their survival. Our data suggests that targeting LAG3-MHCII engagement could be considered as a potentially novel form of anti-CLL immunotherapy.

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