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ORIGINAL PAPER

T-helper cell regulation of CD45 phosphatase activity by galectin-1 and CD43 governs chronic lymphocytic leukaemia proliferation

John F. Imbery[1,2] | **Julia Heinzelbecker**[1,2] | **Jenny K. Jebsen**[1,2] | **Marc McGowan**[1,2] | **Camilla Myklebust**[1,2] | **Nunzio Bottini**[3] | **Stephanie M. Stanford**[3] | **Sigrid S. Skånland**[2,4] | **Anders Tveita**[1,2] | **Geir E. Tjønnfjord**[2,5] | **Ludvig A. Munthe**[1,2] | **Peter Szodoray**[1,2] | **Britt Nakken**[1,2]

[1]Department of Immunology, Oslo University Hospital, Oslo, Norway

[2]Faculty of Medicine, KG Jebsen Centre for B Cell Malignances, Institute of Clinical Medicine, University of Oslo, Oslo, Norway

[3]Division of Rheumatology, Allergy and Immunology, Department of Medicine, University of California, San Diego, La Jolla, California, USA

[4]Department of Cancer Immunology, Institute for Cancer Research, Oslo University Hospital, Oslo, Norway

[5]Department of Haematology, Oslo University Hospital, Oslo, Norway

Correspondence
John F. Imbery, Department of Immunology, Oslo University Hospital, Rikshospitalet, Sognsvannsveien 20, 0372 Oslo, Norway.
Email: j.f.imbery@medisin.uio.no

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Summary

Chronic lymphocytic leukaemia (CLL) is characterised by malignant mature-like B cells. Supportive to CLL cell survival is chronic B-cell receptor (BCR) signalling; however, emerging evidence demonstrates CLL cells proliferate in response to T-helper (Th) cells in a CD40L-dependent manner. We showed provision of Th stimulation via CD40L upregulated CD45 phosphatase activity and BCR signalling in non-malignant B cells. Consequently, we hypothesised Th cell upregulation of CLL cell CD45 activity may be an important regulator of CLL BCR signalling and proliferation. Using patient-derived CLL cells in a culture system with activated autologous Th cells, results revealed increases in both Th and CLL cell CD45 activity, which correlated with enhanced downstream antigen receptor signalling and proliferation. Concomitantly increased was the surface expression of Galectin-1, a CD45 ligand, and CD43, a CLL immunophenotypic marker. Galectin-1/CD43 double expression defined a proliferative CLL cell population with enhanced CD45 activity. Targeting either Galectin-1 or CD43 using silencing, pharmacology, or monoclonal antibody strategies dampened CD45 activity and CLL cell proliferation. These results highlight a mechanism where activated Th cells drive CLL cell BCR signalling and proliferation via Galectin-1 and CD43-mediated regulation of CD45 activity, identifying modulation of CD45 phosphatase activity as a potential therapeutic target in CLL.

KEYWORDS
B cells, CD43, CD45, chronic lymphocytic leukaemia, galectin-1

Abbreviations: ANOVA, analysis of variance; BCR, B-cell receptor; CLL, chronic lymphocytic leukaemia; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats associated with Cas9 endonuclease; exGal-1, extracellular galectin-1; mAb, monoclonal antibody; pCAP, phosphorylated coumaryl amino propionic acid; PBMCs, peripheral blood mononuclear cells; POLR2A, RNA polymerase II subunit A; siRNA, small interfering RNA; Th, T-helper.

INTRODUCTION

Chronic lymphocytic leukaemia (CLL) is a malignancy characterised by accumulation of mature-like clonal $CD5^+$ B cells. CLL cell survival and accumulation is highly dependent on B-cell receptor (BCR) signalling and support from cells in the surrounding microenvironment.[1–4] To this end, new therapies targeting downstream BCR signalling, like ibrutinib,[5] have improved patient outcome over chemotherapy-based treatments. Nevertheless, these therapies are not curative and emphasise a need for new therapeutic options in CLL. Additionally, it is unclear how microenvironmental help may impact the BCR signalling pathway. However, we recently discovered T-helper (Th) cell interaction with mature B cells via CD40-CD40L enhanced BCR signalling potential by regulating the B-cell CD45 protein tyrosine phosphatase (PTP) activity.[6] Furthermore, in CLL cells, responses to CD40 ligation are reduced in patients undergoing BCR therapy,[7–9] suggesting a reciprocal cross-talk between BCR signalling and the CD40 pathway.

CD45 is a PTP present on all cells of the haematopoietic lineage, except erythrocytes, and is essential for proper functioning of immune cells.[10] One key role of CD45 is to regulate T and B cell antigen receptor signalling by activating kinases of the Src-family through removal of an inhibitory phosphate group[11,12] and as mentioned briefly above, CLL cells are dependent on BCR signalling for activation, proliferation, and survival.[1] The dynamic regulation of CD45 phosphatase activity occurs, in part, through the influence of various binding partners. Of particular interest is galectin-1, a member of a larger class of carbohydrate-binding proteins known as lectins. Galectin-1 is well documented as being a CD45 ligand[13–15] with the ability to modulate its activity.[16,17] We recently connected surface galectin-1 expression to CD45 activity regulation in B cell activation and plasma cell differentiation.[6] Interestingly, galectin-1 is also a ligand for CD43,[13] which is a cell-surface glycoprotein found on CLL cells[18] and is established as a useful marker in the diagnostic immunophenotyping of CLL.[19] Furthermore, galectin-1 modulated dendritic cell signalling by co-clustering CD43/CD45 on the plasma membrane, which recruited pSyk and induced dendritic cell migration.[20] While one report has demonstrated elevated serum levels of galectin-1 in patients with CLL,[21] there is minimal exploration into galectin-1 and CD43's connection to CD45 function and activity for CLL cells.

Chronic lymphocytic leukaemia cells in the periphery have an anergic-like phenotype induced by chronic BCR signalling,[22] but with an impairment in apoptotic machinery prolonging survival.[23,24] Nevertheless, anatomical tissue sites can exist as proliferative centres for CLL cells.[25] Emerging evidence shows the microenvironment is critical for transitioning the CLL cell into a pro-proliferative state.[3] Especially important to this transition are contributions from Th cells. Early reports demonstrated infiltration by Th cells into proliferative sites[26] and their co-localisation with the proliferating CLL cells.[27–29] Furthermore, CLL cells present antigen and attract specific Th cells important for CLL cell activation and proliferation,[30,31] which was both contact and CD40L-dependent.[31] We also demonstrated xenotransplantation of CLL-derived peripheral blood mononuclear cells (PBMCs) alongside autologous CLL-specific Th cells into mice supported CLL cell proliferation in vivo.[31] As a whole, this highlights a growing body of work emphasising a critical role for Th cells in CLL disease progression.

Although Th cells are becoming an acknowledged contributor to CLL cell activation, the mechanism governing Th cell-mediated CLL cell proliferation remains inadequately explored. We previously identified Th cell signals upregulated CD45 phosphatase activity and rescued BCR signalling in anergic human B cells.[32] Furthermore, we showed Th cells governed high CD45 phosphatase activity in memory B cells, which enhanced BCR signalling and was essential for their effective differentiation into antibody-secreting cells.[6]

Given the overlapping importance of Th cells to healthy, anergic, and CLL cells, we tested the hypothesis that Th-cell regulation of CD45 activity, through galectin-1 and CD43, may be an important mechanistic factor mediating CLL BCR signalling and proliferation in CLL. We used patient-derived CLL PBMCs in a culture system where we activated autologous Th cells and found that galectin-1 and CD43 expression defined a proliferative CLL cell population with enhanced CD45 activity. clustered regularly interspaced short palindromic repeats associated with Cas9 endonuclease (CRISPR-Cas9) gene editing of galectin-1 gene (*LGALS1*) demonstrated galectin-1-mediated regulation of BCR signalling and proliferation. Furthermore, targeting galectin-1 or CD43 using silencing, pharmacology, or monoclonal antibody strategies dampened CD45 phosphatase activity and CLL cell proliferation. Therefore, we suggest modulation of CD45 activity may represent a possible therapeutic target in CLL.

MATERIALS AND METHODS

Study design

This was a hypothesis-driven research article aimed at connecting our laboratory's previous work, which (i) identified a role for Th cells in CLL activation and (ii) identified Th-cell regulation of CD45 activity in anergic and healthy B-cell subsets. To mirror the in vivo CLL disease microenvironment, cell cultures made use of patient-derived material. Patients diagnosed with CLL (Table S1) were recruited after informed consent at the outpatient clinic, Department of Haematology at Oslo University Hospitals, Norway. All ethical approvals were granted by the Regional Ethics Committee for Medical and Health Research Ethics (approval number 2016-947, 2016-1466). Cryopreserved or fresh blood samples from patients with CLL were used. Sample size and experimental end-points were determined based on our previous work in the fields of CLL and B-cell biology and to yield sufficient statistical power. All experimental

data from patients were included except when targeted small interfering RNA (siRNA) introduction did not reduce expression on the protein or genetic level, indicative of unsuccessful siRNA transfection.

Isolation and culture of primary cells

Cell preparations

Blood samples from patients with CLL were prepared using the density gradient medium Lymphoprep™ (Alere Technologies) for isolation of PBMCs. The resulting PBMC layer was removed, washed in phosphate-buffered saline (PBS), and red blood cells lysed via treatment with sterile filtered ammonium chloride potassium buffer lysing solution. PBMCs were subsequently placed directly into culture media (RPMI-1640 supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 1× non-essential amino acids, 50 nM of the antioxidant 1-thiglycerol, and 12 μg/ml of the antibiotic gentamicin) or cryopreserved for later use.

Cell culture

The $CD8^+$ T cells were depleted from patient-derived CLL PBMCs using $CD8^+$ Dynabeads™ (ThermoFisher Scientific). CD8-depleted PBMCs had the frequency of activated Th cells increased with the addition of Dynabeads Human T-Activator CD3/CD28 (Gibco, used according to manufacturer's specifications) and 20 u/ml interleukin-2 (Sigma-Aldrich), referred to as 'stimulated' hereafter. Conversely, control CD8-depleted PBMCs received no stimulation, referred to as 'unstimulated' in the remaining text. Following a 72–96 h incubation, 1×10^6 PBMCs were plated into 1-ml wells. Pharmacological inhibitors of CD45 (PTP CD45 inhibitor; Calbiochem) and galectin-1 (Calixarene OTX008; Axon Medchem), monoclonal CD43 antibody (DF-T1, ProSci Incorporated; 1G10, L60, BD Biosciences; MEM-59; ThermoFisher Scientific), or vehicle or isotype control were added at the indicated concentrations and PBMCs incubated for an additional 24–72 h prior to flow cytometric analysis.

Positive selection of CLL cells

When necessitated by the experimental setup, CLL cells could be directly isolated from PBMCs by positive selection with $CD19^+$ microbeads (Miltenyi Biotec). Positive selection proceeded according to manufacturer's recommendations. Briefly, PBMCs were passed through a magnetic-activated cell sorting (MACS) separator, with $CD19^+$ CLL cells retained on the magnet and unlabelled cells flowed through. The $CD19^+$ CLL cells were released from the magnet, creating a pure CLL cell population for culturing.

Flow cytometry antibodies

The following antibodies (clone, company) were used for flow cytometric analysis at concentrations recommended by the manufacturer: CD4-Alexa Fluor 488 (OKT4; eBioscience), Phospho Syk (Tyr348)-PE (moch1ct; eBioscience), Galectin1-Alexa Fluor 647 (GAL1/1831; Novus Biologicals), Phospho Lck (Tyr 394)-PE (A18002D; BioLegend), Phospho Lck (Tyr505)-PE (A17013A; BioLegend), Phospho BTK (Tyr223)-PE (A16128B; BioLegend), Phospho ERK1/2 (Thr202, Tyr204)-PerCP-eFluor™ 710 (MILAN8R; Invitrogen), CD5-PerCP/Cyanine5.5 (L17F12; BioLegend), CD43-PE/Cy7 (CD4310G7; BioLegend), Ki67-Brilliant Violet 711 (Ki-67; BioLegend), CD19-PE/Cy7 (HIB19; BioLegend), CD43-PE (CD4310G7; BioLegend).

Flow cytometry-based CD45 phosphatase activity assay

Cellular CD45 activity was measured by taking advantage of a novel method developed by our collaborators[33] and used previously by our laboratory.[6,32] Briefly, a phosphotyrosine mimic, phosphorylated coumaryl amino propionic acid (pCAP), was incorporated into a cell permeable peptide. The peptide contains a sequence, for simplicity called SP1, derived from a known CD45 substrate (Lck) and pCAP-SP1 was confirmed to be CD45 specific.[33] Once in the cell, active CD45 dephosphorylates pCAP-SP1, releasing a brightly fluorescent component (excitation 405 nm) easily captured on a flow cytometer using a Pacific Blue emission filter. Thus, the amount of fluorescence from dephosphorylated pCAP-SP1 directly correlated to CD45 activity. The dephosphorylated pCAP-SP1 derivative is fixable, unaffected by permeabilisation, and therefore could be combined with staining for flow cytometry surface and intracellular targets described below.

Flow cytometry

Prior to antibody staining, dead cells were labelled with Live/Dead Fixable Near-IR Dead Cell Stain Kit (Excitation 750, Emission 775; ThermoFisher Scientific) for 15 min at room temperature and excess dye washed away. Following the wash, antibodies for staining surface targets were diluted in fluorescence-activated cell sorting (FACS) buffer and applied to cells for 30 min on ice. Unattached surface antibodies were removed with a wash. A Transcription Factor Buffer Set (BD Pharmigen) optimised for staining of intracellular targets for flow cytometric analysis was used to fix/permeabilise cells for 45 min at 4°C. Afterward, cells were washed twice in a perm/wash buffer from the same set. Antibodies for intracellular targets were diluted in perm/wash buffer, applied to cells for 45 min at 4°C, and washed twice prior to analysis on an Attune NxT Acoustic Focusing Cytometer (ThermoFisher Scientific). All analyses were done using FlowJo Cytometric Software (TreeStar, Inc.).

Gene silencing using siRNA

Chronic lymphocytic leukaemia cells were co-cultured with activated Th cells for 72–96 h, positively selected from culture using CD19 microbeads, and re-suspended in PBS at 2.5×10^5 cells/10 µl. In all, 1.5 µM of CD43 siRNA (Silencer Select; ThermoFisher Scientific) or 1.5 µM scrambled siRNA (Silencer Select Negative Control; ThermoFisher Scientific) was added to the cell suspension and introduced via electroporation (1400 V, 10 ms, 3 pulses) with the Neon™ Transfection System (Invitrogen). Electroporated cells were quickly placed into pre-warmed RPMI media and stained for flow cytometric analysis 72 h post-transfection.

Real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated from purified CLL cell cultures using Trizol reagent (Life Technologies). Reverse transcription and quantitative PCR was performed using the Taqman®RNA-to-C_T™ 1-Step Kit (Life Technologies) on the StepOnePlus™ System (Life Technologies): reverse transcription at 48°C for 15 min, then 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 1 min at 60°C in 15 µl reaction volumes with 10 ng RNA as template/well. All PCR reactions were done in triplicates. Controls containing no template or no reverse transcriptase enzyme were included in each run. Taqman primers and probes for human *CD43* and RNA polymerase II subunit A (*POLR2A*) were chosen as they do not amplify genomic DNA. Gene expression was quantified by the comparative threshold cycle method and normalised to expression of the human housekeeping gene *POLR2A*.

Confocal microscopy

Following $CD19^+$ isolation, CLL cells were briefly incubated with 10 µM pCAP-SP1. Phosphatase activity quenching and cellular fixation was achieved using 10 mM sodium orthovanadate/2% paraformaldehyde for 10 min. The CLL cells were washed and adhered to Superfrost Plus microscopy slides via centrifugation (Shandon Cytospin 4; Thermo Scientific) at 800 rpm (approximately 72 *g*) for 4 min. Slides dried overnight at 4°C. A perm/wash buffer optimised for permeabilisation and primary/secondary antibody dilution was made from Transcription Factor Buffer Set (BD Pharmigen) according to manufacturer's specifications. The CLL cells were permeabilised for 15 min at room temperature with gentle shaking and subsequently incubated with primary antibodies against CD45 (mouse anti-human, 1:100), galectin-1 (goat anti-human, 1:25), and CD43 (rabbit anti-human, 1:50) at 37°C for 1 h. Excess primary antibody was washed off and secondary antibody applied at room temperature for 45 min (donkey anti-mouse Alexa Fluor 488, donkey anti-goat Alexa Fluor 555, donkey anti-rabbit Alexa Fluor 647; 1:500). The CLL cells were washed to remove excess secondary antibody, mounted with ProLong Gold Antifade, sealed with nail polish, and images acquired sequentially on an Olympus FV1000 confocal microscope with a 60×NA1.35 UPlanSAPO oil objective. Image processing took place in ImageJ (W. S. Rasband, National Institutes of Health, Bethesda, MD, USA) where fluorophore intensity was measured using the RGB profiler tool and corrected for background fluorescence.

Statistical analysis

All statistical analyses were computed using GraphPad Prism (GraphPad Software, Inc.). The specific statistical tests are indicated in the figure legends. To ensure appropriateness of the selected statistical test, the normality of the data distributions were first analysed with the D'Agostino-Pearson omnibus normality test or the Shapiro–Wilk's normality test. Resultant values are represented as individual data points or mean (±SD). A $p < 0.05$ was taken as statistically significant: $^*p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$. Additionally, when applicable for two-way analysis of variance comparing between Th and CLL cells: black asterisks denote statistical significance between Th and CLL cells, red asterisks denote statistical significance of CLL cells versus control, and blue asterisks denote statistical significance of Th cells versus control.

RESULTS

CD45 activity, antigen receptor signalling, and proliferation is upregulated upon Th–CLL cell collaboration

Given the known involvement of Th cells in supporting CLL survival and proliferation, in addition to Th-cell upregulation of CD45 activity in anergic[32] and normal mature B cells,[6] initial experiments assessed whether Th cells could similarly regulate CD45 activity in CLL cells. To recapitulate the in vivo activation of CLL Th cells, we increased the frequency of activated autologous Th cells ('stimulated', see Materials and methods section). Patient characteristics can be found in Table S1 and the flow cytometry gating strategy in Figure S1. Both stimulated CLL and Th cells upregulated CD45 activity in comparison to their unstimulated counterparts (Figure 1A,B upper panels). Additionally, no significant upregulation of autofluorescence was observed from either culture condition in the absence of the CD45 activity probe (Figure 1A,B upper panels). Analogously, analysing the frequency of CD45 $activity^+$ cells revealed significant increase in both CLL and Th cells over background fluorescence (Figure 1A,B lower panels). To link CD45 activity with BCR signalling in CLL cells, similarly to normal B cells, we included phosphoflow analysis of pSyk. CLL cell stimulation resulted in a significant increase of CD45 activity and pSyk co-expression in comparison to unstimulated CLL cells (Figure 1C, upper panels). Furthermore, the $pSyk^{hi}$

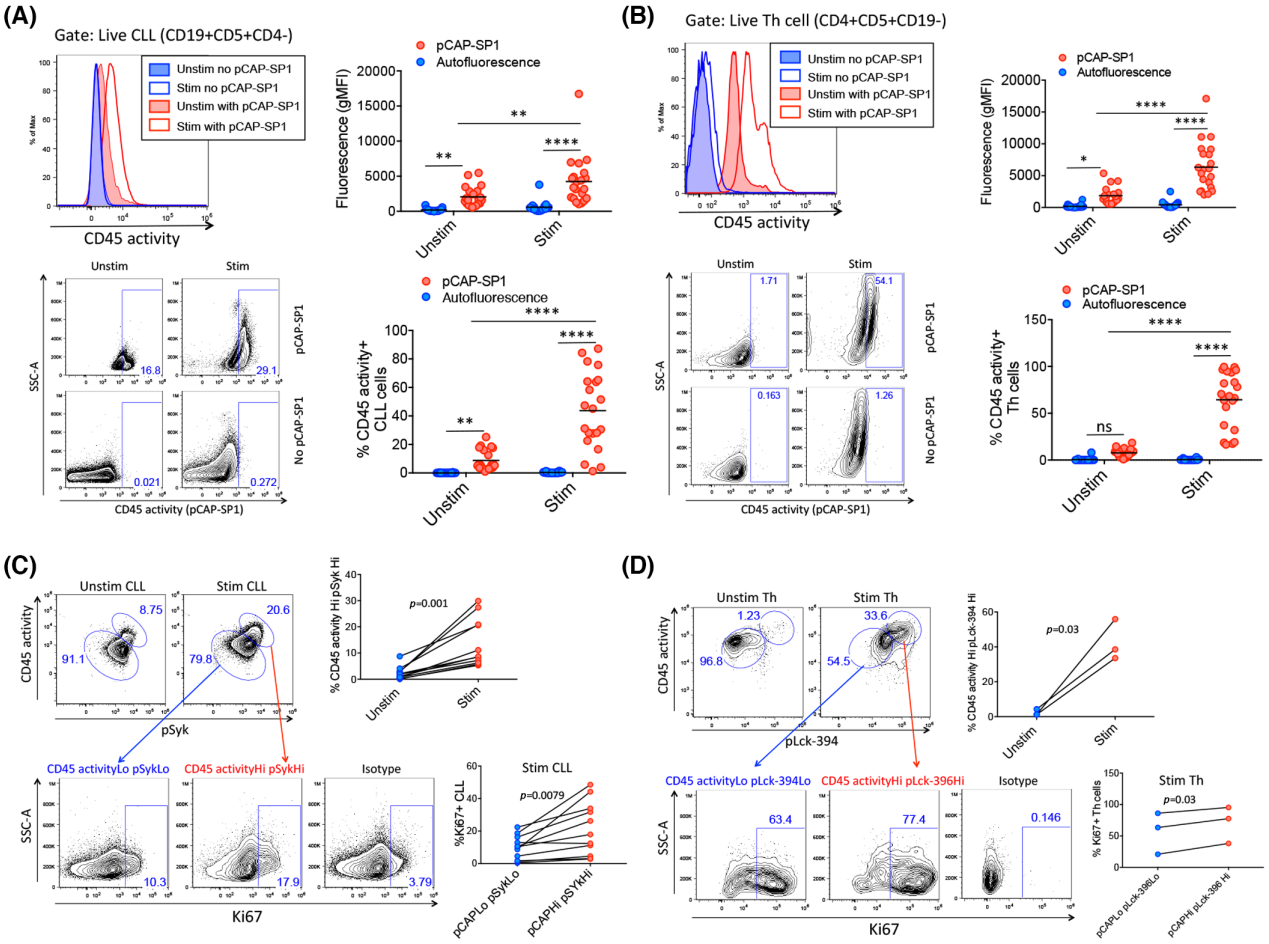


FIGURE 1 CD45 activity, antigen receptor signalling, and proliferation is upregulated upon Th–CLL cell collaboration. (A, B) Stimulated CLL and Th cells displayed higher CD45 activity in comparison to their unstimulated counterparts. Minimal autofluorescence was also observed from both culture conditions when the pCAP-SP1 (CD45 activity) probe was omitted. Included are representative flow cytometry histograms and graphs with means ± SD of pCAP-SP1 gMFI (upper panels). Additionally, there was a higher percentage of CD45 activity^{+} stimulated CLL and Th cells in comparison to their unstimulated counterparts (lower panels). There was no change in the percentage of CD45 activity^{+} CLL and Th cells when the pCAP-SP1 probe was omitted from either culture condition indicating the specificity of this assay. Included are representative flow cytometry scatterplots and graphs means ± SD of %CD45 activity^{+} (lower panels). (C, D) Representative flow cytometry dot plots associated increasing CD45 activity with heightening activity of antigen-signalling kinases upon stimulation. Furthermore, gating on pSyk^{hi}/CD45 activity^{hi} (CLL cells, C) or pLck^{hi}/CD45 activity^{hi} (Th cells, D) revealed increased proliferation (Ki67^{+}) compared to CD45 activity^{lo} populations. Graphs show values for %pSyk^{hi}/CD45 activity^{hi} CLL cells (C, upper panel) and %pLck^{hi}/CD45 activity^{hi} Th cells (D, upper panel) in unstimulated versus stimulated cultures. Each pairing represents one patient. Additionally, graphs show values for %Ki67^{+} within pSyk^{hi}/CD45 activity^{hi} versus pSyk^{lo}/CD45 activity^{lo} CLL cells (D, lower panel), and %Ki67^{+} within pLck^{hi}/CD45 activity^{hi} versus pLck^{lo}/CD45 activity^{lo} Th cells (D, lower panel). Each pairing represents one patient. Statistical significance was defined by two-way ANOVA (A, B), Wilcoxon matched-pairs signed rank test (C, upper), or Student's paired *t*-test (C, lower, D). Data is representative of 21 CLL patient samples (A, B), 11 patient samples (C), and three patient samples (D). *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; ****$p \le 0.0001$. ANOVA, analysis of variance; CLL, chronic lymphocytic leukaemia; gMFI, geometric mean of fluorescence intensity; Th, T-helper. [Colour figure can be viewed at wileyonlinelibrary.com]

and CD45 activity^{+} population harboured the highest levels of the proliferation marker Ki67 (Figure 1C, lower panels). Similarly, activation of Th cells also induced CD45 activity together with activation of Lck kinase (pLck-394) (Figure 1D, upper panels), and downregulation of the inactive Lck form (pLck-505) (Figure S2). Th cells with increased CD45 activity and active Lck kinase correspondingly exhibited a modestly higher proliferative level (Figure 1D, lower panels). These data link CD45 activity with downstream antigen receptor signalling and proliferation in CLL and autologous Th cells, in agreement with our previous findings on non-malignant B cells.[6]

Interrogation of the CD45 signalling pathway using pharmacological inhibition of CD45 phosphatase produced significant cell death in stimulated CLL and Th cells (Figure 2A,B, upper panels). However, the remaining gated live CLL cell population showed significantly reduced levels of proliferation (Figure 2A, lower panels), while the remaining Th cells did not display a pronounced reduction in proliferative capacity (Figure 2B, lower panels). This highlights the importance of the CD45 pathway for CLL cell proliferation and for cell survival in both Th and CLL cells. Ultimately, results presented here link Th cell help to increasing CLL cell CD45 activity, BCR receptor signalling, and proliferation.

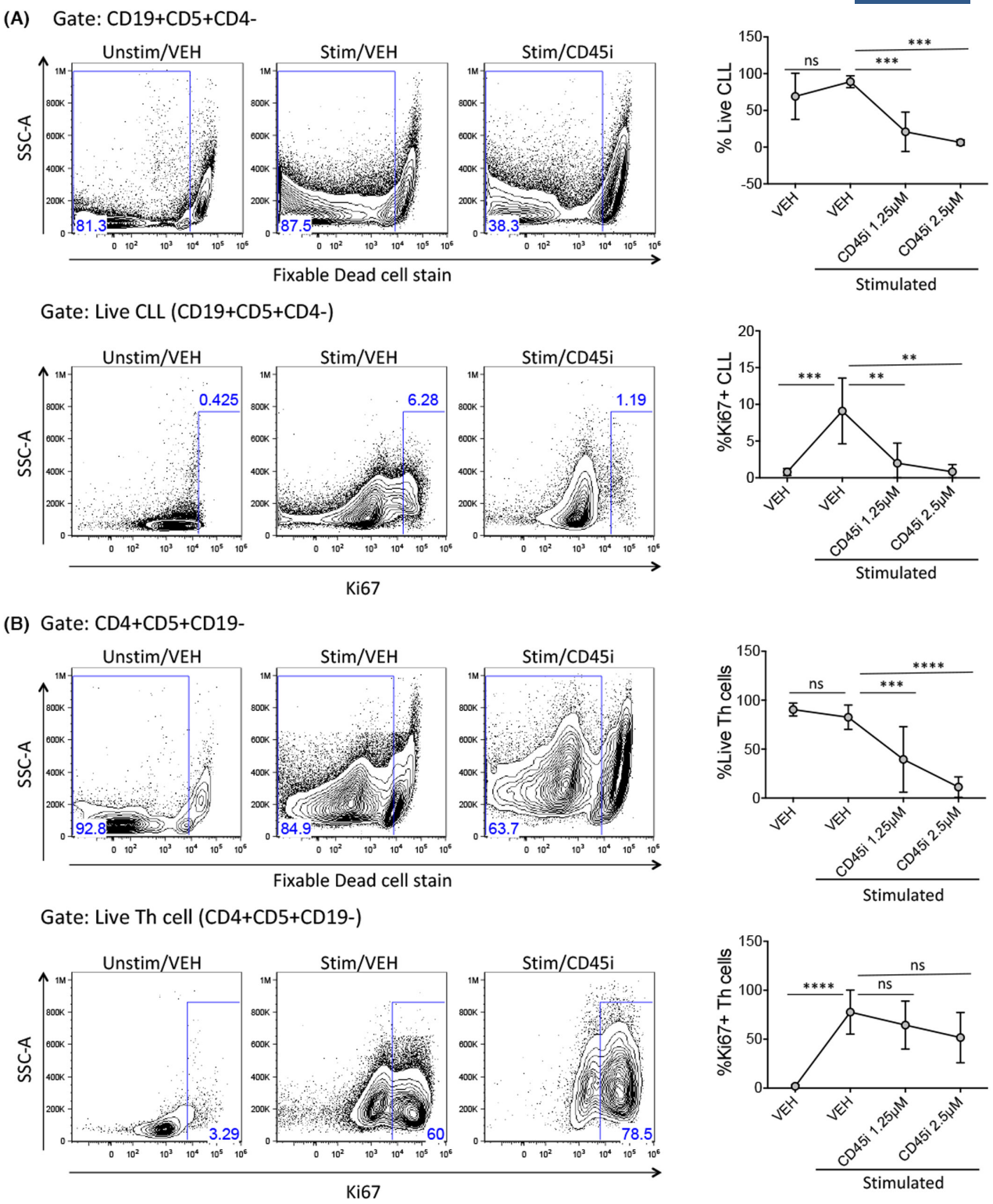


FIGURE 2 Inhibition of CD45 activity induced cell death and reduced CLL proliferation. (A, B) Use of the PTP CD45 inhibitor induced cell death (viability stain, upper panels) in both CLL and Th cell populations but reduced proliferation (Ki67$^+$, lower panels) only in the remaining live CLL cells. Graphs show means ± SD %live or %Ki67$^+$ in CLL (A) or Th cells (B). Statistical significance was defined by Kruskal–Wallis test with Dunn's correction (A, B lower panel) or one-way ANOVA with Sidaks's multiple comparison correction (B, upper panel). Data is representative of 10 CLL patient samples. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$. ANOVA, analysis of variance; CLL, chronic lymphocytic leukaemia; PTP, protein tyrosine phosphatase; Th, T-helper. [Colour figure can be viewed at wileyonlinelibrary.com]

Increased CD45 activity in CLL and Th cells is associated with surface galectin-1 expression

As CD45 is ubiquitously expressed on immune cells, and pharmacological inhibitors may have on-target toxicities, we investigated alternative approaches for regulation of CLL cell CD45 activity. We recently connected Th-cell signals to galectin-1 regulation of CD45 activity as a mechanism for B-cell activation and differentiation into plasma cells.[6] Consequently, we hypothesised that the Th-cell regulation of CD45 activity in CLL cells could also, in part, be galectin-1 mediated.

To test this, we used flow cytometric analysis of surface stained galectin-1 and identified the membrane localisation of galectin-1 and CD45 using confocal microscopy. Th-cell mediated stimulation of CLL cells increased galectin-1 surface expression (Figure 3A) and the galectin-1$^+$ population was associated with the highest CD45 activity (Figure 3B). As the corresponding isotype control for the galectin-1 antibody was not commercially available, we tested the antibody specificity in a previously characterised Raji B-cell line where the gene encoding galectin-1 (*LGALS1*) was edited with CRISPR-Cas9 technology[6] (Figure S3A). Confocal microscopy revealed areas of co-localisation between galectin-1 and CD45 in primary unstimulated CLL cells (Figure 3C), suggesting galectin-1 is also a ligand for CD45 in CLL cells. Furthermore, confocal images showed heterogeneous galectin-1 expression in CLL cells, confirming results first characterised by flow cytometry. Similar flow cytometric results were obtained from Th cells as they displayed a robust increase in surface galectin-1 expression upon activation (Figure 3D). The galectin-1 expressing Th-cell population also harboured the highest CD45 activity (Figure 3E). Further experiments addressed the source of galectin-1 in our culture system by assessing the intracellular expression of galectin-1 in CLL and Th cells. Galectin-1 was more consistently upregulated in Th cells (Figure S3C), while upregulation of intracellular galectin-1 occurred only in a subset of CLL cells (Figure S3B). Importantly, this suggests Th cells may be the primary source of galectin-1 surface binding for CLL cells in our co-culture system.

In summary, an activating ligand for CD45, galectin-1, had increased surface expression on both CLL and Th cells upon stimulation. Furthermore, galectin-1 co-localised with CD45 in CLL cells and correlated with increasing CD45 activity, suggesting CD45 phosphatase activity could be regulated by galectin-1 surface binding.

Proliferating CLL and Th cells have increased CD45 activity, galectin-1, and CD43 expression

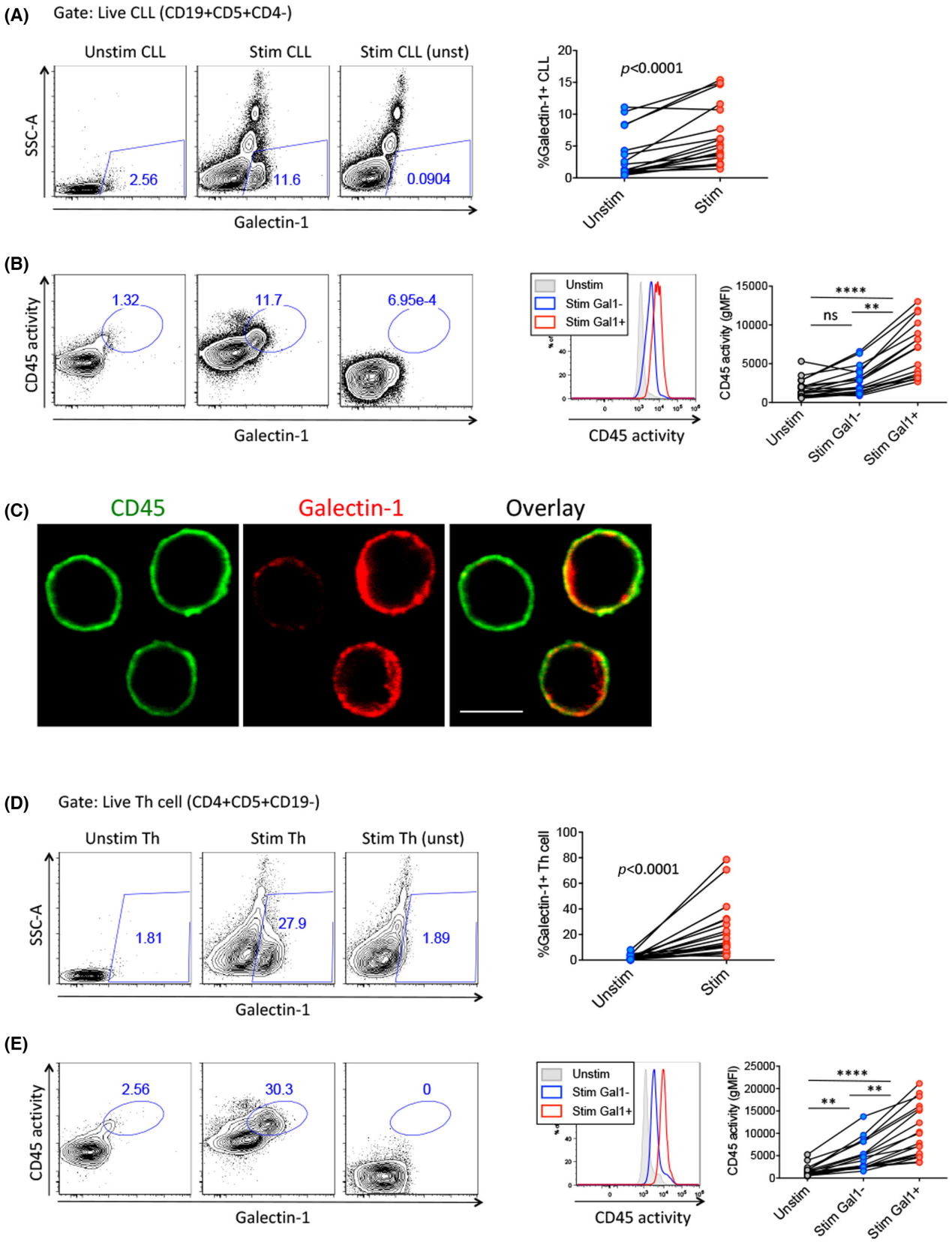
To clarify the significance of increasing surface galectin-1 expression and CD45 activity in stimulated CLL-Th cell co-cultures, we investigated these markers and their association with the proliferative output of CLL and Th cells. Included as an additional candidate marker was CD43. While CD43 is linked to a variety of T-cell functions including calcium signalling, adhesion, activation, and survival,[34–38] its role remains inadequately addressed in B cells, and by extension, CLL cells. However, given its diverse role in T cells, and expression on CLL cells,[18] we hypothesised it may also regulate the downstream output (e.g., proliferation) of CLL cells.

We found that stimulated proliferating Ki67$^+$ CLL cells were associated with the highest CD45 activity, and the highest CD43 and galectin-1 surface expression (Figure 4A,B), suggesting a role for these proteins in CLL proliferation. Results obtained in Th cells were largely analogous to CLL cells, in that proliferating Th cells displayed high CD45 activity, and high galectin-1 and CD43 surface expression (Figure 4C,D). In contrast to CLL cells, CD45 activity, galectin-1, and CD43 levels in Th cells were generally associated with activation rather than proliferating cells, highlighting these factors as more specific markers for proliferating CLL cells (Figure 4E). To ascertain a snapshot of the in vivo CD45 activity of proliferative CLL cells, we analysed the CXCR4^{dim}CD5^{bright} subpopulation shown previously to harbour the largest percentage of proliferative CLL cells in the peripheral blood.[39,40] This proliferative subpopulation had significantly higher CD45 activity in comparison to the resting (CXCR4^{bright}CD5^{dim}), largely non-proliferative subpopulation (Figure 4F). Collectively, we identified CD45 activity, galectin-1 surface binding, and CD43 surface expression associated with proliferating CLL cells, suggesting their involvement in regulation of CD45 activity and downstream cell function.

Blocking galectin-1 binding with OTX008 inhibited both CLL and Th cell proliferation

Having demonstrated that proliferating CLL and Th cells are associated with high galectin-1 surface expression, we investigated pharmacological targeting of galectin-1 as a method to reduce proliferation. To do so, we

FIGURE 3 Increased CD45 activity in CLL and Th cells correlated with surface galectin-1 expression. Representative flow cytometry dot plots show increased galectin-1 surface staining in stimulated CLL and Th cells (A, D, unst = unstained control), which correlated with increasing CD45 activity (B, E). Graphs show paired individual values from patients for %galectin-1$^+$ CLL cells (A) or Th cells (D) in unstimulated versus stimulated samples. Furthermore, graphs show paired individual values from patients for CD45 activity (gMFI) in unstimulated, stimulated galectin-1$^-$, and stimulated galectin-1$^+$ CLL (B) and Th cell (E) populations. Statistical significance was calculated by Student's paired *t*-test (A), Wilcoxon matched-pairs signed rank test (D), or Friedman test with Dunn's correction (B, E). Data are representative of 18 CLL patient samples. (C) CLL cells isolated from patient PBMCs display CD45 (green) and galectin-1 (red) staining. Co-localisation (yellow) is evidenced in the overlay, scale bar is 5 μm. $^{**}p \leq 0.01$; $^{****}p \leq 0.0001$. CLL, chronic lymphocytic leukaemia; gMFI, geometric mean of fluorescence intensity; PBMCs, peripheral blood mononuclear cells; Th, T-helper. [Colour figure can be viewed at wileyonlinelibrary.com]



made use of OTX008,[41] an allosteric galectin-1 inhibitor shown to effectively down-modulate tumour growth.[42–44] Delivery of OTX008 to the co-culture system resulted in a concentration-dependent decrease in proliferation of both stimulated CLL and Th cells (Figure 5A,B). We also assessed the effectiveness of galectin-1 inhibition based on the immunoglobulin heavy chain variable region (IGHV) mutational status (unmutated [U] and mutated

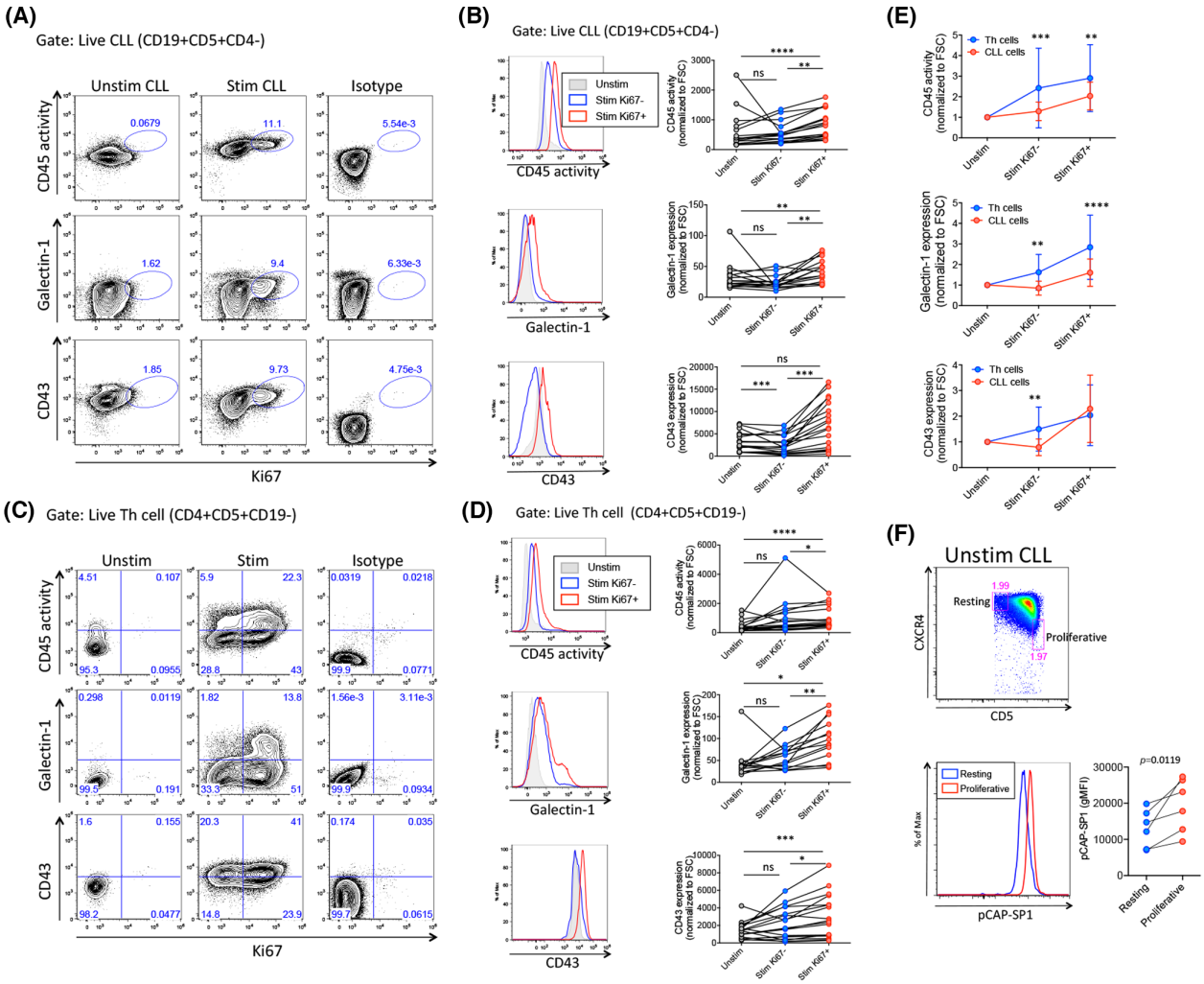


FIGURE 4 Proliferating CLL and Th cells have increased CD45 activity, galectin-1, and CD43 expression. Representative flow cytometry dot plots showing proliferation ($Ki67^{+}$) associated with increasing CD45 activity (upper panels), galectin-1 surface staining (middle panels), and CD43 surface expression (lower panels) in CLL cells (A) or Th cells (C). Graphs show paired individual values (normalised to size) from patients for gMFI CD45 activity (upper panels), galectin-1 surface staining (middle panels), and CD43 surface expression (lower panels) in gated live CLL cells (B) and Th cells (D). (E) Galectin-1 was more robustly increased on proliferating Th cells versus proliferating CLL cells. (F) the proliferative ($CXCR4^{dim}CD5^{bright}$) CLL cell subpopulation in peripheral blood had higher CD45 activity in comparison to the non-proliferative, resting subpopulation ($CXCR4^{bright}CD5^{dim}$). Included is a representative flow cytometry dot plot and histogram. Graphs show means ± SD. Statistical significance was defined by Friedman test with Dunn's correction (B upper and middle panels, D), repeated-measures one-way ANOVA with Sidak's correction (B lower panel), two-way ANOVA (E), or Student's paired *t*-test (F). Data are representative of 18 (A–E) and six (F) CLL patient samples. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$. ANOVA, analysis of variance; CLL, chronic lymphocytic leukaemia; gMFI, geometric mean of fluorescence intensity; Th, T-helper. [Colour figure can be viewed at wileyonlinelibrary.com]

[M]) of CLL cells. Results found similar effectiveness of OTX008 in decreasing the proliferative capacity of CLL and Th cells across both patient subgroups (Figure 5A,B). Moreover, treatment with OTX008 resulted in a decrease of the galectin-1 surface expressing populations in both CLL and Th cells, with a concomitant decrease in CD45 activity (Figure 5C,D). We additionally observed an OTX008 concentration-dependent decrease in both the percentage of Th cells and an associated increase in frequencies of dead Th and CLL cells from stimulated co-cultures (Figure S4B,C,E,F,H). Non-stimulated CLL and Th cells were largely unaffected by treatment with similar concentrations of OTX008 (Figure S4A,C,D,F,G), likely reflecting low galectin-1 expression levels.

To ensure OTX008 effects were specific to galectin-1 inhibition, we tested OTX008 effects in Raji wildtype and LGALS1 knockout (KO) cell lines. Wildtype Raji B cells where highly sensitive to OTX008 treatment, displaying a reduction both in viability (Figure S5A,B) and proliferation (Figure S5C,D). Interestingly, the Raji LGALS1 KO cell line displayed a modest reduction in viability (Figure S5A,B) in response to OTX008 but the proliferative signature remained unchanged (Figure S5C,D). Thus, OTX008's reduction in proliferation is specific to galectin-1 inhibition but changes to cell viability may be attributable to an off-target effect of the inhibitor at high concentrations. Additional results with the Raji LGALS1 KO cell line also revealed decreased levels of BCR signalling molecules pSyk, pErk, and pBTK in

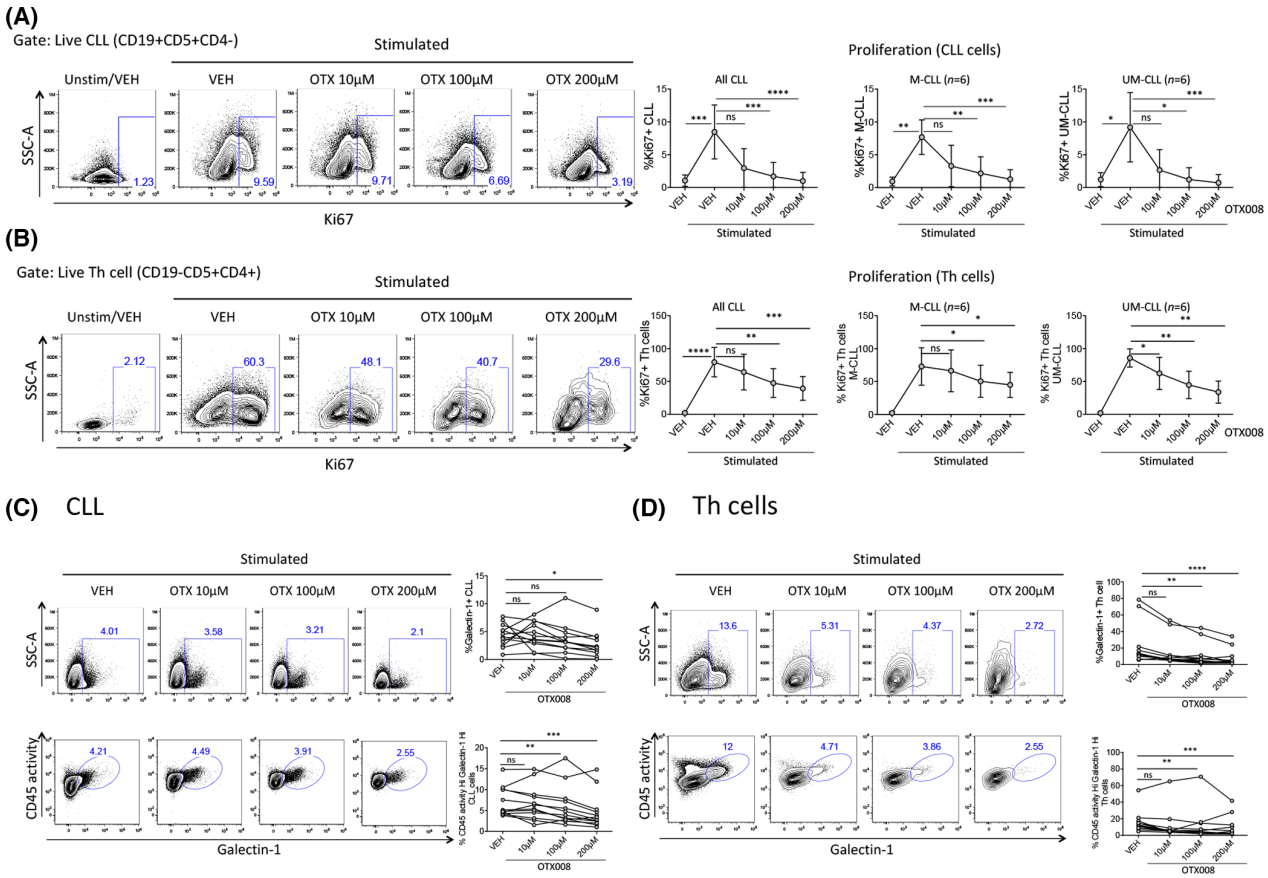


FIGURE 5 Blocking galectin-1 binding with OTX008 inhibited both CLL and Th cell proliferation. (A, B) Representative flow cytometry dot plots demonstrate treatment with OTX008 reduced proliferation in both stimulated CLL cells (A) and Th cells (B). Patients were additionally subdivided based on the mutational status of the immunoglobulin heavy chain (M = mutated, UM = unmutated) revealing similar results. Graphs show means ± SD. (C, D) Representative flow cytometry dot plots reveal increasing concentrations of OTX008 reduced galectin-1 surface staining (upper panels), with a subsequent reduction in the galectin1$^+$/CD45activity$^+$ (lower panels) populations in both stimulated CLL cells (C) and Th cells (D). Graphs show means ± SD. Statistical significance was calculated by Friedman test with Dunn's correction (a left and right panels, C, D) and repeated-measures one-way ANOVA with Dunn's correction (a middle panel, B). Data are representative of 12 CLL patient samples. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$. ANOVA, analysis of variance; CLL, chronic lymphocytic leukaemia; Th, T-helper. [Colour figure can be viewed at wileyonlinelibrary.com]

comparison to wildtype (Figure S6A,B), demonstrating a role for galectin-1 in BCR signalling alongside its previously characterised role in CD45 activity[6]

Overall, our results highlight galectin-1 as a novel target to reduce both CLL and Th cell proliferation, while sparing resting cells.

Direct effect of OTX008 on CLL cell proliferation

As CLL cells are activated and proliferate in response to specific Th cells,[31] the observed reduction in CLL cell proliferation after OTX008 treatment may be an indirect effect resulting from death of activated Th cells. Thus to elucidate the possible direct effect of OTX008, CLL cells were isolated following Th stimulation and placed in culture with subsequent addition of OTX008 or vehicle (Figure 6A). We observed a concentration-dependent decrease in CLL cell proliferation with OTX008 treatment (Figure 6A upper panels, Figure 6B) and a concomitant reduction in CD45 activity$^+$/Ki67$^+$ population (Figure 6A lower panels), confirming a direct effect of OTX008 on CLL cells. CLL cell viability also remained stable across the varying OTX008 concentrations (Figure 6C,D).

Galectin-1 and CD43 expression defines a proliferating CLL cell population with elevated CD45 activity

Initial results linked increasing surface galectin-1, CD43 expression, and high CD45 activity to proliferating CLL cells (Figure 4). While each candidate marker was assessed individually, CD43 also serves as a galectin-1 binding partner[13] and localises with CD45 for galectin-1-mediated signalling in dendritic cells.[20] Given the relationship of CD43 to galectin-1 and CD45, and the demonstrated link between galectin-1 expression, high CLL CD45 activity (Figure 3), and regulation of CLL proliferative output (Figure 6A,B), additional experiments examined CD43/galectin-1 co-expressing populations. We hypothesised the role of CD43

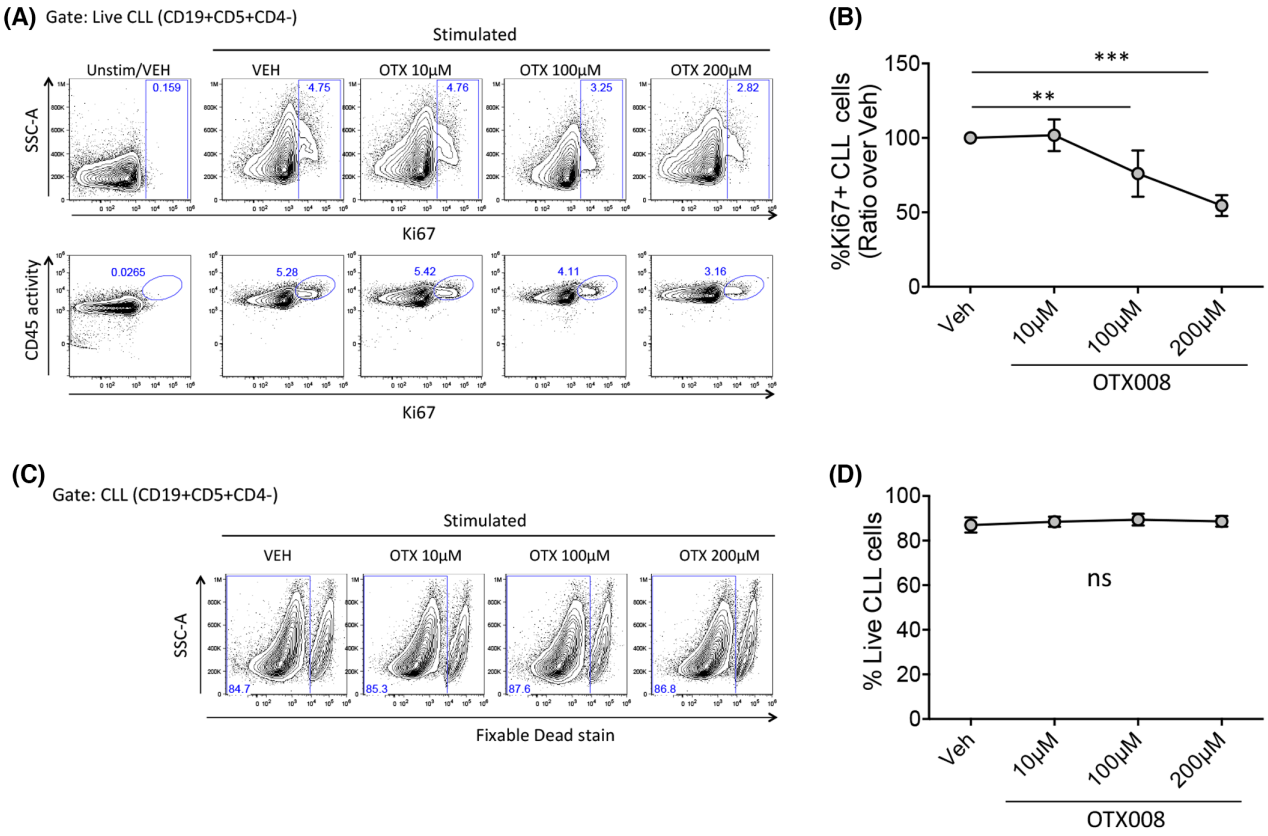


FIGURE 6 Direct effect of OTX008 on CLL cell proliferation. (A) Representative flow cytometry dot plots reveal a reduction in isolated CLL cell proliferation with OTX008 treatment (upper panels) and a concomitant reduction in the Ki67$^+$/CD45 activity$^+$ population (lower panels). (B) Graph shows means ± SD of %Ki67$^+$ CLL cells (ratio over Veh). (C) OTX008 did not induce cell death in isolated CLL cells. (D) Graph shows means ± SD of %live CLL cells. Statistical significance was defined by repeated-measures one-way ANOVA with Dunn's correction. Data are representative of eight CLL patient samples. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$. ANOVA, analysis of variance; CLL, chronic lymphocytic leukaemia. [Colour figure can be viewed at wileyonlinelibrary.com]

in modulating CLL proliferative output may be via CD43 regulation of CD45 activity.

Flow cytometric analysis of Th-stimulated CLL cells showed an increase in the percentage of CD43^{hi}/Gal1$^+$ and CD43^{hi}/CD45 activity$^+$ populations in comparison to unstimulated CLL cells (Figure 7A,B). In addition, stimulated CD43^{hi}/Gal-1$^+$ CLL cells displayed a significantly larger proliferative signature and higher CD45 activity than their CD43^{lo}/Gal-1$^-$ expressing counterpart did (Figure 7C,D). To confirm possible regulation of CD45 activity by CD43, we introduced siRNA directed towards CD43 in primary CLL cells and confirmed a significant downregulation of CD43 surface expression with a 22% CD43 silencing efficiency and 28% downregulation at the mRNA level (Figure S7A–C). Importantly, we observed a concomitant downregulation of CD45 activity in stimulated CLL cells treated with CD43 siRNA compared to control siRNA (Figure 7E). Finally, results obtained from flow cytometry showing co-expression of CD43/galectin-1 were extended using confocal microscopy on isolated CLL cells. Confocal images showed areas of co-localisation between CD43, CD45, galectin-1, and CD45 activity (pCAP-SP1 probe) (Figure 7F). Analysis of the Pearson correlation coefficient between CD43/CD45 of 11 CLL cells revealed a value of 0.770, indicating high degree of co-localisation. Collectively, our data provided evidence for CD45 activity regulation by CD43 in CLL cells.

Treatment with an anti-CD43 antibody downregulated surface CD43 expression and specifically dampened CLL cell proliferation in vitro

To further interrogate the functional consequences of CD43 inhibition, we administered an anti-CD43 monoclonal antibody (mAb; clone DF-T1) to stimulated CLL and Th cell collaborative cultures. We observed a concentration-dependent inhibition of proliferation in CLL cells, but not in Th cells (Figure 8A,B). We furthermore observed significant downregulation of CD43 surface expression in both CLL and Th cell subsets (Figure 8C,D), suggestive of a dependence on CD43 surface expression in CLL cells for proliferation, but not in Th cells. Downmodulation of CD43 also corresponded with decreased pSyk activation in CLL cells (Figure 8C,D), possibly as a reflection of CD43's regulation of CD45 activity. Surveying percentages of CLL and Th cell subsets within live cells showed a slight increase in the Th cell subset with increasing concentrations of anti-CD43 mAb, while CLL

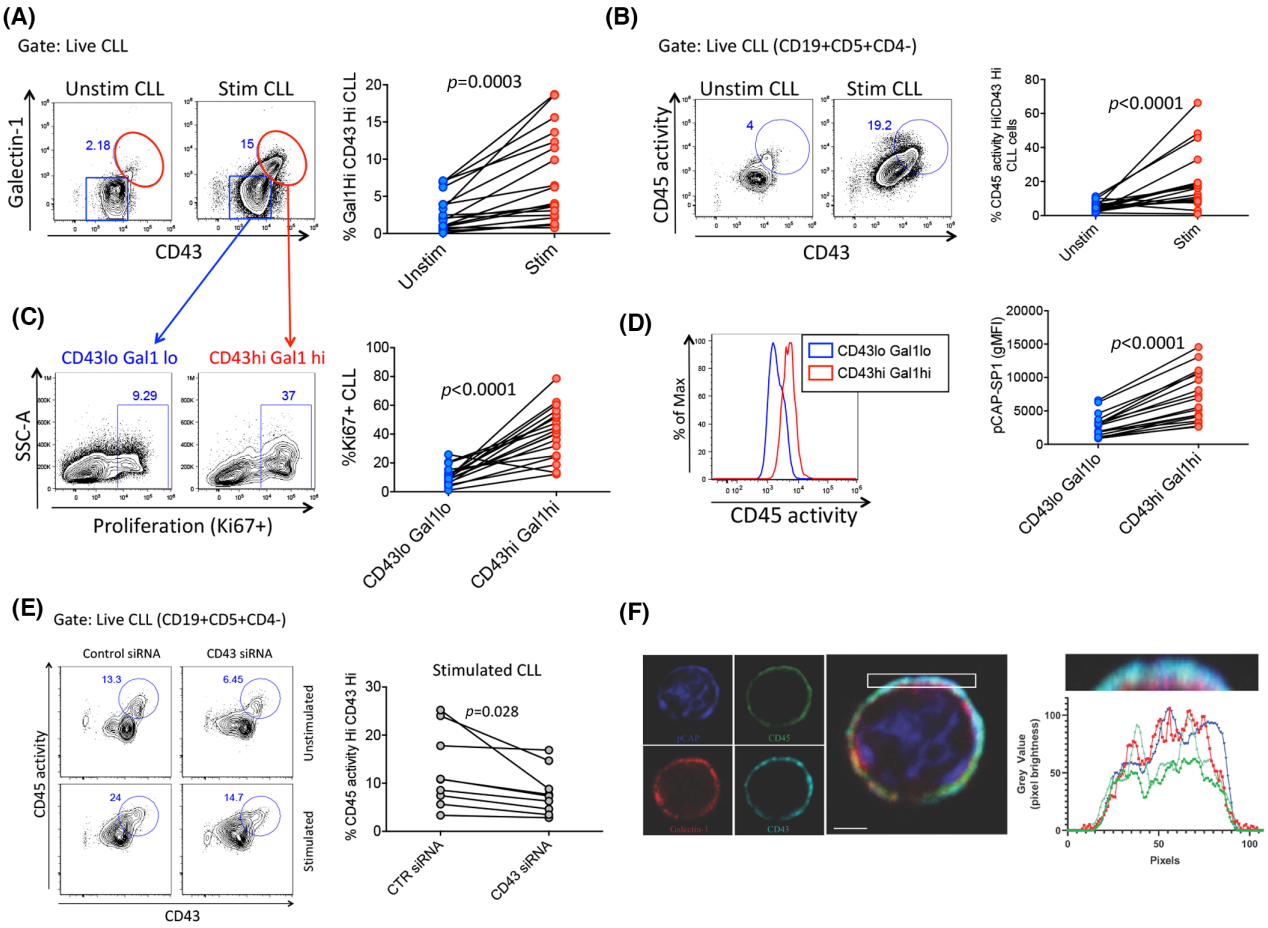


FIGURE 7 Galectin-1 and CD43 expression defined a highly proliferating CLL cell population with elevated CD45 activity. (A) Representative flow cytometry dot plots illustrate an increase in the CD43^{hi}/galectin-1^{+} population in stimulated CLL cells. Graph shows paired individual values from patients for galectin-1^{+}/CD43^{hi} CLL cells between unstimulated and stimulated cultures. (B) Flow cytometry dot plots further illustrate an increase in the CD43^{hi}/CD45 activity^{hi} population in stimulated CLL cells. Graph shows paired individual values from patients for CD43^{hi}/CD45activity^{hi} CLL cells between unstimulated and stimulated cultures. (C) Gating on the CD43^{hi}/galectin-1^{+} population revealed higher levels of proliferation (Ki67^{+}) versus the CD43^{lo}/galectin-1^{lo} CLL cells. Graph shows paired individual values from patients within gated CD43^{hi}/galectin-1^{hi} versus CD43^{lo}/galectin-1^{-} CLL cells. (D) CD43^{hi}/galectin-1^{+} population had higher CD45 activity than CD43^{lo}/galectin-1^{-} in stimulated CLL cells. Included is a representative flow cytometry histogram. Graph shows paired individual values from patients for CD45 activity (gMFI) within CD43^{hi}/galectin-1^{hi} versus CD43^{lo}/galectin-1^{lo} CLL cells. (E) Introduction of CD43 siRNA reduced the CD43^{hi}/CD45 activity^{hi} population in stimulated CLL cells. Graph shows paired individual values from patients for CD43^{hi}/CD45 activity^{hi} CLL cells in control siRNA versus CD43 siRNA treated stimulated CLL samples. Statistical significance was defined by Student's paired *t*-test (A, C–E) and Wilcoxon matched-pairs signed-rank test (B). Data are representative of 18 (A, C, D), 20 (B), and nine (E) CLL patient samples. (F) CLL cells were positively isolated from patient-derived PBMCs, exposed to pCAP-SP1 (fluorogenic CD45 activity probe, blue), and immunolabelled for CD45 (green), galectin-1 (red), and CD43 (cyan). In the quadruple overlay (scale bar 10 μm) a region of interest was selected, with the corresponding graph displaying fluorophore intensity (*y*-axis) at a given pixel number (*x*-axis). CLL, chronic lymphocytic leukaemia; gMFI, geometric mean of fluorescence intensity; PBMCs, peripheral blood mononuclear cells; siRNA, small interfering RNA. [Colour figure can be viewed at wileyonlinelibrary.com]

cells remained constant (Figure S8A). This increase with the anti-CD43 mAb in Th cells is consistent with previous reports demonstrating CD43 as a negative regulator of Th cell proliferation.[36,45] Furthermore, monitoring cell death revealed no significant change with increasing anti-CD43 mAb treatment compared to isotype control (Figure S8B). Analysing CD43 surface expression together with CD45 activity or galectin-1 revealed a concomitant downregulation of these proliferative CD43^{hi}/CD45 activity^{hi} and CD43^{hi}/galectin-1^{+} populations in CLL cells (Figure 8E,F). Testing of additional anti-CD43 mAb clones (1G10, MEM59, and L60) in the stimulated collaborative culture system showed that the inhibitory effect on CLL cell proliferation was clearly dependent on the clone's ability to downregulate CD43 surface expression, CD43^{hi}/CD45 activity^{hi} and CD43^{hi}/galectin-1^{+} populations, and pSyk activation (Figure S9A–D). Additionally, in the anti-CD43 mAb treatment that downregulated CD43 (clone 1G10 but not MEM59), we observed a concomitant decrease in galectin-1 surface expression in CLL but not Th cells (Figure S9D). This suggested CD43 and galectin-1 may be physically linked on CLL cells. Further analyses showed no significant effect of the anti-CD43 mAbs on CLL or Th cell death (Figure S9D) and the anti-CD43 mAb clones did not block binding of the flow cytometry antibody (Figure S9E). To our knowledge, these data provide the first evidence that downregulating CD43 surface

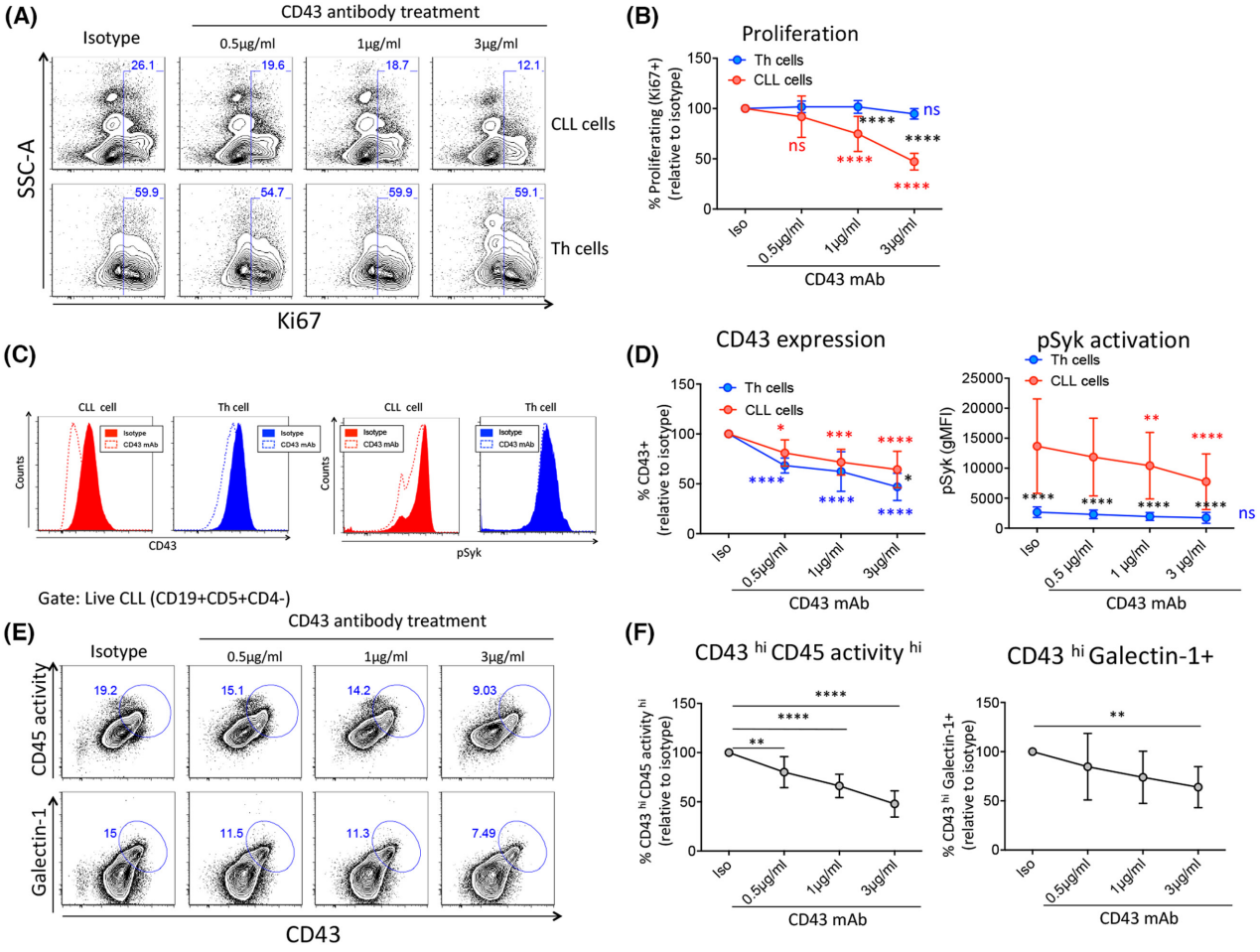


FIGURE 8 Treatment with an anti-CD43 antibody downregulated surface CD43 expression and specifically dampened CLL cell proliferation in vitro. (A) Flow cytometry dot plots illustrate changes in the proliferative (Ki67$^+$) capacity of stimulated CLL (upper panels) and Th cells (lower panels) with increasing concentration of monoclonal anti-CD43 antibody. Control cultures were treated with isotype antibody. (B) Graph shows means ± SD percentage proliferating cells relative to control. CLL cells had reduced proliferation in comparison to Th cells. (C) Representative flow cytometry histograms showing decreased CD43 expression and pSyk activation in control and anti-CD43 treated stimulated CLL cells (red histograms, left panel) and Th cells (blue histograms, right panels). (D) Graph shows means ± SD percentage CD43 expression (gMFI) relative to control and overall pSyk activation (gMFI). Both stimulated Th and CLL cells had decreased surface CD43 expression, while CLL cells had a corresponding reduction in pSyk activation. (E) Representative flow cytometry dot plots illustrating a decrease in the stimulated CLL CD43^{hi}/CD45 activity^{hi} and CD43^{hi}/galectin-1$^+$ populations upon anti-CD43 antibody treatment compared to control. (F) Graph shows means ± SD percentage of CD43^{hi}/CD45 activity^{hi} and CD43^{hi}/galectin-1$^+$ populations relative to control in stimulated CLL cells. Statistical significance is defined by two-way ANOVA (B, D) and repeated-measures one-way ANOVA with Dunnett's correction (F). Data are representative of nine (B, left panel D, left panel F), seven (right panel D), and eight (right panel F) CLL patient samples. *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; ****$p \le 0.0001$. ANOVA, analysis of variance; CLL, chronic lymphocytic leukaemia; gMFI, geometric mean of fluorescence intensity; Th, T-helper. [Colour figure can be viewed at wileyonlinelibrary.com]

expression reduces CD45 activity and specifically inhibits CLL cell proliferation without significantly affecting Th cell proliferation.

DISCUSSION

Co-ordination of signalling cross-talk between B and Th cells is a hallmark of humoral immunity. For CLL, recent evidence has implicated Th cells as important drivers for converting CLL cells into a pro-proliferative state,[4,30,31] and our results identify a mechanism contributing to this conversion. We propose activated Th cells from the microenvironment upregulate the CD45 phosphatase activity of CLL cells via galectin-1 and/or CD43. This change in CD45 activity has subsequent important implications for downstream CLL BCR signalling and proliferation. *Ex vivo* culture experiments utilising either an inhibitor of galectin-1 or monoclonal antibodies directed towards CD43 significantly reduced CLL cell proliferation by dampening CD45 activity and thus identified CD45 activity modulation as a possible novel therapeutic target in CLL.

Galectin-1 is a previously identified CD45 ligand, and we report increasing levels of surface galectin-1 expression correlated with increasing CD45 activity$^+$ in CLL cells. Furthermore, we linked increasing CD45 activity$^+$ to activated Syk, implicating a CD45 and galectin-1 pathway important for disinhibition of BCR signalling. We previously

demonstrated that Th signals induced activation of Syk kinase without engaging the BCR in a CD45-dependent manner.[32] Thus, our present results are likely a reflection of tonic CLL BCR signalling. Treatment with the steric galectin-1 inhibitor (OTX008) furthered evidence in favour of a causal relationship between galectin-1 surface binding and BCR signalling output through CD45 phosphatase activity regulation as it decreased the CD45 activity^{+}/Ki67^{+} population. Experiments performed in Raji wildtype and Raji LGALS1 KO cells strengthened evidence both for galectin-1-dependent proliferation and modulation of BCR signalling, in addition to previously demonstrated regulation of CD45 activity.[6] Increased galectin-1 binding and heightened BCR signalling is not unexpected, as it supports a previous report describing elevated galectin-1 serum levels in patients with CLL and galectin-1-dependent lowering of the BCR signalling threshold.[21] Furthermore, the major source of galectin-1 in these patients was from monocytes and nurse-like cells.[21] We identify Th cells, which were not previously investigated, as the likely source of galectin-1 in our culture system. Thus, we propose Th cells, along with nurse-like cells, contribute to a supportive microenvironmental niche for CLL cell activation via galectin-1 secretion and subsequent modulation of BCR signalling and proliferation through increasing CD45 phosphatase activity.

Interestingly, another study on galectin levels in CLL found galectin-1 unchanged, galectin-3 reduced, and galectin-9 elevated in patient serum compared to healthy individuals.[46] The disparity between these two studies regarding galectin-1 is not readily apparent but could be linked to glycosylation status of the binding partners as only serum galectin levels were analysed. However, galectin-3 and galectin-9 may warrant further study in our system as both these galectins are shown to bind CD45,[47,48] although galectin-9 was identified as a negative regulator of BCR signalling.[49] Ultimately, the net regulation of CD45 phosphatase activity by galectins may characterise an observed CLL population that upregulated CD45 activity upon stimulation, but was galectin-1 negative.

To assess the potential clinical usefulness of galectin-1 modulation, we subdivided patients based on their IGHV mutational status for further analysis. Traditionally, patients with U-CLL (≤2% variation from germline) have a clinically worse prognosis[50,51] and shorter duration of complete remission before relapse[52] compared to their M-CLL (≥2% variation from germline) counterparts. OTX008 reduced the proliferative capacity of CLL cells in both U-CLL and M-CLL. Furthermore, we did not observe any differences between U-CLL and M-CLL in additional parameters such as level of galectin-1 surface expression or CD45 activity (unpublished). These results emphasise, regardless of prognostic indicators, the promising potential of (i) a translatable mechanism for CLL cell activation and (ii) universally targeting galectin-1 modulation of CD45 activity as a potential target for therapy of patients with CLL.

Activated T cells upregulate galectin-1,[53–55] yet its precise role in T-cell signalling has remained enigmatic. A majority of evidence favours a galectin-1-mediated pathway that induces apoptosis of T cells[14,56,57] that is reliant on CD43 expression[35] and may or may not be CD45 dependent.[56,58] We also report activated Th cells upregulate galectin-1 and connect this ligand to the increasing CD45 activity of activated Th cells. However, these CLL patient-derived Th cells seemingly used increasing galectin-1 in a pro-proliferative capacity, as the CD45 activity^{+}/pLck^{394+} population correlated with higher levels of proliferation. Furthermore, activated Th cells were sensitive to death following use of OTX008, suggesting a dependence on galectin-1 binding for survival and strengthening an anti-apoptotic role for galectin-1 in our culture system. Perhaps, the 'tuning' of CD45 phosphatase activity by galectin-1 is the determining factor of the Th cell fate.

Additional insights into the observed anti-apoptotic effect of galectin-1 can also be derived from extracellular galectin-1 (exGal-1)'s differential impact on Th cell subpopulations. ExGal-1 inhibited cytokine production and triggered apoptosis of activated Th1 and Th17 cells, while Th2 cells did not apoptose and instead upregulated cytokine production following treatment with exGal-1.[59] Elsewhere, in classical Hodgkin lymphoma, Reed–Sternberg cells overexpress and secrete galectin-1 to maintain a Th2-prominent microenvironment.[60] Thus in summary, the pro-survival effect we observed in CLL-derived Th cells could be linked to exGal-1-dependent Th2 skewing in our culture system. However, characterising Th cell subsets in CLL has to date yielded conflicting results as both Th1- and Th2-enriched microenvironments have been proposed.[61–66]

One report highlighted different roles of exGal-1 and intracellular galectin-1.[54] Here, activated Th cells retained their increasing galectin-1 intracellularly, which in turn sensitised these cells to apoptosis upon binding by exGal-1 secreted by the microenvironmental niche.[54] Given CLL cells had inconsistent modulation of intracellular galectin-1 in our activated culture system, and that activated Th cells had surface bound galectin-1 when cultured alone (unpublished observation), our results support an autocrine-like mechanism of action for galectin-1 on Th cells from patients with CLL. Our discovered mechanism of action could represent either (i) the ability of Th cells under CLL pathological conditions to modulate their use and secretion of galectin-1; (ii) differences in stimulation paradigms, i.e., CD3/28 beads for our culture system versus phytohaemagglutinin for Deak et al.[54] or (iii) differences in the sensitivity of methods used for surface detection of galectin-1.

CD43 is used as an important marker, among others, for immunophenotypical identification of CLL cells.[19] We show the novel finding that a CD43^{hi}/Gal-1^{+} population identifies a proliferating subset of CLL cells with high CD45 activity. To our knowledge, this is the first association of CD43 with downstream signalling properties in CLL cells, and silencing of CD43 by siRNA strategies

confirmed regulation of CD45 activity. Mechanistically, our working hypothesis is galectin-1 serves as a bridging protein between CD43 and CD45, and all together, their interaction is important for CD45 phosphatase activity regulation. However, further work is needed to unquestionably demonstrate galectin-1's dependency on CD43. Nevertheless, results in dendritic cells of CD43/CD45 co-clustering following human recombinant galectin-1 treatment,[20] reduction in galectin-1 surface expression upon anti-CD43 mAb treatment, and confocal co-localisation experiments strengthens this notion.

Particularly interesting was use of mAbs directed against different CD43 epitopes, which resulted in varying degrees of modulation in downstream CLL cell proliferation, but invariably left the Th cell compartment largely unaffected. That mAbs against CD43 epitopes produced differing downstream results is not surprising, as this phenomenon was observed with Th cells.[67] In Th cells, this was due to epitope-specific activation of distinct downstream signalling pathways and induction of distinct cytokine profiles.[67] In contrast, anti-CD43-mediated dampening of CLL cell proliferation was dependent on downregulation of surface CD43, presumably by antibody mediated receptor internalisation. Interestingly, anti-CD43 mAb treatment led to downregulation of surface CD43 also in the Th cell compartment; however, without affecting Th cell proliferation, suggesting surface CD43 was not essential for Th cell proliferation in our collaborative cultures. It is tempting to speculate that this effect was due to the CD43-associated downregulation of surface galectin-1 in CLL cells, which did not occur in Th cells. Ultimately, CD43 may have relevance beyond diagnostic immunophenotyping, and the use of a CD43 mAb for regulation of CD45 phosphatase activity may be a promising therapeutic target as it preferentially dampened CLL cell proliferation even in the presence of activated Th cells.

One of the major underlying themes in this paper was upregulation of CD45 activity in CLL cells occurred in a co-culture system when we increased the frequency of activated Th cells. Firstly, this extends reports of a pivotal role for Th-cell help in CLL[3,30,31] and strengthens support for Th cells as drivers of CLL cell activation.[68] Secondly, upregulation of CD45 activity in the presence of activated Th cell-derived signals also appears to be a translatable mechanism of Th-cell regulation of B cells, as it reflects our observations in anergic[32] and normal mature B-cell systems.[6] However, the result of increasing CD45 activity on B-cell output is then likely dependent on the particular B-cell subset. Nevertheless, given its increasing translatability, modulation of CD45 phosphatase activity presents an intriguing therapeutic target.

Growing evidence supports CD45 phosphatase, and by extension its regulation by binding partners such as galectins and CD43, as an increasingly relevant therapeutic target in haematological malignancies. For example, cell lines mimicking Burkitt lymphoma[17] and anaplastic large cell lymphoma[69] demonstrated changes in CD45 activity via galectin-1, which influenced the downstream activation states of these cells. Furthermore, galectin-3 exerted an anti-apoptotic effect on large B-cell lymphoma cells through changes in CD45 activity.[70] Additionally, increasing galectin-1 serum levels correlated with advanced clinical stage in Hodgkin lymphoma,[71] and galectin-1 supported multiple myeloma cell survival in culture.[72] In these studies, if the effect of galectin-1 occurred as result of changes in CD45 activity remains unknown though highly probable. While targeting CD43 as a method to reduce CD45 activity has not been previously described, immunomodulation of CD43 has been proposed as a therapeutic target in non-B-cell leukaemias.[73,74] Overall, our results are consistent with the literature in that there is an escalating demand for interrogating CD45 phosphatase activity as a potential therapeutic target for CLL and beyond, although it is not without limitations.

Arguably the most limiting aspect of these results is the broad expression of CD45 among immune cells. Thus, specific and direct therapeutic regulation of CD45 phosphatase activity only in CLL cells may be difficult to achieve. To this end, we investigated alternative approaches and discovered both galectin-1 and CD43 as regulators of CD45 activity. Galectin-1 and CD43 are also expressed on Th cells, and thus we provided a side-by-side comparison to CLL cells following use of our reagents. However, one additional caveat is although we tried our best to recapitulate the in vivo activation of Th cells, use of CD3/28 activator beads and interleukin 2 could represent a robust stimulus that may mask effects of the inhibitors on Th cells. Going forward, establishing in vivo xenotransplantation experiments using mice, where we transplant CLL PBMCs and autologous Th cells alongside our inhibitors, will be an important validation step for CD45 activity regulation as a new therapeutic target in CLL.

AUTHOR CONTRIBUTIONS

John F. Imbery designed and performed experiments, analysed the data, and wrote the manuscript. Julia Heinzelbecker, Jenny K. Jebsen, Marc McGowan, and Camilla Myklebust performed experiments. Nunzio Bottini and Stephanie M. Stanford designed a vital new reagent (CD45 phosphatase activity probe). Anders Tveita and Julia Heinzelbecker generated the Raji LGALS1 KO cell line. Geir E Tjønnfjord recruited patients and collected samples. Sigrid S. Skånland provided patient samples. Ludvig A. Munthe designed experiments. Peter Szodoray conceptualised the hypothesis, designed experiments, reviewed the data. Britt Nakken conceptualised the hypothesis, designed experiments, reviewed and analysed the data, compiled figures. All authors edited and reviewed the manuscript.

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CONFLICT OF INTEREST

None.

ORCID

John F. Imbery https://orcid.org/0000-0002-0809-3550

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