

# KIR3DL2 may represent a novel therapeutic target in aggressive systemic peripheral T-cell lymphoma

Only a few humanized monoclonal antibodies (mAb) are available to treat patients with peripheral T-cell lymphomas (PTCL), with the exception of anti-CCR4 (mogamulizumab) and anti-CD30 antibody-drug conjugate (Brentuximab-vedotin). Lacutamab, a first-in-class humanized anti-KIR3DL2 cytotoxicity-inducing mAb which has demonstrated *in vitro* antitumor activity by antibody-dependent cell-mediated cytotoxicity (ADCC) and encouraging clinical activity in a phase I clinical trial (dose-escalation plus expansion cohort) that enrolled relapsed/refractory advanced cutaneous T-cell lymphomas (CTCL) patients (*clinicaltrials.gov. Identifier: NCT02593045*),<sup>1</sup> is under phase I evaluation (*clinicaltrials.gov. Identifier: NCT03902184*). Aside from adult T-cell leukemia lymphomas (ATL) and CTCL, KIR3DL2 expression has not yet been investigated in large cohorts of PTCL patients and the possible modulation of KIR3DL2 by therapeutic agents has not been addressed in this context. We found that KIR3DL2 was expressed at the protein level in several aggressive PTCL entities and could be efficiently targeted using lacutamab *in vitro*. The regulation of KIR3DL2 expression is controlled by DNA methylation in PTCL, and chemotherapy (gemcitabine and oxaliplatin) could increase lacutamab efficacy through the modulation of KIR3DL2 expression.

KIR3DL2 is an inhibitory receptor of the killer immunoglobulin-like receptor (KIR) family, clonally expressed in normal NK cells and sub populations of  $\alpha\beta$  and  $\gamma\delta$  T cells, as are the other KIR.<sup>2</sup> KIR3DL2 is expressed by the neoplastic T cells of Sézary syndrome (SS)<sup>3</sup> and other CTCL.<sup>4</sup> We have also recently shown that KIR3DL2 is the only KIR recurrently expressed in ATL, mostly in acute subtypes.<sup>5</sup>

Following the finding of *KIR3DL2* mRNA overexpression in some PTCL, especially in PTCL-not otherwise specified (PTCL-NOS) (n=16/50) and ALK-anaplastic large cell lymphomas (ALCL) (n=6/25), as compared to controls (*data not shown* and unpublished) using RNA sequencing, we performed immunohistochemistry and flow cytometry using specific anti-KIR3DL2 mAb. The anti-KIR3DL2 13E4 mAb (Innate Pharma, Marseille, France) was used for flow cytometry. Several anti-KIR3DL2 antibodies were used for immunohistochemistry: two specific anti-KIR3DL2 mAb (clones MOG1-MK323-12B11 [12B11] and MOG1L-P3-R4D-H5 [H5]; Innate Pharma), allowing the staining of frozen and formalin-fixed paraffin-embedded (FFPE) tissue sections, respectively, or the commercially available 5.133 clone, which recognizes KIR3DL2, as well as KIR3DL1 and other KIR (Miltenyi Biotec, Paris, France), for frozen sections only. We showed KIR3DL2 protein expression in 95 of 252 (38%) PTCL (Figures 1A-D; Table 1; *Online Supplementary Figure*

S1), including 20 of 88 (23%) angio-immunoblastic TCL (AITL), 21 of 51 (42%) PTCL-NOS, nine of 29 (31%) ALCL (including 5/13 ALK<sup>+</sup> and 5/16 ALK<sup>-</sup> ALCL), nine of 20 (45%) enteropathy-associated TCL (EATL), nine of nine (100%) monomorphic epitheliotropic intestinal TCL (MEITL), eight of 21 (38%) nasal-type NK/T-cell lymphomas (NK/TCL), 13 of 17 (76%) hepatosplenic TCL (HSTL), and none of eight T-cell prolymphocytic leukemia (TPLL) samples. Nearly half of the 92 KIR3DL2<sup>+</sup> PTCL (44 cases) showed high expression (>50% KIR3DL2<sup>+</sup> cells) by immunohistochemistry, a feature common to HSTL (9/10), MEITL (6/9) and PTCL-NOS (11/38). The neoplastic nature of two AITL and two HSTL cases could be demonstrated by double immunofluorescence tests (Figure 1C). We conducted a concordance study between three observers and three centers on 12 selected cases to test the validity of the H5 clone (see legend of Table 1 for details) to address KIR3DL2 expression on FFPE samples. The inter-observer agreement was considered to be good with an estimated weighted  $\kappa$  ranging from 0.65 to 0.78 depending on the readers, and an excellent International Consensus Classification (0.9, 95% confidence interval [CI]: 0.82-0.95), as detailed in the *Online Supplementary Figure S2*.

The role of KIR3DL2 in lymphomagenesis is a subject of debate, as it has been suggested that it may increase cell survival in SS by inhibiting the activation-induced cell death (AICD)<sup>6</sup> or conversely may lead to apoptosis through the engagement of CpG oligodeoxynucleotides (ODN CpG).<sup>7</sup> In PTCL, KIR3DL2 expression may reflect the proposed derivation of NKTCL, MEITL, and HSTL from innate immune cells with constitutive expression of KIR3DL2. However, the reason why KIR3DL2 may be found in nodal PTCL, such as AITL or PTCL-NOS, likely derived from cells of the adaptive immune system, as are SS and ATL, is still unclear. Several lines of evidence suggest that KIR are induced upon T-cell activation. KIR<sup>+</sup> CD4<sup>+</sup> T cells can be identified in various chronic inflammatory diseases such as rheumatoid arthritis, coronaritis or spondylarthritis.<sup>2,8</sup> In these T-cell subsets, KIR were shown to act as costimulatory or co-inhibitory receptors of the T-cell receptor (TCR), as they may do in SS cells.<sup>9</sup> In accordance with these findings, inhibitory KIR have been shown to down-regulate the TCR-signaling pathway and promote resistance of human and mouse T cells to AICD.<sup>10</sup> Consistent with this hypothesis, murine PTCL showed NK cell-like reprogramming of neoplastic murine T cells toward NK cells, with expression of various NK receptors due to chromatin remodeling in a mouse model of PTCL, driven by chronic TCR stimulation based on homeostatic prolif-

**Table 1.** KIR3DL2 expression using immunohistochemistry and flow cytometry in the peripheral T-cell lymphoma cohort.

PTCL	Whole sample, N=252 N (%)	FCM, N=63 N (%)	IHC, N=189 N (%)	Total, N=252 %
			<b>KIR3DL2<sup>+</sup></b>	
PTCL,NOS	51 (20)	3/13 (23)	18/38 (47)	42
AITL	88 (35)	7/21 (33)	13/67 (19)	23
ALCL, ALK <sup>+</sup>	13 (5)	0/2	5/11 (45)	38
ALCL, ALK <sup>-</sup>	16 (6)	1/5 (20)	4/11 (36)	31
HSTL	17 (7)	3/7 (43)	10/10 (100)	76
NK/TCL	21 (8)	0/1	8/20 (40)	38
EATL	20 (8)	0	9/20 (45)	45
MEITL	9 (4)	0	9/9 (100)	100
LGL	9 (4)	5/8 (62)	1/1	67
TPLL	8 (3)	0/6	0/2	0

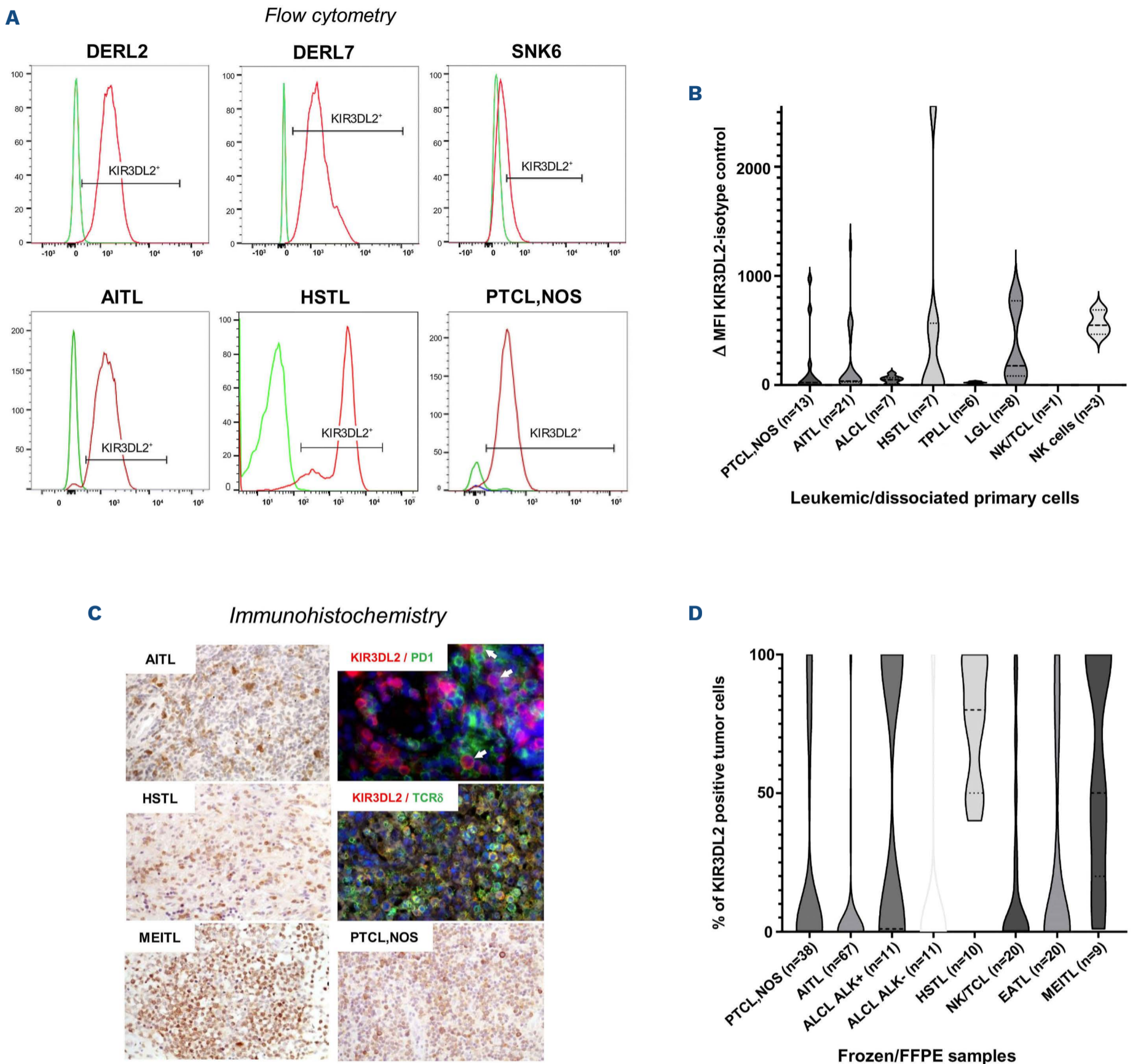
PTCL: peripheral T-cell lymphoma; FCM: flow cytometry; IHC: immunohistochemistry; PTCL,NOS: PTCL not otherwise specified; AITL: angio-immunoblastic T-cell lymphoma (TCL); ALCL: anaplastic large-cell lymphoma; EATL: enteropathy-associated TCL; MEITL: monomorphic epitheliotropic intestinal TCL; NKTCL: nasal-type NK/TCL; HSTL: hepatosplenic TCL; LGL: large granular lymphocytic; TPLL: T-cell prolymphocytic leukemia. FCM was applied to viable cell suspensions of frozen PTCL samples (spleen, lymph nodes, liver, blood) from the CeVI collection of the Carnot Institute CALYM (N=55), maintained in dimethyl sulfoxide and fresh blood samples of patients in a leukemic phase from our institution (N=8). Cases were considered to be positive if the  $\Delta$  mean fluorescence intensity (MFI) between the specific anti-KIR3DL2 13E4 monoclonal antibody (Innate Pharma) and isotype control was superior to 100. For IHC, frozen (N=73) or formalin-fixed paraffin-embedded (FFPE) (N=122) PTCL tissue samples (N=195 samples from 189 patients), including 6 for which there were both frozen and FFPE material, were retrieved from the biobank of the multicentric T-cell lymphoma consortium (TENOMIC) of the Lymphoma Study Association (LYSA). The positivity threshold for KIR3DL2 expression was set to 5% of positive tumor cells.

eration of p53<sup>-/-</sup> T cells.<sup>11</sup> Moreover, we recently demonstrated that HTLV-1 infection triggered KIR3DL2 expression by normal CD4<sup>+</sup> cells after 7 days of cell culture, suggesting that HTLV-1 influences KIR3DL2 expression either through infection-dependent chronic antigenic stimulation, viral integration, or the transcriptional activity of viral proteins.<sup>5</sup> Thus, in PTCL, it is conceivable that KIR3DL2, expressed either because of lineage properties or downstream of chronic antigenic stimulation, promotes PTCL cell survival through its inhibitory functions on the TCR pathway leading to reduced AICD.

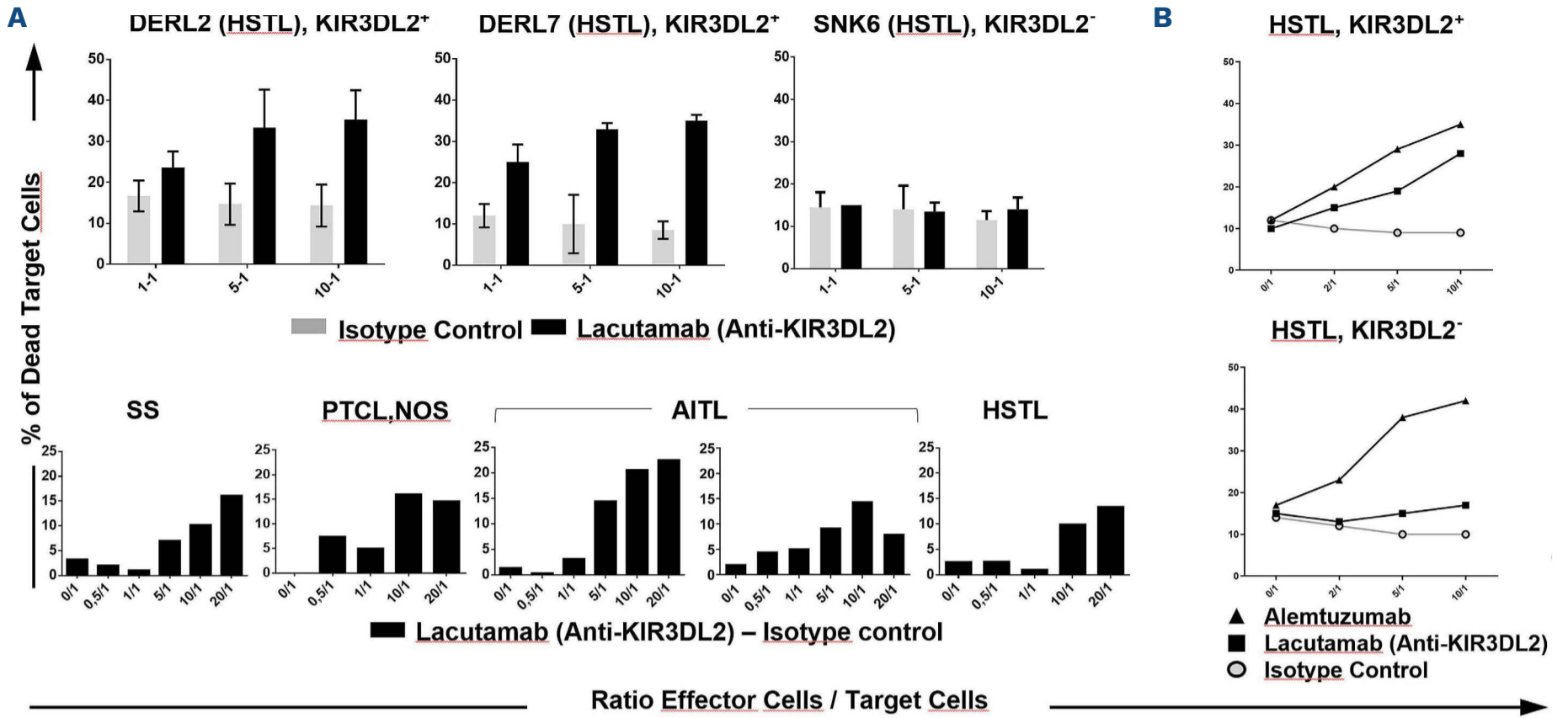
We performed heterologous ADCC assays using lacutumab on various PTCL cell lines (KIR3DL2<sup>+</sup> HSTL lines DERL2, DERL7 and KIR3DL2<sup>-</sup> NKTCL-derived SNK6 line), as well as primary cells from KIR3DL2<sup>+</sup> AITL (n=2), HSTL (n=2), PTCL-NOS (n=1), one KIR3DL2<sup>-</sup> HSTL together with SS cells used as positive controls. We observed effector/target ratio-dependent cytotoxic activity against KIR3DL2<sup>+</sup> DERL2 and DERL7 HSTL-derived cells, but not against KIR3DL2<sup>-</sup> SNK6 cells and the primary KIR3DL2<sup>-</sup> cells from a HSTL. Similar results were obtained with the primary KIR3DL2<sup>+</sup> lymphoma cells from AITL, PTCL-NOS and HSTL patients (Figures 2A, B). Unfortunately, autologous assays from the primary cell samples could not be performed due to insufficient quantity of cells.

Deciphering the molecular mechanisms that drive KIR3DL2 expression in neoplastic T cells of PTCL and CTCL is important, because it may pave the way to innovative combination therapies, thus improving the efficiency of

lacutumab. In normal NK and T lymphocytes, KIR expression was previously shown to be regulated at the epigenetic level, mainly through DNA methylation of CpG islands.<sup>12</sup> However, the mechanisms that drive KIR3DL2 expression in other PTCL have never been explored. We, thus, investigated the potential role of DNA methylation in modulating *KIR3DL2* expression in PTCL cells and tumors. We found lower promoter methylation of KIR3DL2 CpG islands in KIR3DL2<sup>+</sup> (HUT78, DERL2, DERL7) than KIR3DL2<sup>-</sup> (MyLa and SNK6) cells by bisulfite sequencing. We obtained similar results for primary lymphoma cells and patients tumors (HSTL and EATL tumor samples and PBMC from one PTCL-NOS) (Figures 2C, D). Overall, the median level of methylated CpG islands was significantly lower in KIR3DL2<sup>+</sup> than in KIR3DL2<sup>-</sup> samples (49.2 vs. 83.9%;  $P < 0.0001$ ). Though a more extensive analysis of all different PTCL subtypes is needed, our data suggest that CpG methylation at the promoter locus may participate in the regulation of KIR3DL2 expression. The ability of 5-azacytidine to induce or increase KIR expression in normal NK cells<sup>13</sup> and both CD4<sup>+</sup> and CD8<sup>+</sup> T cells<sup>12</sup> *in vitro* has been previously shown. Using a similar approach, 5-azacytidine treatment allowed us to increase KIR3DL2 expression at the cell surface of HUT78 cells, expanding our findings in ATL,<sup>5</sup> and to induce it in the MyLa (Figures 2E, F) but not SNK6 cell lines, the former being constitutively KIR3DL2<sup>+</sup> and the two later KIR3DL2<sup>-</sup>. This suggests that KIR3DL2 silencing by promoter methylation in PTCL may be reversible. Importantly, the level of CpG methylation post-treatment



**Figure 1. KIR3DL2 expression in peripheral T-cell lymphoma subtypes.** (A) KIR3DL2 protein expression (red line) by flow cytometry using the specific anti-KIR3DL2 13E4 monoclonal antibody (mAb) (Innate Pharma) in the DERL2 and DERL7 hepatosplenic T-cell lymphoma (HSTL)-derived and SNK6 nasal-type NK/TCL (NKTCL)-derived cell lines and peripheral blood mononuclear cells (PBMC) from 3 peripheral TCL (PTCL) patients (angio-immunoblastic TCL [AITL], HSTL, PTCL not otherwise specified [PTCL-NOS]) in a leukemic phase, on which antibody-dependent cell-mediated cytotoxicity (ADCC) assays could be performed, compared to staining with an isotype-matched control mAb (green line). (B) Violin plots of KIR3DL2 protein expression using flow cytometry in the 63 PTCL. (C) KIR3DL2 protein expression by immunohistochemistry (IHC) in formalin-fixed paraffin-embedded (FFPE) samples from representative patients with AITL, HSTL, monomorphic epitheliotropic intestinal TCL (MEITL), and PTCL-NOS, showing scattered positive atypical cells in the AITL sample, with co-expression of PD1 (green) in a few cells (double immunofluorescence). Diffuse expression of KIR3DL2 by neoplastic cells was observed by IHC in the HSTL, MEITL and PTCL-NOS samples, as confirmed by double immunofluorescence with co-expression of KIR3DL2 (red) and TCR- $\delta$  (green) in most cells of the HSTL sample. Cell nuclei counterstained with DAPI appear in blue. Double immunofluorescence and chromogenic staining experiments (PD-1/KIR3DL2 and TCR- $\delta$ /KIR3DL2) were performed on FFPE tissue using the Opal™ Reagent Pack system (Akoya Biosciences, Marlborough, USA) or BOND -III Autostainer (Leica Microsystems, Newcastle upon Tyne, UK), respectively. (D) Violin plots of KIR3DL2 protein expression obtained from IHC analyses of 186 PTCL tissue samples. The semi-quantitative evaluation of the proportion of positive cells was independently performed by 2 pathologists (JB and NO).



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**Figure 2. *In vitro* assays for lacutamab and KIR3DL2 promoter methylation levels in peripheral T-cell lymphoma samples and cell lines.** (A) Targeted *ex vivo* antitumor efficiency of lacutamab against several peripheral T-cell lymphoma (PTCL) cell lines (upper panel) and primary PTCL cells (lower panel) in antibody-dependent cell-mediated cytotoxicity (ADCC) assays at various effector/target (E/T) ratios with either lacutamab or isotype control monoclonal antibody (mAb) (1 µg/mL each). The results are expressed as the percentage of dead cells in the presence of lacutamab relative to dead cells in the presence of the isotype control for each population at a given E/T ratio. Dead cells were measured using the Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) on a LSR Fortessa X20 instrument (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (TreeStar). All experiments were performed in triplicate for cell lines. For each set of data at different E/T ratios we used the same donor, but different donors were used across the different cell lines and primary samples studied. (B) Targeted *ex vivo* antitumor efficacy of lacutamab against one KIR3DL2<sup>+</sup> (upper panel) and one KIR3DL2<sup>-</sup> (lower panel) hepatosplenic TCL (HSTL) primary samples, assessed with the same donor. (C) Percentage of methylated CpG islands of the *KIR3DL2* gene promoter in KIR3DL2<sup>+</sup> PTCL samples (3 cell lines, peripheral blood mononuclear cells [PBMC] from 1 Sézary syndrome (SS), 7 HSTL and 2 enteropathy-associated TCL [EATL] frozen tumor samples) relative to that of KIR3DL2<sup>-</sup> cases (2 cell lines, PBMC from 1 SS, and 2 EATL frozen tumor samples). Analysis of the *KIR3DL2* promoter methylation patterns was performed after bisulfite treatment of the *KIR3DL2* promoter followed by plasmid ligation for clonal amplification and direct Sanger sequencing, as previously described,<sup>12</sup> using primers generating a 353-base pair amplicon covering the *KIR3DL2* promoter region (forward primer ATTTTATGTGTGAGAGGTTGGATTTGAG; reverse primer CTCCATCCCCACTCTCCCTCCCTCTAT). The mean percentage of methylation across all CpG island from each clone (sum of %mCpG1-15/15) is shown for each sample. The median level of CpG island methylation was compared between KIR3DL2<sup>+</sup> and KIR3DL2<sup>-</sup> samples and the statistical significance assessed using the non-parametric Mann-Whitney U test with Prism 6.0 software (GraphPad). (D) CpG island methylation in tumor samples from examples of KIR3DL2<sup>+</sup> PTCL patients (left panels), and the KIR3DL2<sup>-</sup> SNK6 and KIR3DL2<sup>+</sup> DERL PTCL cell lines (right panels). CpG island methylation is higher in KIR3DL2<sup>-</sup> samples. (E) Increase in KIR3DL2 expression in the MyLa cell line after pretreatment with 5-azacytidine for 7 days (at 2, 5, and 10 µM), as determined by flow-cytometry. (F) CpG island methylation of the *KIR3DL2* gene promoter at baseline and after treatment with 5-azacytidine (5 µM), determined after bisulfite treatment.

did not change in the SNK6 line, suggesting resistance to the 5-azacytidine hypomethylation effect (*data not shown*). To note, although weak KIR3DL2 expression could be induced in MyLa cell line, this did not increase its sensitivity to lacutamab. KIR3DL2 expression is, at least in part, epigenetically regulated at the level of the gene promoter in PTCL, as in normal NK and T cells.<sup>12</sup> Of note, we recently demonstrated that KIR3DL2 is also upregulated by gene promoter hypomethylation, but required TAX viral protein expression in ATL neoplastic T cells.<sup>5</sup> The role of other gene expression regulators, including transcription factors and other key epigenetic players, such as chromatin remodeling systems and histone modifications, or signal transducers is yet to be studied. Importantly, the role of histone acetylation and methylation was shown to be involved in KIR expression in normal NK cells.<sup>14</sup>

Targeting KIR3DL2 may, thus, represent a promising new therapeutic approach for patients with KIR3DL2-expressing PTCL that is refractory/resistant to conventional chemotherapy. We also provide a rationale for combination approaches with epigenetic modifiers, such as the Food and Drug Administration-approved 5-azacytidine, currently being assessed in a phase III study for relapsed AITL patients (*clinicaltrials.gov. Identifier: NCT03593018*). Chemotherapy regimens, especially with gemcitabine and oxaliplatin, which are widely used in relapsed/refractory PTCL patients, are already known to modulate cell-surface receptors expression.<sup>15</sup> We found that pretreatment of the KIR3DL2<sup>+</sup> HUT78 cell line with gemcitabine + oxaliplatin (GemOx) increased the level of KIR3DL2 expression and sensitivity to lacutamab (*Online Supplementary Figure S3*). In this respect, the KILT phase II clinical trial will aim to evaluate the combination of lacutamab with gemcitabine + oxaliplatin (Gemox) in patients with KIR3DL2-ex-

pressing relapsed/refractory PTCL (*clinicaltrials.gov. Identifier: NCT04984837*).

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### Disclosures

HS, CB, VP, FG and LG are employees of Innate Pharma and Amandine Decroos received financial support from Innate Pharma for her PhD. HS, LG and CB are senior directors and have an ownership interest (including patents) in Innate Pharma. AD, MC, JB, NO, PG and OH received research grants from Innate Pharma. The other authors have no conflicts of interest to disclose.

### Contributions

AD performed the ADCC experiments and part of the KIR3DL2 immunostaining (TENOMIC Cohort), participated in the analyses of *KIR3DL2* promoter methylation, analyzed the data, and revised the paper. MC participated in the design of the study, KIR3DL2 immunostaining, analyzing of the data, and writing and revision of the paper. JB performed part of the KIR3DL2 immunostaining, analyzed the results, and revised the paper. LLh and VA performed and analyzed the results of the flow cytometry studies. MB participated in the interobserver correlation study on KIR3DL2 expression in FFPE samples. SC performed and analyzed the results of the flow cytometry studies (KIR3DL2 and KIR3DL1 on the CeVi

cohort) and revised the paper. VP and LP designed the experiments and performed the analyses of *KIR3DL2* promoter methylation. LLa and NM provided technical support for the KIR3DL2 staining by immunohistochemistry with the 5.133, MOG1-MK323-12B11, and MOG1L-P3-R4D-H5 mAb and LLa performed the double staining experiments by immunofluorescence for confocal imaging. JG provided technical support for the ADCC experiments. HS, CB, and LGa provided the specific antibodies to KIR3DL2 for the immunohistochemistry, flow cytometry, and ADCC experiments and participated in writing and revising the paper. LGe participated in the design of the study and in writing and revising the paper. SCa performed the statistical analysis regarding interobserver correlations on KIR3DL2 expression on FFPE samples. LdL reviewed PTCL included in the study. PG participated in the design of the study and writing and revising the paper. OH participated in the design of the study and writing and revising the paper. NO designed the study, analyzed the data, and wrote the paper.

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### Data-sharing statement

Data presented in this manuscript are available upon request.

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