# Title: CD27 agonist antibodies mediate clinical responses through intratumoral stimulation in B-cell malignancies: multicenter RiVa trial

**Running Title**: Anti-CD27 immunostimulation in lymphoma


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## Statement of translational relevance

Immunotherapy with agonist antibodies has yet to achieve clinical approval for cancer; the reason behind their limited success remains unclear. The RiVa trial (NCT03307746) evaluated the combination of varlilumab (an anti-CD27 T-cell agonist) and rituximab (a tumor-depleting CD20 antibody) in participants with relapsed/refractory B-cell non-Hodgkin lymphoma (B-NHL). The combination was safe, but clinical responses were modest. Our bulk and single-cell transcriptomic analyses confirmed T and innate immune cell activation by varlilumab in patients' tumors. Clinical responses were associated with pre-treatment inflamed tumor signatures and higher expression of CD27-expressing T and NK cells, alongside activated γδ T-cell signatures. Thus, CD27 agonistic antibodies can overcome an immunosuppressive tumor microenvironment to drive clinically meaningful anti-tumor activity, but their efficacy depends on sufficient intratumoral target expression and pre-existing immune-primed tumors. Selecting the right patients is crucial to unlocking the clinical potential of agonist antibodies.

## Abstract

*Purpose*:

Varlilumab is a CD27 agonist antibody, delivering a T-cell costimulation. Preclinical studies show that agonistic CD27 antibodies can activate intratumoral T-cells to release chemokines and cytokines to augment macrophage-dependent tumor killing induced by CD20 antibodies, i.e. rituximab, in B-cell lymphoma. This clinical trial evaluated the safety and efficacy of rituximab and varlilumab in patients with previously treated B-cell non-Hodgkin lymphoma (B-NHL).

*Patient and Methods:*

This multicenter phase IIa trial recruited patients with relapsed or refractory CD20+ B-NHL. Patients were randomized to Arm A or B. All received rituximab on Day 1 of Cycles1-6, and varlilumab on Day 2 (Arm A) or Day 8 (Arm B) of Cycle 1, and Day 2 of Cycles 3 and 5. Tumor biopsies were collecting pre-treatment and on-treatment (after varlilumab in Arm A and before varlilumab in Arm B). The primary objective was to assess safety and anti-tumor activity.

*Results*:

Twenty-seven participants were evaluable, with modest overall response and disease control rates of 15.4% (4/27) and 38.8% (8/27), respectively. Intratumoral bulk RNA sequencing analysis demonstrated that adding varlilumab to rituximab enhanced CD4+ T-cell infiltration and increased T- and innate-cell signatures; inflamed tumor signatures were observed pre-treatment in responders. Single-cell analysis further showed that higher levels of CD27-expressing T and NK cells, along with activated γδ T-cell signatures, were associated with response, whereas CD27-expressing B cells correlated with non-response.

*Conclusions*:

Rituximab and varlilumab show modest activity. However, CD27 agonist antibodies may elicit meaningful anti-tumor responses when tumors express sufficient intratumoral targets and exhibit existing immune priming.

## Introduction

Monoclonal antibody-based immunotherapies have transformed the treatment landscape of multiple cancer types in the last two decades (reviewed in (1)). Monoclonal antibodies (mAb) can elicit various mechanisms of action. These include binding to and killing tumor cells through mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC) and direct cell death induction for antibodies such as anti-CD20 (rituximab) and anti-HER2 (trastuzumab) (2). These mAbs can also induce anti-tumor immunity by inhibiting immune checkpoints e.g. anti-CTLA-4 (ipilimumab) and anti-PD-L1/PD-1 (nivolumab, pembrolizumab) (2); or stimulating anti-tumor immunity by triggering signaling through immune receptors (e.g. tumor necrosis factor receptor superfamily (TNFRS) members such as CD40, OX40, 4-1BB, GITR and CD27). However, despite over 300 clinical trials investigating immunostimulating/agonist mAb, none have received clinical approval (3). These antibodies (Ab) show potent T-cell immunostimulatory effects *in vitro* or in murine models, but in human studies, on-treatment tumor biopsies demonstrating the ability of these mAb to stimulate intratumoral T cells within the tumor microenvironment (TME) is less certain (1). As such, the mechanism behind the failure of this class of mAb to induce clinically significant disease remission in patients is unknown but may include insufficient T-cell agonism within an immunosuppressive TME.

Regardless of the lack of clinical success, TNFRS agonists remain a topic of significant interest because clinical responses to immune checkpoint inhibitors are often partial and may not be long-lasting (4). CD27 is a TNFRS member; it is constitutively expressed on all T-cell subsets, memory B cells and a small proportion of NK cells (5). Ligation of CD27 on T cells by its ligand, CD70, expressed on activated antigen presenting cells, augments T-cell priming, proliferation, survival and effector function. Apart from its co-stimulatory effects, CD27 possesses two key attributes that make it an appealing therapeutic target: first, its constitutive expression on T cells ensures availability of sufficient target for stimulation; second, stimulation of CD27 is contingent on prior T-cell receptor signaling, minimizing the risk of uncontrolled T-cell stimulation and associated immune toxicity. In addition, our preclinical studies demonstrate that T-cell stimulation with an agonistic anti-CD27 mAb induces T cells to release chemokines and cytokines such as IFNγ, CCL3, CCL4 and CCL5. This, in turn, activates macrophages and promotes their infiltration

into the TME (6). These macrophages have enhanced ability to mediate antibody-dependent cellular phagocytosis (ADCP) of tumor cells when a tumor-depleting antibody such as anti-CD20 is administered in B-cell malignancies. The mechanism of action of anti-CD20 mAb and the principal role of ADCP therein was described previously by our group (7). A similar phenomenon was observed when anti-CD27 was administered in combination with regulatory T-cell (Treg) depleting anti-CTLA-4 in a murine colorectal adenocarcinoma model; where enhanced Treg depletion and increased infiltration of myeloid cells were observed with addition of anti-CD27 (8).

Varlilumab is a fully human, agonistic IgG1 CD27 antibody (9,10). Early-phase studies of varlilumab monotherapy in advanced stage cancers demonstrate that it can be safely administered in patients (11). Modest anti-tumor responses and increased numbers of peripheral blood T-effector memory cells were observed, suggestive of antibody agonism, but direct evidence of intratumoral agonism was not shown. Aside from varlilumab, there is no other published human clinical trial data involving CD27 agonist antibodies, making it a relatively underexplored therapeutic target.

The RiVa trial (NCT03307746) is a phase IIa study assessing the combination of rituximab and varlilumab in patients with relapsed or refractory B-cell non-Hodgkin lymphoma (B-NHL). Whilst this group comprises heterogeneous disease subtypes, they share common therapeutic features, including rituximab sensitivity and a tendency to not respond to immune checkpoint inhibition (12,13). The rationale for combining both mAb follows on from our preclinical observations as described in the earlier paragraph (6,14). Here, we report the clinical and translational data from the study, which includes pre- and on-treatment tumor biopsies, sequenced using bulk and single-cell RNA sequencing (scRNAseq). By comparing *in vivo* intratumoral immune changes with clinical outcomes, we aim to address the following questions: 1) can a CD27 agonist antibody, such as varlilumab, induce intratumoral immunostimulation in patients and 2) will combining anti-CD20-mediated tumor targeting along with T-cell immunostimulation mediate clinically significant anti-tumor responses? If not, what biological factors influence clinical responses?

## MATERIALS AND METHODS

### *EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS*

## Trial design and patients

Coordinated by the Cancer Research UK Southampton Clinical Trials Unit, RiVa is a multicenter, randomized phase IIa trial (NCT03307746) designed for adult patients with relapsed or refractory mature $CD20^+$ B-cell malignancies (low grade: follicular lymphoma (FL) grade 1, 2 or 3a, mantle cell lymphoma (MCL), marginal zone lymphoma (MZL) and lymphoplasmacytic lymphoma (LPL); high grade: diffuse large B cell lymphoma (DLBCL), FL grade 3b or transformed FL subtypes (14). Written informed consent was obtained from all patients prior to patient recruitment and conducted in accordance with the Declaration of Helsinki. The study was ethically approved by the South Central Oxford A Ethics Committee (17/SC/0317), the UK Medicines and Healthcare Products Regulatory Agency (MHRA) and has Health Research Authority approval (IRAS 223132). All patients had relapsed or refractory B-cell lymphoma with no alternative curative options remaining. Study representation is detailed in Supplementary Table S1.

From January 2018 to December 2020, 27 participants were recruited from five hospitals situated in the United Kingdom. Participants were randomized to Arm A or B: all patients were treated with rituximab (375 $mg/m^2$ intravenously (iv)) on D1 of C1 to C6, and varlilumab (3 mg/kg iv) on D2 (Arm A) or D8 (Arm B) of C1, and D2 of C3 and C5 (all participants). Each cycle was two weeks in duration. Mandatory pre-treatment biopsies (within twelve months of study treatment) and where feasible, on-treatment biopsies (D7 or D8 i.e. before administration of D8 varlilumab in arm B) tumor biopsies were collected.

The primary objective was to assess the safety, tolerability and anti-tumor activity of combined rituximab and varlilumab. Secondary objectives included the duration of response, PFS and OS at one year post treatment completion.

## Tumor sample collection

All samples were collected in accordance with Good Laboratory Practice (GLP) and Good Clinical Practice (GCP) guidance on the maintenance of regulatory compliance in laboratories that perform analyses or evaluations of clinical trial samples, as well as the requirements of the UK

Clinical Trials regulations (SI 2044/1031 and SI 2006/1928 as amended), ICH GCP guidelines and the Declaration of Helsinki.

Twenty-two participants had a pre-treatment tumor biopsy, and 21 had on-treatment biopsies either after rituximab alone (9 participants, Arm B) or rituximab and varlilumab (12 participants, Arm A). Fresh tumor cores were placed in 5 mL RPMI supplemented with 1 mM sodium pyruvate, 2 mM glutamine, 1000 µg/mL penicillin, 0.1 mg/mL streptomycin (Gibco, New York) and 10% fetal calf serum, or 5 volumes of RNA*later*™ (ThermoFisher, Ambion, Inc.) buffer immediately and shipped to the central lab (Southampton Experimental Cancer Medicine Centre) overnight. Biopsies were processed for bulk RNA sequencing and in selected cases, by scRNAseq as described below.

**Peripheral blood collection**

Peripheral blood was collected prior to treatment and on-treatment (C1 D1, D2 and D8 and subsequently on D1 on C3-C6 and EOT) in 10 mL lithium heparin tubes (BD, New Jersey) for immunophenotyping by flow cytometry as described below.

In the first 6 participants of Arm A, peripheral blood was also collected in 10 mL serum tubes (BD, New Jersey) for analysis of rituximab and varlilumab pharmacokinetic (PK) analysis.

***METHOD DETAILS***

**Tissue dissociation**

Fresh cores of tumors cores collected in RPMI were minced into 1 $mm^3$ pieces or smaller using a scalpel and added to Liberase™ DL (Roche, Basel) RPMI for 15 minutes at 37 ºC on an orbital shaker at 250 rpm. The sample mixture was then passed through a 70 µm filter, washed four times, red cell lysis (Qiagen) undertaken, and washed twice more. Resultant single cell suspension was cryopreserved in 50% v/v human AB serum (Sigma, Burlington), 40% supplemented RPMI and 10% v/v DMSO (Sigma, Burlington).

**Peripheral blood flow cytometry processing and analysis**

Peripheral blood was thawed and diluted to 100,000 to 200,000 cells per tube, and then incubated the relevant antibody cocktail for T,B, NK cell and myeloid panels for 30 minutes in the dark (T, B, NK: CD45RA, CD56, CD8, CD3, CD19, CCR7 and CD27; myeloid cells: CD3, CD56, CD19, CD45, CD14, CD16, CD11c, HLA-DR, CD32b and CD11b). The Treg Staining Kit (ThermoFisher, Waltham) was utilized prior to antibody incubation as per manufacturer's instructions. Tregs were incubated with antibody cocktail for 40 minutes in the dark (Tregs: FOXP3, FOXP3 isotype, CD4/CD25 cocktail, CD27 and CD3). For T, B, NK cell and myeloid panels, cells were washed in FACS buffer. For the Treg panel, 5 µL FOXP3 was added without washing and further incubated for 40 minutes. Cells were then washed, as was done with the T, B, NK cell and myeloid panels. Acquisition was performed on the FACS Canto II.

**PK processing and analysis**

Peripheral blood collected in plain tubes were allowed to clot for 30-60 minutes at room temperature (RT). The tubes were then centrifuged for 15 minutes at 1500 rpm at 4 ºC and supernatant (serum) aspirated, aliquoted and frozen at -70 ºC and shipped to central labs on dry ice for processing of rituximab and varlilumab concentration quantification.

Quantification of rituximab (15) and varlilumab (11) concentration was undertaken by ELISA as previously described. Briefly, ELISA plates were incubated overnight at 4 ºC with 100 µL of rituximab in coating buffer (15 mM sodium carbonate, 28.5 mM sodium bicarbonate). Plates were blocked and then washed 3 times, with blocking buffer (0.05% PBS-Tween, 1% BSA (w/v)) and wash buffer (0.05% PBS-Tween) respectively. Samples and calibration standards were diluted with blocking buffer. Plates were covered and incubated at room temperature after adding calibration standards to relevant wells, diluted horseradish peroxidase-conjugated antibody (Abcam) to the remaining wells, and then washed three times with wash buffer. Substrate solution was added as per manufacturer's instructions and incubated at RT, away from light sources. After adding stop solution to each well, plates were read with the plate reader.

**Bulk-RNA sequencing and data analysis**

Total RNA was isolated and processed using the Maxwell® RSC simplyRNA Cells and simplyRNA Tissue kits (Promega, Madison) following manufacturer's instructions, and quantified and measured with Bioanalyser (Agilent Biosystems, Santa Clara, RRID:SCR_018043). Sample library preparation was performed and quality checked by the Oxford Genomics Centre (OGC). Adapter-ligated libraries were sequenced by OGC with a 150 bp paired-end read configuration, using the Illumina NovaSeq 6000. All sample sequencing was paired-end. Raw reads were converted to transcript-level counts and TPM (transcripts per million) using Kallisto (v0.46, RRID:SCR_016582) together with Gencode (v31, RRID:SCR_014966) human transcript reference. Counts and TPM were summarized at the gene level using tximport (v1.14, RRID:SCR_016752). Analysis was performed in R, using DESeq2 (v1.38, RRID:SCR_015687) for differential expression analysis, fgsea (v1.24, RRID:SCR_020938) for gene set analysis, and EnhancedVolcano (v1.16, RRID:SCR_018931) for visual representation of differentially expressed genes. CIBERSORTx was used for cell type deconvolution (16). Gene set analysis (17) was performed as follows: genes were ranked by average log fold change and pvalue and provided to the fgsea package, restricting to gene sets >=10 and <=1500 members. The C2 collection (v7.0) from msigDB was used, representing various curated pathways and experimental conditions. Resulting plots were created with ggplot2 (RRID:SCR_014601) in R.

**scRNA sequencing and data processing**

scRNAseq of pre- and on-treatment biopsies were performed using the 10X Genomics (Pleasanton, California) Chromium Single Cell 3' Library & Gel Bead Kit (chemistry version 3.1) as per protocol, from thawed single-cell suspensions. Samples were then sequenced on an Illumina NovaSeq 6000 sequencer by the OGC with a read configuration of 91 bp paired-end. Demultiplexing of sequencing results, barcode processing, read alignment, and UMI counting were performed using the 10X analysis pipeline by Cell Ranger (version 3.2) (18). First, Cell Ranger's mkfastq function was used to demultiplex libraries and convert base call files (sequencing reads) to FASTQ files. Next Cell Ranger's count function was used to perform alignment to the pre-built reference human genome GRCh38-2020A, filter and count unique molecular identifiers (UMI). Samples were further processed using Seurat (version 4.4, RRID:SCR_007322) (19), where cells expressing fewer than 200 or more than 9,000 genes and

more than 10% reads mapping to mitochondrial RNA were excluded from downstream analyses. All doublets that were identified using McGinnis DoubletFinder (20), were removed prior to downstream analyses. RNA counts from each sample were normalized using SCTransform (described by Hafemeister and Satija 2019, RRID:SCR_022146) (21) to ensure a standardized negative binomial regression based on the top 2000 most variable genes and regressed out covariates including cell cycle related genes.

To improve statistical power and facilitate comparative analysis between patient data, all samples were merged and integrated using Seurat's *FindIntegrationAnchors* and *IntegrateData* functions. This integrated dataset was clustered using the Louvain algorithm with resolutions ranging from 0.0 to 0.8 in steps of 0.1, with the optimal resolution selected based on output from clustree (RRID:SCR_016293) (22). Further analysis was performed predominantly in Seurat.

**Visualization, clustering, and identification of cell types**

Unsupervised graph-based clustering algorithm implemented in Seurat v4.4 was used to cluster single cells by their expression. Principal component analysis (PCA) was performed on 3,000 variable genes and the first 50 principal components were used for cell clustering with a resolution parameter of 1. Uniform manifold approximation and projection (UMAP) was performed on the first 27 PCs for visualization in two dimensions. To determine the immune cell populations present in the data, Seurat's *FindAllMarkers* function was utilized to identify differentially expressed genes in each subcluster. Specifically, the MAST algorithm was employed to take into account of expected zero inflation observed in scRNAseq datasets, as well as to incorporate cell-level covariates, such as the number of detected genes (cellular detection rate, CDR), to account for technical variation and cell heterogeneity (23). Only adjusted P values were utilized (at adj P value<0.05). Following this, the identities of immune cell populations (e.g. monocytes, dendritic cells, NK cells, T cells and B cells) were determined based on expression of canonical transcripts observed in output from *FindAllMarkers* and established literature.

**Evaluation of immune subset association with response, *CD27* expression and *CD27* coexpression**

Assessing relative differences in cell proportions per cluster and their association with a positive response (PR), or a negative response (NR), was performed using scProportionTest as described in provided tutorials (24). To evaluate relative *CD27* expression in individual cells per cell cluster, the presence of *CD27* transcripts, if any, per cell were denoted. Then, the average *CD27* count per *CD27* positive cell per cell cluster was calculated and plotted. Levels were given based on natural breaks in data visualization as established literature.

### Cell communication analysis

Potential ligand-receptor interactions were assessed with CellphoneDB version 5 (RRID:SCR_017054) (25). Interactions assessed were of CD27-positive cells/subsets (i.e. T, B and NK cells) split by clinical response (i.e. PR or NR). Because of results reported in previous work (6), macrophages were also included in this analysis. Interactions were filtered at P value<0.05 to identify significant interactions. The CellphoneDB supported ktplots package was utilized to visualize CellphoneDB data, specifically the sum of significant interactions. Significant ligand-receptor mean values generated from the CellphoneDB analysis were further analyzed with CrossTalkeR (26) to determine the direction of interactions, the most influential members (where P value<0.05) of the respective communication network and the ligands of interest per cell type. From this, further scrutinization of both NK and gamma-delta T populations was undertaken. To decipher communication mechanics mediated by CD27 agonism, NK and γδ T cells were further categorized into *CD27*-positive and *CD27*-negative (by transcript) subsets. Their interactions with malignant B cells were assessed and visualized with heatmaps, where only significant interactions were plotted (P<0.05).

## *STATISTICAL ANALYSIS*

### Clinical cohort sample size and analysis

The study was powered using a one-stage Fleming design; an overall response rate (ORR = complete response or partial response at end of treatment assessment using Lugano response criteria) of 13% (p1) and a 40% (p2) rate would mean the treatment warrant further investigation. Using a one-sided significance level of 5%, and 90% power, would require 20 participants in each

of the high- and low-grade subgroups; treatment Arms A and B were combined for analysis of clinical outcomes because the total number of planned doses of rituximab and varlilumab were the same in both arms, and the difference in the timing of varlilumab dosing in C1 was not expected not to influence clinical outcome. As the trial progressed, recruitment was slower than expected and further disrupted by the COVID-19 pandemic. As per the Independent Trial Steering Committee's recommendations, we evaluated whether a reduced sample size could still yield interpretable and meaningful results under the principles of the Fleming design. To this end, it was determined that a reduced sample size of 15 participants per subgroup, observing 5 or more responses (i.e. 33%) would still provide preliminary evidence of clinical benefit.

An *a priori* statistical analysis plan was devised for all clinical outcome analyses. All analyses are stratified by high- or low-grade subgroup and conducted using the intention to treat population, which includes all randomized participants who commenced study treatment.

The first primary endpoint, causality of each adverse event and grading of severity, is summarized by the number and percentage of participants experiencing each event. The second primary endpoint, overall response rate, is summarized as the number achieving an end of treatment response (complete response or partial response) divided by the number with an end of treatment response assessment with those who progressed or died before end of treatment counting as non-response, presented with 90% Clopper-Pearson exact confidence interval (to match the 5% 1-sided significance used in the sample size calculation). Each participant's response to treatment over time is shown using a swimmer plot. Duration of response over follow-up (up to one year from the end of treatment) was summarized using the Kaplan-Meier method. The change in largest lesion size and change in sum of product of diameters of target lesions from end of treatment and best response measurements are presented using waterfall plots. PFS and overall OS is summarized using Kaplan-Meier curves and median follow up duration is calculated using the reverse Kaplan-Meier method; one month is defined as 28 days. Median one-year PFS and OS rates are presented with 90% confidence intervals. Analyses were performed using SAS (version 9.4; Cary, NC) and STATA (version 16.0; College Station, TX, RRID:SCR_012763).

**Experimental data analysis**

Statistical analyses of translational endpoints were analyzed as described in each section above or via using non-parametric Mann Whitney or Kruskal Wallis tests using GraphPad Prism (version 10.2, RRID:SCR_002798; where statistical significance was set at P value < 0.05).

**Data and materials availability:**

All data pertaining to this work are in the manuscript or the supplementary data. Both raw and processed data for bulk RNA-seq and scRNAseq data generated in this study have been deposited and are publicly available in ArrayExpress (RRID:SCR_002964) under E-MTAB-14594 and E-MTAB-14585. No original code was used to analyze these data, except when using different parameters which have been detailed in the methods. Further information and requests should be directed to the corresponding author.

## Results

### Patients and treatment

Twenty-seven participants were enrolled into the RiVa trial: 16 participants with low-grade and 11 with high-grade B-NHL were randomly allocated to either Arm A or B (Fig. S1 and S2). In Arm A, 14 patients received rituximab on day 1 (D1) and varlilumab on day 2 (D2) of cycle 1 (C1) and Arm B, 13 patients received rituximab on D1 and varlilumab on D8 of C1. Treatment was identical for the remaining cycles in both arms, wherein rituximab was administered on D1 of C2-6 and varlilumab on D2 of C3 and C5. The rationale for the initial staggering the dosing of varlilumab between Arm A and B during C1 was to facilitate on-treatment biopsies in participants receiving either rituximab alone or rituximab in combination with varlilumab, as outlined in the Methods section. Each cycle was 14 days in duration. The proportion of high-grade cases in each arm was similar (A: 42.9% (6/14) vs B: 38.5% (5/13)).

Participant demographic and disease characteristics were similar in high- and low-grade groups (Table 1). The median age was 71.0 years (IQR, 62.0 – 77.0) and 59.3% of participants were males. Follicular lymphoma was the most frequent low-grade diagnosis (93.8%) and diffuse large B-cell lymphoma in high-grade cases (72.7%). In the entire cohort, 74% of participants had stage IV disease, and the median number of prior treatment lines was 4 (range, 1 to 13) before

study entry. Furthermore, 63% of participants had rituximab-refractory disease (i.e. disease which progressed on rituximab or relapsed within 6 months of rituximab treatment). Peripheral blood counts are shown in Tables S2 and S3, and peripheral blood and CIBERSORT fraction correlations are shown in Fig. S3.

**Safety and tolerability**

Forty-eight per cent (13/27) of patients received all six cycles of rituximab and all three cycles of varlilumab. Of the 14 patients who withdrew early from treatment, 10 were due to disease progression, 1 due to investigator withdrawal, 2 due to participant withdrawal and 1 due to a breast malignancy being discovered after one cycle. The median inter-cycle time was 14 days (IQR 14 to 14). There were no varlilumab dose reductions. There were 3 participants who required varlilumab dose-delays: 1 due to difficulties in intravenous cannulation, 1 due to non-hematological toxicity and 1 because the ward where treatment was going to be delivered, had to be closed.

Ninety-six per cent (26/27) of participants experienced at least one adverse event (AE) with 74.1% (20/27) experiencing at least a probable, possible or definite treatment-related AE (TRAE) (Table 2). The only TRAE reported by more than 10% of participants were infusion-related reactions (29.6%, 8/27). Sixteen (59.3%) participants had a grade 3 or above AE and in seven participants (25.9%), this was classified as a TRAE. In these 7 participants, 10 TRAEs were reported including 1 case of grade 3 infection, 1 wound infection, 2 cases of grade 3 neutropenia and 1 case of grade 3 febrile neutropenia and 2 cases of grade 3 dyspnea in the same patient. One of these cases was deemed probably related (neutropenia) and the remaining possibility related to treatment; no dose reductions or delays were required for them.

In one participant, a grade 1 pruritic erythematous and macular rash appeared the day after C1D1 rituximab, progressively worsening to grade 3 following the administration of C3. The patient was withdrawn from the study; a skin biopsy demonstrated a drug-induced spongiotic reaction. The rash resolved 100 days after onset. Two months later, the patient was hospitalized with neutropenic sepsis and subsequently developed Stevens-Johnson syndrome, leading to death. The participant had received a bispecific antibody targeting CD3 and CD20 after discontinuing rituximab and varlilumab, and this was considered to be the most likely cause of neutropenic

sepsis. Piperacillin-tazobactam was assigned as the cause of grade 5 Stevens-Johnson syndrome. However, the potential contribution of rituximab and varlilumab to the development of Stevens-Johnson syndrome cannot be ruled out.

## Clinical outcomes

Twenty-three participants had formal radiological disease assessment at the end of treatment. Of the remaining 4, all were withdrawn from treatment prior to completion because of disease progression after one, two and three cycles; and one after the first cycle because of discovery of a new breast malignancy.

Treatment arms A and B were combined for analysis of clinical outcomes because both arms received an identical total number of planned doses of rituximab and varlilumab. The variation in the timing of varlilumab dosing in C1 was intended for exploratory analysis and was not expected to impact clinical outcomes.

By the end of treatment (EOT), 4 participants had achieved a partial response (PR) (2 with low-grade and 2 with high-grade disease) and a further 4 had stable disease (SD) (all low-grade) (Fig. 1A, Table S4). No participants achieved a complete response. Each participant's progress in the trial is summarized in a swimmer plot (Fig. 1B). In summary, the overall response rate at EOT was 15.4% (90% CI 5.4-31.8), and disease control rate 30.8% (16.3-48.7). The median duration of response for partial responders was 9.8 months (range 1.9 months to >1 year); for those with SD, the median duration was 3.5 months (range 18 days to >1 year). Seventeen participants had rituximab-refractory disease – of which 2 achieved a PR and 3 had stable disease, producing a 29.4% disease control rate in this population. Improvement in response was observed in two participants during the follow up period: one participant with SD converted to PR at 2 months and another with PD converted to SD at 6 months.

The study was designed with a 1-year follow-up period after treatment completion. The median duration of follow up for survivors was 14.4 months. Nine patients died during study follow up: 7 from disease progression, 1 from a chest infection and 1 from sepsis and Stevens-Johnson syndrome. Twenty-three participants experienced disease progression. Amongst those with PR, two had rituximab-refractory disease: whilst one participant's disease progressed 9.8

months after initial response, the other continues to be in remission more than four years after treatment. Progression-free survival (PFS) at 12 months was 15% (90% CI 5.4-30.0); median PFS was 1.8 months (90% CI 1.2-3.3); overall survival (OS) at 12 months was 65% (90% CI 47.8-78.3). Median OS was not reached.

**Peripheral pharmacodynamic and pharmacokinetic analysis**

Next, we examined the effect of the addition of varlilumab to rituximab on peripheral blood immune cell subsets (Fig. S4-S6). Time points at which blood biopsies have been taken are shown in Fig. S7A. In general, rapid reduction of peripheral blood B cells was observed after administration of rituximab in both arms, but this was only statistically significant in Arm A - C1D1 to C3D1 median values: 13.47 to 0.08 x $10^6$/L in Arm A and 0.90 to 0.11 x $10^6$/L in Arm B) (Fig. S7B). No significant change was seen in NK (37.33 to 32.68 x $10^6$/L and 64.36 to 45.26 x $10^6$/L in in arms A and B, respectively) and CD8+ T cell numbers (49.84 to 65.25 x $10^6$/L and 46.72 to 39.87 x $10^6$/L in Arms A and B, respectively; Fig. S7C and S7D). However reductions in total CD4+ T cells (117.2 to 28.17 x $10^6$/L and 119.0 to 8.950 x $10^6$/L in Arms A and B, respectively) and T regulatory (Treg) cells were observed with subsequent cycles in both arms; statistical significance was only seen with total CD4+ T cells in Arm A (C1D1 vs EOT: 4.16-fold reduction in median values; Fig. S7E and S7F).

We examined whether clinical responses correlated with changes in peripheral blood immune subset counts or expression of CD27 or CD32b (an inhibitory Fcγ receptor which can down-modulate ADCP on myeloid cells (27)) (Fig. S8 and S9). PR cases were associated with higher proportions of peripheral CD4+ T cells and non-classical monocytes (28) in pre-treatment samples compared to PD cases (Fig. S8F and S9C). No significant differences were observed between CD27 expression on individual lymphocyte populations (Fig. S8K-P), or CD32b in pre-treatment samples (Fig. S9D-F).

Serum levels of rituximab and varlilumab (administered at 375 mg/$m^2$ every 2 weeks and 3 mg/kg every 4 weeks, respectively) were measured in the initial 6 to 8 Arm A participants to assess whether either mAb interfered with their respective pharmacokinetic profile (Fig. S10). Comparable mean serum levels of varlilumab were observed in this study compared to a previous report (29), ranging from 24.91 – 44.89 mg/mL at 30 mins post infusion. Varlilumab was

detectable in the serum of 4/6 participants 4 weeks after the administration of the first dose (mean 4.7 mg/mL).

Peak serum levels of rituximab after administration of the first dose ranged from 72.33 – 1340.91 mg/mL (mean 407.4 mg/mL) and the antibody was detectable two weeks after administration (mean 57.51, range 5.36-121.40 mg/mL), similar to previous reported rituximab pharmacokinetic data (30).

**Varlilumab induces intratumoral immune cell activation**

Matched pre- and on-treatment biopsies were available from 21 participants for bulk RNA sequencing: 9 from Arm A, where rituximab and varlilumab (RV) were administered before the on-treatment biopsy, and 12 from Arm B, where only rituximab (ritux alone) was administered prior to the on-treatment biopsy (varlilumab was given after the biopsy). This design allowed for the evaluation of varlilumab's immunological effects whilst ensuring that both mAbs were administered to all participants. CIBERSORTx deconvolution analysis was applied to estimate the immune cell composition in each biopsy. No significant differences were observed in immune cell fractions between the treatment arms or disease grade (Fig. S11), with B cell, T cell and monocyte/macrophage fractions dominating the fractional estimates in descending order (Fig. 2A). Changes in the proportion of B-cell and macrophage populations after treatment were strongly correlated - wherein greater intratumoral B-cell depletion was associated with greater macrophage infiltration (Spearman r =-0.65, P=0.0013) (Fig. 2B). No significant correlation was observed between intratumoral B-cell depletion and NK infiltration (P=0.09).

Next, changes in immune cell proportions pre- and on-treatment in rituximab and combination arms were compared (Fig. 2C-N). No changes in total B cells, macrophages, NK, Treg and CD8+ T cells were seen, but RV induced a 9.2-fold increase in CD4+ T cell infiltration compared to ritux alone (median 0.0176 vs -0.005, respectively; P=0.0079) (Fig. 2G and 2M).

Gene set enrichment analysis (GSEA) (17) was applied to the on-treatment patient biopsies from each treatment arm, revealing significant normalized enrichment scores (1.97 to 2.52; $P<0.05$) for T and B cell activation, FcγR activation and phagocytosis, and complement activation with RV. In contrast, no enrichment of immune cell activation gene signatures was observed in

the ritux alone arm (Fig. 2O and Table S5). These findings suggest that varlilumab elicits *in vivo* immunostimulatory activity in human B-cell lymphoma tumors.

**Intratumoral features associated with clinical response**

Next, we explored whether bulk or single cell RNA sequencing (scRNAseq) features correlated with clinical responses (Fig. 3). In the bulk RNAseq dataset, the level of intratumoral B-cell depletion in the on-treatment biopsies compared to pre-treatment biopsies correlated with subsequent clinical response; partial responders showed significantly greater B-cell reduction compared to PD cases (PR vs PD: median change -0.05 vs -0.007, P=0.026) (Fig. 3A and 3G). PR cases also demonstrated significantly higher NK (Fig. 3C and 3I, P=0.0319) and total CD4+ T cells (Fig. 3E and 3K; P=0.0098) in on-treatment biopsies compared to those with progressive disease. Using GSEA, we compared the signatures derived by bulk RNAseq between PR and PD cases; signatures associated with TCR, Th1 and interferon signaling pathways were enriched in PR cases (Fig. 3M and Table S6). In contrast, PD cases were enriched in B-cell receptor, phagocytic *FCGR*-mediated signatures.

To investigate intratumoral cell-specific changes, we performed scRNAseq in pre- and on-treatment biopsies from 3 cases with PR and 3 cases with no response (NR: 2 PD and 1 SD at EOT who converted to PD 2 months later). Four cases were available from Arm A (rituximab and varlilumab) and two from Arm B (rituximab) (Fig. 4A). Initially, eight broad immune cell types were identified, including B, T, NK and myeloid cells, which showed relatively similar distributions across all patients and disease groups (Fig. 4B and S12). T and NK cells were further re-clustered separately to characterize them in more detail, including scoring for αβ and γδ T cell canonical genes (31) (Fig. 4C and S13).

Given that CD27 is being targeted, we hypothesized that *CD27* mRNA expression level might correlate with subsequent treatment responses (Fig. 4D and S12C-E). PR cases tended to have more *CD27* expressing T and NK cells than NR cases (PR:NR fold change of: CD4+ naive (Tn): 7.1, CD8+ Teff: 3.2, CD8+ T exhausted (Tex): 1.9, TFH: 3.3, γδ T: 2.6, NK: 1.9 and Tregs: 4.7). NR cases, however, were associated with 2.4-fold more *CD27* expressing B cells (Table S7).

Next, we examined the gene expression of other costimulatory and inhibitory receptors on T and NK cell subsets between PR and NR cases (Table S8). For this, a panel of 72 established co-stimulatory genes underwent unsupervised clustering, forming three broad clusters, as shown in Fig. 4E. No differences in gene expression were observed in NK cells; however significant differences were noted between PR and NR cases in CD8+ T effector cells, a subset of follicular helper T cells with high *CXCL13* expression (henceforth referred to as TFHX13) (32) and most notably in γδ T cells. In γδ T cells, genes associated with cell-cell adhesion were upregulated in NR cases, while genes related to leukocyte activation were upregulated with PR cases. Specifically, γδ T cells in PR cases upregulated other TNFSF and TNFRSF members, including *TNFRSF9* (4-1BB), *TNRSF1B* (TNFR2), *CD70* and *HAVCR1*, *HAVCR2*, *CD80*, *TIMD4* and *CD2* (Fig. 4E and Table S7).

Cell communication analysis was performed to assess pivotal cell-cell interactions between PR and NR cases. Overall, the total number of significant interactions was greater in PR cases compared to NR cases (Fig. S14A), with an increased number of interactions in pre-treatment samples compared to on-treatment samples within the PR group. In pre-treatment PR samples, interactions involving macrophages, TFHX13, proliferating B, NK, CD8+ Tex, γδ T cells were dominant compared to NR cases ($P<0.05$) (Fig. S14B). Notably, in PR cases, multiple strong interactions were observed to arise from macrophages with TFHX13, proliferating B (suspected malignant B cells) and CD8+ Tex, γδ T and NK cells receiving signals. Fewer interactions were observed in on-treatment samples (Fig. S14C), but interactions between TFHX13, γδ T cell, NK and macrophages continue to be prominent in PR cases.

Given the prominence of γδ T and NK interactions in on-treatment PR cases and the variable expression of *CD27* expression on these cells (Fig. S12D), the analysis was repeated after separating NK and γδ T cells based on *CD27* expression (Fig. 4F). The following ligand-receptor pathways showed the highest correlation (based on LRScore) with on-treatment PR cases compared to NR cases: 1) *CCL5* to *CCR1* and *CCR5*, most notably from *CD27*+ γδ T cells but also from CD8+ Tex, and Teff and *CD27*- γδ T cells interacting with macrophages, CD8+ Teff and *CD27*+ γδ T cells as receiving cells, 2) *HLA-B* (on various cell types) interacting with *KIR3DL2* interaction (expressed exclusively on *CD27*+ NK cells), and 3) *PTPRC* (on various cell types) interacting with *CD22* on proliferating B cells (Fig. 4G-H, S14B-F and Table S9).

Communication analyses revealed that both NK and γδ T cells are key players in the cell-cell networks. In view of the prominent interactions and high page rank score observed in γδ T and NK cells in PR cases pre-treatment, we further investigated whether these cells are predicted to interact with malignant B cells (proliferating B cells) and normal B cells (as a comparator). Ranked mean ligand receptor scores (mean LRScore) at $P<0.05$ were categorized into functional groups, irrespective of clinical response group, and are presented in Fig. S14D. In PR cases, *CD27*+ γδ expressed more cell adhesion pathways (*CADM1-CADM1, PECAM1-CD38* and *VCAM1-integrin alpha4beta7 complex*). Inflammatory pathways (*CCL3-CCR1/5, CCL4-CCR5 and CCL5-CCR5)* were prominently featured between γδ T and NK cells and proliferating B cells in PR cases; these interactions were notably absent from NR cases and in normal B-cell interactions. Additionally, *CD27*+γδ T cells upregulated death-inducing pathways (*FASLG-FAS*, *TNFSF10-TNFRSF10A*) in interactions with proliferating B cells in PR cases, compared to NR cases. Altogether, these findings suggest that anti-CD27-dependent clinical anti-tumor responses may, in part be mediated by intratumoral CD27+ γδ T and NK cells.

Finally, as an orthogonal validation of varlilumab's ability to induce T-cell activation, the DEGs identified by scRNAseq, from participants treated with rituximab plus varlilumab vs rituximab alone, were compared with the leading-edge genes from bulk GSEA (Fig. 2O). Multiple T-cell activation-related genes were significantly upregulated (adj $P<0.05$) in both datasets – including *IL6R, PTPRC, ITK, APBB1IP, RHOH, STIM1, FYB1, CD28, DOCK2, FYN, JUN, PRKCB, AKT3* – despite the cohorts being independent (Table S10).

## Discussion

Cancer immunotherapy has seen groundbreaking improvements over the past two decades (33). It is evident that the immune system can be harnessed to restore its anti-tumor activity. However, all current clinically approved anti-cancer antibodies primarily fall into two mechanistic groups: those that directly target the tumor cells and those that block immune checkpoint receptors. Despite promising preclinical data, agonist antibodies have shown only modest anti-tumor activity in patients, with none yet achieving clinical approval (1). But the pursuit of clinically efficacious agonist anti-tumor antibodies should not be abandoned. The limitations of direct-targeting and

checkpoint-inhibiting antibodies – both in efficacy and consistency of responses across malignancies and individuals - highlighting the need for improved approaches (1,33).

Seventy-four percent of our cohort was rituximab-refractory, defined as having disease progression during treatment or relapse within six months after completing a rituximab-containing regimen. In this setting, rituximab monotherapy would not be considered clinically appropriate; therefore, there are no directly relevant published studies available for comparison. However, for context, Sehn et al (34) reported an overall response rate of 33.3% in patients with relapsed/refractory CD20+ low grade lymphoma (n=75) in rituximab-responsive patients with a median of 2 prior lines of treatment (compared to 4 lines of treatment in our study).

Here, not dissimilar to previous studies involving T-cell agonists (1), we observed a modest overall response rate (15.4%) in a cohort of 27 participants treated with rituximab and varlilumab. This improves upon the results of a varlilumab monotherapy study which reported a 0% response rate (3 participants with SD) in 18 participants with B-NHL (29); the improved response rate might be due to co-administration of rituximab but 74% of the population had rituximab-refractory disease and responses were still observed in these participants (11.8%). Further, like Ansell *et al* (29), this population was heavily pre-treated - having been exposed to a median of four previous lines of treatment. Four participants had a PR and in 1 participant, sustained response was observed beyond four years, suggesting that varlilumab can contribute to clinically significant anti-tumor activity in a subset of individuals.

Previous studies demonstrated that varlilumab can be safely administered as a monotherapy and in combination with anti-PD-1 (11,29,35). Whilst safety and tolerability were primary endpoints in RiVa, our expectation was that combined rituximab and varlilumab administration would not change its safety profile because of the antibodies' different modes of action. The overall safety profile is similar to that reported for rituximab alone with infusion-related reactions and fatigue being the commonest adverse events (34). Most participants (74%) experienced grade 1 and 2 TRAE and 7 participants reported no TRAE; the commonest events were infusion-related reactions observed in nearly 20% of participants, fatigue in 15%, and rash, dyspnea and nausea in ~10%. Grade 3 TRAE were reported in 6 participants (~20%) but only 1 required a dose delay and none required a dose reduction. A grade 5 TRAE was observed; Stevens-Johnson syndrome, a rare, severe hypersensitivity reaction involving skin and mucous membranes that is primarily

caused by drugs (36). The underlying disease mechanism is unclear but drug-specific CD8+ cytotoxic T-cells have been implicated. The contribution of varlilumab to development of Stevens-Johnson syndrome is unclear. The participant had received a CD20/CD3 bispecific antibody and antibiotics immediately prior to development of Stevens-Johnson syndrome compared to varlilumab two months previously. It is certainly possible that rituximab and varlilumab may have primed the participant's CD8+ T cells, predisposing the patient to a hypersensitivity reaction with subsequent drug administration.

The initial staggering of varlilumab dosing (administered on D2 of C1 in Arm A and D8 of Arm B) combined with the availability of pre- and on-treatment biopsies, allowed us to examine the intratumoral effects of varlilumab administration with and without rituximab. We reasoned that this variation in varlilumab dosing during C1 would not have a clinically significant impact, since dosing was identical in C2-6. However, all four partial responders were from Arm A. While this may be due to chance given the small sample size, it is also possible that the closer timing of rituximab and varlilumab (D2 following D1) in Arm A enhanced T-cell priming, and that this initial priming step may be more important than previously appreciated.

By bulk RNAseq, evidence of varlilumab agonism was indicated by increased intratumoral infiltration of NK and CD4+ T cells, and activation of T-cell receptor mediated pathways by GSEA, neither of which were observed with rituximab alone. Aligned with our previous murine studies, we also observed activation of FcγR and phagocytosis (6). However, FcγR activation may also be explained by cross-linking of CD27 and FcγR by varlilumab, an IgG1 antibody. On the other hand, intratumoral infiltration of myeloid populations or CD8+ T cells were not evident as observed in mice. This may be accounted for by differences in biopsy timing or drug kinetics between the animal and human studies. However, strong correlation was observed between intratumoral macrophage and B cell abundance, wherein more B-cell depletion was observed with increased macrophages, supporting previous observations suggesting macrophages as the primary effectors of rituximab-mediated B-cell depletion (37). Altogether, the data aligns with our preclinical hypothesis - CD27 agonism can stimulate T cells to activate macrophages to mediate rituximab-dependent tumor cell phagocytosis.

Upregulation of complement activation genes were also detected by GSEA following the addition of varlilumab. Varlilumab contains a wild-type functional human IgG1 Fc that can engage

C1q, suggesting potential for complement-dependent cytotoxicity (CDC). However, CDC was not observed when varlilumab was co-cultured with B-cell lymphoma cell lines in vitro (38). Rituximab is known to induce CDC (2), so the combination of rituximab and varlilumab may have enhanced CDC-mediated killing of tumor cells beyond what is seen with rituximab alone. In addition, varlilumab may contribute to direct tumor killing through other mechanisms. In murine models lacking T and B cells, varlilumab inhibited growth of human B-cell lymphoma cells, and also mediated ADCC in vitro.

We analyzed the immunological changes underlying clinical response or its lack of, to uncover the mechanisms driving response variability. Increased proportions of CD4+ T cells were associated with partial responders in pre-treatment peripheral blood and tumor. This population comprised mainly CD27+ cells and thus may be preferentially targeted by anti-CD27. Whilst CD27 expression levels on CD4+ T cells did not correlate with clinical response per se, scRNAseq analysis showed that PR cases had more *CD27* mRNA expressing T cells. Conversely, NR cases showed more *CD27*-expressing B cells. The expression of CD27 on normal memory B cells and B-NHL cells is well-described but the functional consequence of receptor ligation is less clear. On normal B cells, *in vitro* CD27 stimulation led to increased antibody production, plasma cell differentiation and CD70 expression (39-41). It is further hypothesized that CD70-CD27 (on T cells) interaction might enhance germinal center proliferation. Thus, in addition to B cells posing as a sink for varlilumab, there is a theoretical risk that the antibody might drive malignant B-cell proliferation, however no significant effects were observed on circulating B cells in previous clinical studies of varlilumab (11,29,35). Our GSEA analyses from bulk RNAseq showed upregulation of B-cell receptor pathway signatures but there was significant increase in B-cell fractions on CIBERSORTx analysis. Further single-cell analysis of the malignant B-cell population was impeded as VDJ sequencing was not undertaken to differentiate normal from malignant B cells.

No correlation was observed between the number of CD4+ T or NK cells in pre-treatment samples and clinical responses in the scRNAseq dataset. This may have been due to the low number of single-cell cases sequenced. However, gene expression and cell communication analyses across immune cell subsets yielded intriguing findings. In pre-treatment samples from PR cases, macrophages and TFHX13 cell were particularly prominent. Both cell types are known to support tumor growth in lymphoma (42,43). Their association with PR cases suggest that these

tumors may be more depending on support from surrounding immune cells than those in NR cases, potentially making them more susceptible to eradication through immunomodulation. In addition, we observed an unexpected influence from γδ T cells in our scRNAseq data – an interesting observation given the previous described association between γδ T cells and favorable outcomes across 25 malignancy types (44) and more recent observations on the role of γδ T cells in influencing response to checkpoint inhibition (45). γδ T cells segregated with response on analysis of co-stimulatory and inhibitory genes and contributed significantly to the cell communication analysis. Numerous genes associated with the regulation of leucocyte activation genes were increased in γδ T cells of pre-treatment, responder cases including *TNFRSF1B* (TNFR2), *TNFRSF9* (4-1BB), *CD27*, *PDCD1* (PD-1) and *LAG-3*. By cell communication analysis, upregulation of *CCL3*, *CCL4*, *CCL5* and *IFNG* pathways were observed in the CD27+ γδ T cell fraction of PR cases. These genes were also upregulated in CD8+ effector T cells in our earlier preclinical B-cell lymphoma studies (6). Altogether, we hypothesize that the presence of activated, intratumoral γδ T cells might contribute to an effective, anti-tumor response by anti-CD27 agonists. Aligned with this, De Barros *et al* (46) previously demonstrated that co-culture of γδ T cells with CD70 promoted survival, proliferation and secretion of Th1 cytokines. However, these data require cautious interpretation and validation as our scRNAseq was undertaken from the 3' end, resulting in suboptimal sequencing of TCR genes. Further, these data are limited by its small sample size.

There are several possible reasons why only modest clinical efficacy is observed with varlilumab (and rituximab) despite evidence of intratumoral agonism. First, the dosing of varlilumab may have been suboptimal to drive effective T-cell agonism. The first-in-human study of varlilumab (11) demonstrated that 1 mg/kg saturated receptors on T-cells for at least a month. CD27 receptor occupancy was not evaluated in RiVa, and whilst the mean serum level did not differ vastly from previous studies (11,29) (4.7 mg/mL vs ~ 10 mg/mL), serum varlilumab was undetectable in 2/6 participants four weeks after administration, suggesting that the patients may have benefited from a more frequent dosing schedule than 4-weekly. Second, deepening of clinical responses were observed beyond the end of treatment assessment (4 weeks after administration of cycle 6 drugs), indicating that additional cycles of treatment may have induced a higher response rate. Third, our preclinical studies demonstrate that varlilumab is a relatively weak agonist. It is an IgG1 antibody, which has higher affinity for activating Fcγ receptor cells, leading to potential

depletion of CD27-expressing effector T cells. The first-in-human study of varlilumab (11) reported a trend towards peripheral Treg depletion and this was similarly observed in RiVa with peripheral Tregs and CD4+ T cells. No significant depletion was observed with peripheral CD8+ T cells or intratumoral T cells, which may be accounted for by differences between the on-biopsy treatment timing (a week after varlilumab) compared to repeated peripheral blood sampling until the end of treatment. Further, varlilumab binds to a more membrane-proximal epitope than some other clones - this prevents it from rapidly dissociating and binding to other trimers to form higher order oligomers, to trigger receptor signaling (8). Altogether, we propose that future studies should explore either: 1) the use of more potent CD27 agonist antibodies - achievable through Fc-engineering, increased valency or alternative epitope targeting - which may overcome the dependence on high levels of CD27 expression; or 2) selection of patients with greater intratumoral CD27-expressing T cells to improve clinical responses. Further, given anti-CD27 agonists can augment Fc-mediated immune effector killing and boost T-cell stimulation, its combined use with other anti-CD20 antibodies such as obinutuzumab (34) or CD20/CD3-engaging bispecific antibodies (47) may be worth clinical exploration.

Our study has several limitations. Although clinical investigators were asked to consider pseudoprogression due to immune cell infiltration as a potential explanation for tumor enlargement, there was no requirement for follow-up imaging or biopsy to confirm this. As a result, pseudoprogression could not be definitively ruled out as a cause of early disease progression. The RiVa trial included a small sample size, with only three matched pre- and on-treatment cases analyzed by scRNAseq. This reflects the technical challenges of obtaining high-quality, viable single-cell tumor biopsies. Nevertheless, robust methodologies underpin our data: 6,697-11,230 cells (median 10,511) were profiled per sample, ensuring strong statistical power at the single-cell level, multi-sample integration to increase resolution and control for sample-level variability. Similar small-scale single-cell cases have successfully generated initial valuable insights (48-50). Importantly, key findings, such as the association between pre-treatment T-cell activation signatures and response to treatment, were also observed in a larger cohort of bulk RNAseq data (n=21), lending support to the scRNAseq results. While we acknowledge that the single-cell data are exploratory and require further validation, this study offers the only available dataset to date characterizing the intratumoral effects of CD27 stimulation in patients. These mechanistic insights

will inform the clinical development of CD27 and other TNFRS agonists, for which more than 65 trials are currently ongoing (51).

Our findings demonstrate that CD27 agonist antibodies, such as varlilumab, can overcome the immunosuppressive tumor microenvironment to drive T-cell activation - particularly in individuals with pre-existing immune-primed tumors – leading to meaningful anti-tumor responses. These results parallel those observed with immune checkpoint inhibitors, where immune-inflamed tumors correlate with better clinical responses (52,53). Moreover, unlike strategies solely focused on enhancing anti-tumor CD8+ T-cell cytotoxicity, we validate our previous murine data, showing that T-cell stimulation can be leveraged to augment antibody-dependent cellular phagocytosis by tumor-depleting antibodies.

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**Author contributions:**

S.H.L. was responsible for the conceptualization, design, funding acquisition, participant enrolment and treatment, data analysis and drafted the manuscript, L.E.B. and M.J.R-Z. analyzed translational data and drafted the manuscript, A.H.T. and A.C. processed samples and analyzed data, A.G. analyzed data, P.McK., W.O., K.L., P.M., R.L., A.J.D., P.W.M.J. and G.P.C. enrolled and treated patients, T.K., M.Y. and G.G. contributed to study design, L.S. analyzed clinical data and drafted the manuscript, K.T analyzed clinical data, JC, C.W., N.K. and Z.K. collected and analyzed clinical data. M.S.C and A.A contributed and adjusted the manuscript. All authors were involved in writing and revising the manuscript. All authors have full access to the data, participated in reviewing and approved the final version prior to submission. S.H.L., L.E.B., G.G. and L.S. verified the raw data and had final responsibility for the decision to submit the manuscript for publication.

**List of Supplementary Materials**

RIVA_Paper_Supplemental_Information.pdf. *Figures S1–S14* and *Tables S1-S10*

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# FIGURE LEGENDS

## Table 1. Baseline demographic and disease characteristics of participants

| | Low Grade B-NHL (*n* = 16) | High Grade B-NHL (*n* = 11) | Total (*N* = 27) |
|---|---|---|---|
| Median age, years (range) | 68.0 (54.0-80.0) | 71.0 (49.0-87.0) | 71.0 (49.0 – 87.0) |
| Gender, *n* (%)<br>Male<br>Female | <br>9 (56.3)<br>7 (43.8) | <br>7 (63.6)<br>4 (36.4) | <br>16 (59.3)<br>11 (40.7) |
| Mean body mass index, $kg/m^2$ (range) | 26.0 (19.4-35.1) | 26.9 (20.0-36.4) | 26.4 (19.4-36.4) |
| Lymphoma classification, *n* (%)<br>Mantle cell lymphoma<br>Follicular lymphoma (grade 1,2 or 3a)<br>Follicular lymphoma (grade 3b)<br>Transformed follicular lymphoma<br>Diffuse large B-cell lymphoma | <br>1 (6.3)<br>15 (93.8)<br>0<br>0<br>0 | <br>0<br>0<br>2 (18.2)<br>1 (9.1)<br>8 (72.7) | <br>1 (3.7)<br>15 (55.6)<br>2 (7.4)<br>1 (3.7)<br>8 (29.6) |
| Stage, *n* (%)<br>I<br>II<br>III<br>IV | <br>0<br>1 (6.3)<br>3 (18.8)<br>12 (75.0) | <br>0<br>0<br>3 (27.3)<br>8 (72.7) | <br>0<br>1 (3.7)<br>6 (22.7)<br>20 (74.1) |
| Presence of B symptoms, *n* (%)<br>Yes<br>No | <br>1 (6.3)<br>15 (93.8) | <br>2 (18.2)<br>9 (81.8) | <br>3 (11.1)<br>24 (88.9) |
| Presence of extranodal disease, *n* (%)<br>Yes<br>No | <br>11 (68.8)<br>5 (31.3) | <br>8 (72.7)<br>3 (27.3) | <br>19 (70.4)<br>8 (29.6) |
| ECOG Performance status, *n* (%)<br>0<br>1<br>2 | <br>5 (31.3)<br>9 (56.3)<br>2 (12.5) | <br>5 (45.5)<br>5 (45.5)<br>1 (9.1) | <br>10 (37.0)<br>14 (51.9)<br>3 (11.1) |
| Median number of prior systemic treatments (range) | 4.0 (1.0-13.0) | 3.0 (1.0-7.0) | 4.0 (1.0-13.0) |
| Response to last line of treatment, *n* (%)<br>Relapsed<br>Refractory | <br>12 (75.0)<br>4 (25.0) | <br>8 (72.7)<br>3 (27.3) | <br>20 (74.1)<br>7 (25.9) |
| Rituximab-refractory, *n* (%)<br>Yes<br>No | <br>9 (56.3)<br>7 (43.8) | <br>8 (72.7)<br>3 (27.3) | <br>17 (63.0)<br>10 (37.0) |

[1] *Race/ethnicity or geography was not collected for this trial*

960 **Table 2. Overall safety summary of AEs experienced by participants**

| | Low Grade B-NHL (n=16) | High Grade B-NHL (n=10) | Total (n=26) |
|---|---|---|---|
| Number of participants who experienced at least one AE, *n* (%) | 16 (100) | 10 (90.9) | 26 (96.3) |
| Number of participants who experienced at least one TRAE, *n* (%) | 12 (75.0) | 8 (72.7) | 20 (74.1) |
| Number of participants affected by the following TRAEs, *n* (%)[1] | | | |
| Neutropenia | 1 (6.3) | 1 (9.1) | 2 (7.4) |
| Nausea | 2 (12.5) | 1 (9.1) | 3 (11.1) |
| Fatigue | 2 (12.5) | 2 (18.2) | 4 (14.8) |
| Infusion-related reaction | 5 (31.3) | 3 (27.3) | 8 (29.6) |
| Blood alkaline phosphatase increased | 0 | 1 (9.1) | 1 (3.7) |
| Dyspnea | 2 (12.5) | 0 | 2 (7.4) |
| Pruritis | 1 (6.3) | 0 | 1 (3.7) |
| Rash | 2 (12.5) | 1 (9.1) | 3 (11.1) |
| Worst grade of AE experienced in each participant, *n* (%) | | | |
| Grade 1 | 2 (12.5) | 1 (9.1) | 3 (11.1) |
| Grade 2 | 3 (18.8) | 4 (36.4) | 7 (25.9) |
| Grade 3 | 8 (50.0) | 3 (27.3) | 11 (40.7) |
| Grade 4 | 2 (12.5) | 0 | 2 (7.4) |
| Grade 5 | 1 (6.3) | 2 (18.2) | 3 (11.1) |
| No AE | 0 | 1 (9.1) | 1 (3.7) |
| Worst grade of TRAE experienced in each participant, *n* (%) | | | |
| Grade 1 | 3 (18.8) | 3 (27.3) | 6 (22.2) |
| Grade 2 | 5 (31.3) | 2 (18.2) | 7 (25.9) |
| Grade 3 | 3 (18.8) | 3 (27.3) | 6 (22.2) |
| Grade 4 | 0 | 0 | 0 (0.0) |
| Grade 5 | 1 (6.3) | 0 | 1 (3.7) |
| No TRAE | 4 (25.0) | 3 (27.3) | 7 (25.9) |
| Nature of grade ≥3 TRAE experienced in each participant, *n* (%) | | | |
| Infection | 1 (6.3) | 0 | 1 (3.7) |
| Neutropenia | 1 (6.3) | 1 (9.1) | 2 (7.4) |
| Febrile neutropenia | 0 | 1 (9.1) | 1 (3.7) |
| Dyspnea | 1 (6.3) | 0 | 1 (3.7) |
| Pruritis | 1 (6.3) | 0 | 1 (3.7) |
| Rash | 1 (6.3) | 0 | 1 (3.7) |
| Stevens-Johnson syndrome | 1 (6.3) | 0 | 1 (3.7) |
| Wound infection | 0 | 1 (9.1) | 1 (3.7) |

961 [1] *TRAEs occurring in 5% or fewer participants are not shown*

962 **Figure 1. Change in size of tumor burden from baseline to end of treatment**

963 **(A)** Tumor burden is represented by % change in sum of perpendicular diameters (SPD) of
964 measured legions from pre-treatment to end of treatment. Assigned clinical response is denoted by
965 the different colors. Blue stars denote participants that were rituximab-refractory prior to trial
966 entry. **(B)** Swimmer plot of recruited patients and their response to treatment over time, detailing
967 disease grade, final response achieved and any recorded deaths.

968

969 **Figure 2. Effects of rituximab +/- varlilumab on intratumoral immune cell composition**

970 **(A)** Tumors were biopsied prior to treatment, RNA sequenced, and data deconvolved using
971 CIBERSORTx. Shown here are the CIBERSORTx proportions, represented as a percentage, of
972 each cell type, in Arm B (rituximab, ritux), and Arm A (combination of rituximab and varlilumab,
973 RV). **(B)** Correlation between the change in total macrophages (Y axis) and total B cells (X axis)
974 is shown. ***P<0.001 by Pearson correlation,***P<0.001. **(C-N)** As in (A) but comparisons
975 between pre- and on-treatment biopsies were performed in each treatment arm. (C-H) show the
976 matched patient CIBERSORTx fractions for pre-treatment and on-treatment in total B cells (C),
977 total macrophages (D), total NK cells (E), total CD8+ T cells (F), total CD4+ T cells (G) and
978 regulatory T cells (H). (I-N) show the change (difference between on-treatment and pre-treatment
979 value) in matched CIBERSORTx fractions of different cell types between pre- and on-treatment
980 biopsies for each treatment arm: total B cells (I), total macrophages (J), total NK cells (K), total
981 CD8+ T cells (L), total CD4+ T cells (M) and regulatory T cells (N). **(O)** Gene set enrichment
982 analysis from on-treatment biopsies in patients treated with rituximab and varlilumab compared to
983 rituximab alone, demonstrates enrichment in T cell activation, B cell activation, FcγR activation
984 and phagocytosis, and complement activation. Gene sets are colored by (publication) source.

985

986 **Figure 3. The biological parameters between cases based on clinical response**

987 **(A-L)** Comparisons between pre- and on-treatment biopsies in progressive disease (PD), stable
988 disease (SD) and partial response (PR) in different CIBERSORTx fractions. (A-F) show the
989 matched patient CIBERSORTx fractions in pre-treatment and on-treatment for each response
990 group: total B cells (A), total macrophages (B), total NK cells (C), total CD8+ T cells (D), total
991 CD4+ T cells (E) and regulatory T cells (F). (G-L) show the change (difference between on-
992 treatment and pre-treatment value) in matched CIBERSORTx fractions of different cell types

between pre- and on-treatment biopsies for each response group: total B cells (G), total macrophages (H), total NK cells (I), total CD8+ T cells (J), total CD4+ T cells (K) and regulatory T cells (L). Mann-Whitney (PR vs PD) *P<0.05, **P<0.00. (M) The gene set enrichment analysis from pre-treatment biopsies in PR compared to PD. Gene sets are colored by publication source.

**Figure 4. Analysis of single-cell transcriptomes based on clinical response**

**(A)** The illustration depicting the cohort of 6 participants' (3 partial responders (PR) and 3 non-responders (NR)) pre- and on-biopsies analyzed by scRNAseq. **(B)** UMAP projection of all cells clusters from PR and NR cases, where each dot is a cell, and each color denotes a cluster. Clustering depicts eight broad cell types. **(C)** UMAP projection of the further subdivisions of T lymphocytes from PR cases and NR cases; inset image shows CD4:CD8 expression where increased ratio correlates with CD4 expression (dark orange) and decreased expression ratio correlates with CD8 expression (light orange). **(D)** Feature plot showing the density of *CD27* transcripts in PR, NR, pre- and on-treatment biopsies. Each cell is represented by a dot. The higher the density of CD27+ cells (i.e. cells with CD27 transcripts), the darker the region. **(E)** shows K-means clusters of scaled average expression of candidate co-stimulation and inhibitory receptor genes in T cell and NK subsets. 72 candidate T cell co-stimulation genes formed 3 clusters with differential biological activities. $-\log_{10}$(Bonferroni adjusted P values) are shown for gene ontology enrichment. *C-2 includes *CD27* and *CD70* genes. Gene expression is z-score scaled. **(F)** Significant cell-cell interactions in comparative analysis between NR on-treatment (blue arrows) and PR on-treatment (orange arrows) biopsies. The cell types with significant contribution are illustrated by the nodes, and the size of the node denotes their importance in the overall network. Arrow thickness defines the percentage of cells interacting from that node. **(G)** and **(H)** show Sankey plots depicting ligand to receptor interactions between different cell types via either CCL5 (ligand) to CCR5 (receptor) (G) or PTPRC (ligand) and CD22 (receptor) (H) in a comparative analysis between NR and PR on-treatment biopsies. Sankey plots show interactions from a given source cell type (the cell producing the signal) to target cells (the cell receiving the signal) via the specified ligand-receptor interaction. Links in orange depict interactions more prominent in PR, and those in blue are more prominent in NRs. The scale indicate a ligand-receptor score (LRScore) as defined by CrossTalkeR.