TRANSLATIONAL SCIENCE

Association of baseline soluble immune checkpoints with the risk of relapse in PR3-ANCA vasculitis following induction of remission

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/ard-2022-222479).

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Received 15 March 2022
Accepted 2 August 2022

To cite: Gamerith G, Mildner F, Merkel PA, *et al*. *Ann Rheum Dis* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/annrheumdis-2022-222479

ABSTRACT

Objectives We investigated whether soluble immune checkpoints (sICPs) predict treatment resistance, relapse and infections in patients with antineutrophil cytoplasm antibody (ANCA)-associated vasculitis (AAV).

Methods Plasma sICP concentrations from available samples obtained during conduct of the RAVE trial were measured by immunoabsorbent assays from patients with either proteinase 3 (PR3) or myeloperoxidase (MPO)-ANCA vasculitis and were correlated with clinical outcomes, a set of biomarkers and available flow cytometry analyses focusing on T cell subsets. Log-rank test was used to evaluate survival benefits, and optimal cut-off values of the marker molecules were calculated using Yeldons J.

Results Analysis of 189 plasma samples at baseline revealed higher concentrations of sTim-3, sCD27, sLag-3, sPD-1 and sPD-L2 in patients with MPO-ANCA vasculitis (n=62) as compared with PR3-ANCA vasculitis (n=127). Among patients receiving rituximab induction therapy (n=95), the combination of lower soluble (s)Lag-3 (<90 pg/mL) and higher sCD27 (>3000 pg/mL) predicted therapy failure. Twenty-four out of 73 patients (32.9%) in the rituximab arm reaching remission at 6 months relapsed during follow-up. In this subgroup, high baseline values of sTim-3 (>1200 pg/mL), sCD27 (>1250 pg/mL) and sBTLA (>1000 pg/mL) were associated with both sustained remission and infectious complications. These findings could not be replicated in 94 patients randomised to receive cyclophosphamide/azathioprine.

Conclusions Patients with AAV treated with rituximab achieved remission less frequently when concentrations of sLag-3 were low and concentrations of sCD27 were high. Higher concentrations of sTim-3, sCD27 and sBTLA at baseline predicted relapse in patients treated with rituximab. These results require confirmation but may contribute to a personalised treatment approach of AAV.

WHAT IS ALREADY KNOWN ON THIS TOPIC?

⇒ Disease recurrence is common in patients with antineutrophil cytoplasm antibody (ANCA)-associated vasculitis, especially in those with proteinase 3 (PR3)-ANCA positivity, ear, nose and throat involvement, normal kidney function, *Staphylococcus aureus* nasal carriage, a rise in ANCA titres, a negative-to-positive transition of the ANCA test, and treatment choice and duration.

⇒ Besides PR3-ANCA vasculitis, recurrence of $CD19^+$ B cells and return of ANCA positivity, robust biomarker predicting relapse in patients undergoing rituximab induction therapy has not been identified in previous studies.

WHAT THIS STUDY ADDS?

⇒ This exploratory study first identified high sCD27 and low sLag-3 at baseline as promising biomarkers for primary resistance to rituximab in ANCA-associated vasculitis (AAV).

⇒ In patients with AAV treated with rituximab and achieving remission, lower concentrations of sTim-3, sCD27 and sBTLA were linked to disease relapse and to lower infection rates.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY?

⇒ Early risk stratification based on easy to measure baseline soluble immune checkpoint patterns will help to identify individuals with primary rituximab resistance, later disease recurrence and infectious complications. However, our finding requires confirmation in independent prospective cohorts, before using this in daily clinical practice.

INTRODUCTION

ANCA-associated vasculitis (AAV) is characterised by distinct clinical phenotypes, granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis and can further be distinguished by the presence of either antibodies to proteinase 3 (PR3) or myeloperoxidase (MPO).[1] In spite of improved management and new therapies, up to 38% of patients with AAV fail to achieve remission at 6 months[2] and up to 89% experience a relapse at 5 years of follow-up,[3] depending on the definition of remission and the therapies used to maintain remission and the duration thereof. The identification of predictors of failure to induce remission

or relapse according to treatment is relevant to treatment selection and follow-up. Recognised risk factors for relapse include disease parameters such as PR3-ANCA vasculitis, GPA, higher presenting estimated glomerular filtration rate, *Staphylococcus aureus* nasal carriage, ANCA positivity at the time of completion of induction therapy, an ANCA titre rise or a negative-to-positive transition and previous relapses[3 4] and management parameters including the choice of induction and maintenance therapy.[3] The recurrence of ANCA after negativity and B-cell recurrence are predictors of relapse in PR3-ANCA vasculitis following rituximab (RTX) induction therapy,[5] but more accurate predictors are needed.

The Rituximab vs Cyclophosphamide for ANCA-Associated Vasculitis (RAVE) trial found non-inferiority of RTX compared with a combination of cyclophosphamide (CYC) for induction followed by azathioprine for the maintenance of remission.[2] Unsupervised analysis of serum samples indicated that CXCL13, matrix metalloproteinase-3 and tissue inhibitor of metalloproteinase-1 best discriminated active AAV from remission.[6] Such translational findings are pivotal to better understand disease pathogenesis. However, different analyses and/or biomarkers may be needed to predict disease recurrence following remission. In RAVE, 30.1% of patients achieving remission at 6 months relapsed during the follow-up period of 18 months.[7] To date, circulating biomarkers predictive of relapse have not been identified in prospective studies and would be valuable in determining which patients would eventually profit from maintenance therapy concepts.

Analysis of the transcriptome in autoimmune diseases, including AAV, has revealed a CD8 T-cell exhaustion signature is associated with improved relapse-free survival (RFS).[8] T-cell exhaustion is characterised by increased surface expression of immune checkpoint (ICP) molecules programmed cell death protein 1 (PD-1) and lymphocyte activation gene 3 (Lag-3).[8 9] Membrane-bound ICPs, such as membrane-bound T-cell immunoglobulin and mucin domain 3 (mTim-3) and mLag-3, have inhibitory functions, their expression on T cells dampens effector T-cell response, and its blockade worsens autoimmunity,[9 10] while others are mainly involved in immune system activation such as mCD27.[11] Soluble forms of these immune checkpoints (sICPs) are alternatively spliced or cleaved from the surface of immune cells implicated in the pathogenesis of AAV, such as $CD4^+$ and $CD8^+$ T cells and B cells.[9 12] Little is known about specific biological functions of sICPs, and specifically in vasculitis where a tight regulation of immune system homeostasis (activation vs inhibition) is needed to balance between disease control and infectious complications. In this context, reversal of T-cell exhaustion by immune checkpoint inhibitors (ICIs) has been linked to new onset of vasculitis.[13]

This study measured plasma concentrations of 14 sICPs in PR3- and myeloperoxidase (MPO)-ANCA vasculitis to determine if they were associated with outcomes in the RAVE trial, especially induction of remission and sustained remission. Concentrations were correlated with a published set of experimental biomarkers[14 15] and flow cytometry analyses of T-cell phenotypes.

Table 1 Baseline characteristics of patients according to their ANCA serotype are summarised

Baseline characteristics	ANCA serotype		FDR-adjusted p-value
	PR3 (n=127)	MPO (n=62)	
Age, years	51 (47–54)	60 (53–69)	0.0005
Gender			0.004
Male	76 (59.8)	22 (35.5)	
Female	51 (40.2)	40 (64.5)	
BMI (kg/m^2)	27.6 (27–29)	26.6 (25–28)	0.061
Phenotype			0.0002
GPA	123 (96.9)	19 (30.6)	
MPA	4 (3.1)	42 (67.7)	
Systemic	84 (66.1)	30 (48.4)	0.028
Newly diagnosed	48 (37.8)	43 (69.4)	<0.0001
Outcome			0.016
CR	54 (42.5)	42 (67.7)	
Relapse after CR	37 (29.1)	6 (9.7)	
No CR	36 (28.3)	14 (22.6)	
Kidney failure	56 (44.1)	43 (69.4)	0.002
Alveolar haemorrhage	34 (26.8)	12 (19.4)	0.297
BVAS/WG	8 (7–9)	8 (7–9)	0.805
Creatinine clearance (mL/min/1.73 m^2)	92.0 (83.1–99.8)	46.0 (34.2–69.9)	0.0002
Steroids prior randomization	108 (88.5)	58 (95.1)	0.179
Concentrations of sICP			
sTim-3 (pg/mL)	1264.50 (950.6–1429)	2664.84 (2299–3345)	<0.0001
sCD27 (pg/mL)	1205.59 (988.9–1616)	5658.77 (3222–8215)	<0.0001
sIDO (pg/mL)	48.42 (31.77–73.64)	46.58 (28.44–112.01)	0.671
sLag-3 (pg/mL)	144.70 (117.3–177.7)	287.21 (167.3–428.6)	0.0277
sBTLA (pg/mL)	1116.39 (875.6–1623)	1575.76 (1099–2679)	0.105
sPD-1 (pg/mL)	36.97 (30.56–46.35)	54.79 (43.13–78.91)	0.0004
sPD-L2 (pg/mL)	4848.31 (4174–5404)	8713.78 (6522–11796)	<0.0001

Median with 95% CI or the absolute count with the percentage within the outcome group are displayed. Kidney function at baseline was better preserved in PR3-ANCA vasculitis, while complete remission was more frequent in MPO-ANCA vasculitis, with fewer relapses observed in MPO-ANCA vasculitis and MPA. The concentrations of selected soluble immune checkpoints are highlighted. Concentrations were in general lower in PR3-ANCA vasculitis in comparison to MPO-ANCA vasculitis.
ANCA, antineutrophil cytoplasm antibody; BMI, body mass index; BTLA, B- and T-lymphocyte attenuator; BVAS/WG, Birmingham Vasculitis Activity Score/Wegener's granulomatosis; CD, cluster of differentiation; CR, complete remission; FDR, false discovery rate; GPA, granulomatosis with polyangiitis; IDO, indoleamine 2,3-dioxygenase; Lag-3, lymphocyte activation gene 3; MPA, microscopic polyangiitis; MPO, myeloperoxidase; PD-1, programmed cell death protein 1; PD-L2, programmed cell death-ligand 2; PR3, proteinase 3; sICP, soluble immune checkpoint; Tim-3, T-cell immunoglobulin and mucin domain 3.

METHODS

Study cohort and design

Details about the study cohort are available in the online supplemental appendix. This study used baseline plasma samples, stored at −80°C, of 189 patients (75.1% GPA, 24.3% MPA and one with no clear phenotype) included in the RAVE trial.[2 7] Subsequent samples at 6 months (n=145) and 18 months (n=87) were used to assess longitudinal regulation of sICPs. Sustained remission was defined as Birmingham Vasculitis Activity Score for Wegener Granulomatosis (BVAS/WG) of 0. Relapse was defined as BVAS/WG>0 after patients achieved remission.

Laboratory analyses

The plasma concentrations of the sICPs were quantified using a commercially available multiplex immunoassay (Life Technologies; ProcartaPL HU-IMM 96T: EPX140-15803-901), which measures the soluble concentrations of B-and T-lymphocyte attenuator (BTLA), glucocorticoid-induced TNFR-related protein (GITR), herpesvirus entry mediator (HVEM), indoleamine 2,3-dioxygenase (IDO), LAG3, PD1, PDL1, PDL2, TIM3, cluster of differentiation (CD)27, CD28, CD80, CD137 and CD152 (CTLA4/cytotoxic T-lymphocyte-associated antigen 4). We followed the manufacturer' protocols with slight adaptation applying a 7-point standard dilution series. Patient samples were run in duplicates with 25 µL of plasma each and were measured using a Magpix (Luminex). Data were analysed by the xPONENT 4.2 software (Luminex). The given values represent the mean of the duplicates given in picograms per millilitre. Specific protocols of peripheral blood mononuclear cell isolation and flow cytometry analyses (antibodies and gating strategies) are given in the online supplemental appendix (online supplemental figure 1 and online supplemental table 1 and protocol).

Statistical analysis

Median values were used for descriptive statistics of quantitative variables, and percentages were used for qualitative variables. In addition, Spearman correlation was used to determine the correlations between sICP concentrations and selected clinical features, and between concentrations of sICPs with concentrations of previously described biomarkers.[6] Mann-Whitney U test was used to compare concentrations of sICPs between different subgroups (ie, relapse vs remission). False discovery rate correction proposed by Benjamini and Hochberg was used to adjust the p-values. The optimal cut-off was calculated with the area under the receiver operating characteristic (ROC) curve (AUC) and the Yeldons J. Survival data were analysed using Kaplan-Meier curves and Cox proportional hazard regression modelling for univariate and multivariate analyses. Multivariate analysis was performed incorporating those biomarkers and clinical features with a p-value lower than 0.05. Cox regression modelling was used to adjust for creatinine clearance, as calculated by the Modification of Diet in Renal Disease equation. Statistical analyses were performed using SPSS (IBM SPSS Statistics 27) and Graph Pad Prism 8.

RESULTS

General clinical characteristics

Baseline plasma samples of 189 out of 197 patients randomised to participate in the RAVE trial were available and analysed. Participants had a median age of 52.0 years. One hundred and twenty-seven participants (67.2%) were PR3-ANCA and 62 (32.8%) MPO-ANCA positive. Clinical baseline characteristics are summarised in online supplemental table 2. Plasma concentrations of sTim-3, sCD27, sLag-3, sPD-1 and sPD-L2 were lower in patients with PR3-AAV compared with MPO-AAV (table 1).

Relationship between sCD27 concentrations and sLag-3 expression at baseline and remission achievement with rituximab induction

A minority of patients in the trial failed to achieve remission: 10 of 95 (10.5 %) patients randomised to RTX and 9 randomised to CYC/AZA. These patients were considered refractory to treatment. Ten patients in the RTX arm who failed induction therapy had lower plasma sLag-3 concentrations at baseline (64.47 pg/mL (95% CI: 38.20 to 487.4) than those who successfully achieved remission (162.99 pg/mL (95% CI: 136.65 to 284.20), p=0.024); this treatment-refractory group also showed a trend for higher plasma concentrations of sCD27 (4416.87 (95% CI: 988.9 to 19900) vs 1312.56 (95% CI: 996.6 to 2612) pg/mL, p=0.054). Only two out of six patients (33.3 %) who presented with a low sLag-3 (cut-off 90 pg/mL, based on ROC analysis) and a high sCD27 (cut-off >3000 pg/mL, based on ROC analysis) achieved remission, while 88.7% (n=47/53) patients with either sLag-3 high or sCD27 low and 100% (n=36/36) with sLag-3 high and sCD27 low achieved remission (p<0.001) (figure 1A). These findings were not observed in the CYC/AZA arm (figure 1B).

Relationship between baseline soluble immune checkpoint concentrations and sustained remission after rituximab induction

In the RTX arm, 24 of 73 patients (32.9%) relapsed with a median RFS time of 5.64 months. This group of relapsing patients had lower baseline plasma concentrations of sTim-3, sCD27 and sBTLA compared with patients who had sustained

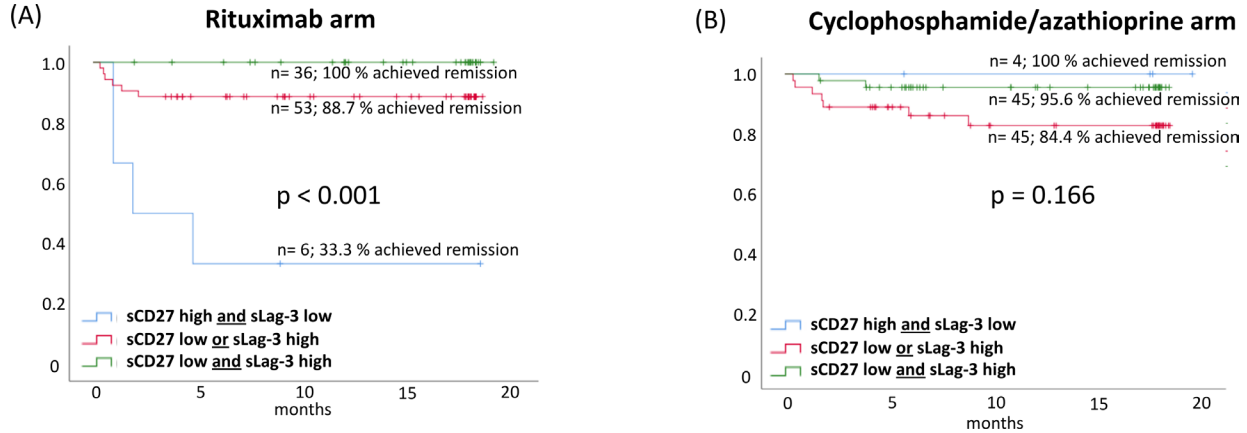

Figure 1 Nineteen patients included in our analysis had treatment failure, of whom 10 received rituximab and 9 were treated with cyclophosphamide as induction and azathioprine as maintenance therapy. (A) A combination of higher sCD27 and lower sLag-3 associated with treatment failure in the rituximab arm. Only two out of six patients achieved remission in this group (p<0.001). (B) The same combination did not predict treatment failure in patients receiving cyclophosphamide/azathioprine. CD, cluster of differentiation; sLag-3, soluble lymphocyte activation gene 3.

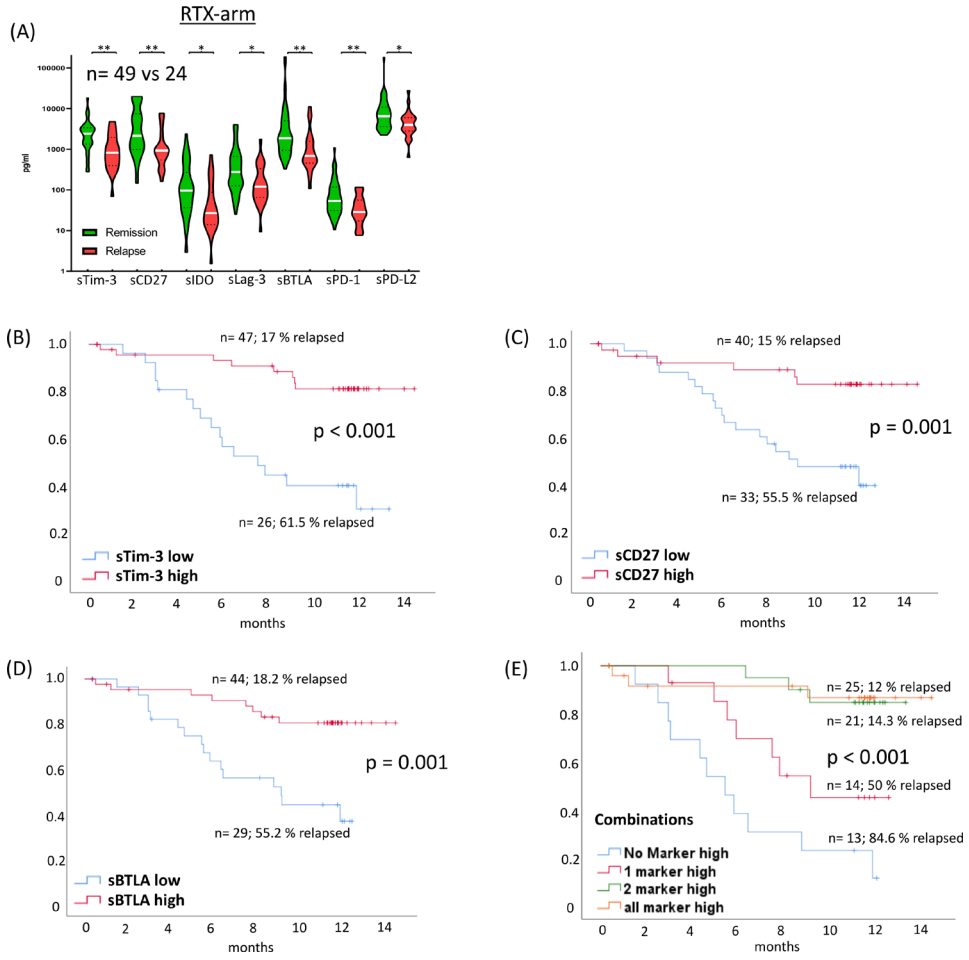

Figure 2 Different baseline plasma concentrations of immune checkpoints comparing patients in sustained complete remission (green) versus relapsed patients (red) receiving rituximab. Significant differences are marked by * for p<0.05 and ** for p<0.005 (A). Kaplan-Meier curves for the sustained remission rates in rituximab (RTX)-treated patients in months from the time point of complete remission achievement. Relapse defined the event. Patients were divided according to the baseline plasma concentrations of (B) sTim- 3, (C) sCD27 and (D) sBTLA. (E) displays the combination of those three markers in RTX-treated patients. BTLA, B- and T-lymphocyte attenuator; CD, cluster of differentiation; IDO, indoleamine 2,3-dioxygenase; Lag-3, lymphocyte activation gene 3; PD-1, programmed cell death protein 1; PD-L2, programmed cell death-ligand 2; Tim-3, T-cell immunoglobulin and mucin domain 3.

remission. Plasma concentrations of relapsing versus non-relapsing patients were: 821.97 (95% CI: 471.4 to 1453) versus 2417.58 (95% CI: 1620 to 3035) pg/mL (p=0.001) for sTim-3, 928.49 (95% CI: 572.2 to 1229) versus 2153.67 (95% CI: 1310 to 4961) pg/mL (p=0.001) for sCD27 and 685.27 (95% CI: 476.4 to 1476) versus 1870.44 (95% CI: 1359 to 2928) pg/mL (p=0.001) for sBTLA (figure 2A). Lower concentrations of sTim-3 (<1200 pg/mL), sCD27 (<1250 pg/mL) and sBTLA (<1000 pg/mL) were associated with more relapses in RTX-treated patients (61.5% vs 17%, p<0.001; 55.5% vs 15.0%, p=0.001; 55.2% vs 18.2%, p=0.001; figure 2B–D; table 2), but no difference was found in the CYC/AZA arm (online supplemental figure 6). A stronger correlation of these plasma sICP with relapse was obtained by combining more than one of these biomarkers. Patients with low concentrations of two or three plasma concentrations of these biomarkers relapsed in 50% (n=14) and 84.6% (n=13) of the cases, respectively, with a HR of 4.9 (95% CI: 1.720 to 3.918, p=0.022) and 11.7 (95% CI: 3.209 to 42.298, p<0.001), while patients with one or no plasma level had a relapse rate of under 15% (figure 2E). When further dividing these analyses into ANCA subtypes, we observed that this relapse survival benefit was seen in PR3-ANCA patients and GPA as opposed to patients with MPO-ANCA and MPA, which were hampered by small sample sizes (online supplemental figure 2A–L). Further subcohort analyses for newly diagnosed versus relapsing patients, no or prior steroid use before sampling, with or without acute kidney failure, male and female patients further support the relevance of sTim3, sCD27 and sBTLA in RTX-treated patients, even though some of these findings lack significance and were impacted by low numbers (online supplemental figure 3A–X and table 3). Similar subcohort analyses in CYC/AZA-treated patients are presented in online supplemental figures 7–9.

Baseline soluble immune checkpoint concentrations correlate with clinical characteristics

Higher age, BVAS/WG score, newly diagnosed vasculitis, steroid exposure and infectious complications correlated positively with sICP concentrations. Age correlated with sTim-3 (r=0.331, p<0.001), sCD27 (r=0.287, p<0.001), sPD-L2 (r=0.173, p=0.017) and sIDO (r=−0.233, p=0.001). BVAS/WG score and new diagnosis of vasculitis both correlated with sTim-3 (r=0.205, p=0.005 and r=0.268, p<0.001), sCD27 (r=0.236, p=0.001 and r=0.257, p<0.001) and

Table 2 Baseline characteristics of patients according to durable complete remission or relapse and received therapy.

Durable complete remission?	RTX arm		P-value	FDR-adjusted p-value	CYC/AZA arm		P-value	FDR-adjusted p-value
	Yes (n=49)	No (n=24)			Yes (n=47)	No (n=19)		
Age, years	55 (48–66)	57 (50–66)	0.986	1.0	53 (47–58)	49 (42–60)	0.346	0.582
Gender, female	29 (74.4)	10 (25.6)	0.159	0.337	19 (40.4)	8 (42.1)	0.900	0.982
BMI (kg/m^2)	25.7 (24.8–28.3)	26.8 (23.9–28.7)	1.0	1.0	28.8 (27.0–30.7)	29.3 (24.9–35.3)	0.772	0.879
Serotype			0.039	0.108			0.006	0.036
PR3	29 (59.2)	20 (83.3)			25 (53.2)	17 (89.5)		
MPO	20 (40.8)	4 (16.7)			22 (46.8)	2 (10.5)		
Systemic	27 (55.1)	13 (54.2)	0.94	0.995	32 (68.1)	12 (63.2)	0.701	0.869
Newly diagnosed	30 (61.2)	7 (29.2)	0.01	0.044	28 (59.6)	8 (42.1)	0.197	0.394
Kidney failure	27 (55.1)	11 (45.8)	0.456	0.657	30 (63.8)	7 (36.8)	0.045	0.116
Alveolar haemorrhage	14 (28.6)	6 (25.0)	0.748	0.869	6 (12.8)	7 (36.8)	0.026	0.094
BVAS/WG	8 (7–10)	8 (6–9)	0.578	0.771	8 (7–9)	7 (5–10)	0.623	0.801
Creatinine clearance (mL/min)	59.1 (42.4–73.0)	82.2 (64.3–109)	0.033	0.105	73.8 (63.5–93.7)	103.3 (89.0–141.9)	0.035	0.105
Steroids prior randomization	42 (89.4)	20 (83.3)	0.766	0.869	43 (91.5)	16 (84.2)	0.385	0.582
Concentrations of sICP								
Tim-3 (pg/mL)	2417.58 (1620–3035)	821.97 (471.38–1453)	0.001	0.012	1698.06 (1226–2338)	1330.44 (528.4–2610)	0.388	0.582
CD27 (pg/mL)	2153.67 (1310–4961)	928.49 (572.2–1229)	0.001	0.012	2417.27 (1440–3305)	1659.20 (990.0–3027)	0.368	0.582
IDO (pg/mL)	96.58 (56.65–129.02)	26.97 (14.21–73.64)	0.005	0.036	41.16 (20.1–66.8)	57.22 (31.2–288.1)	0.102	0.245
Lag-3 (pg/mL)	275.9 (158.7–456.0)	120.51 (71.13–244.9)	0.008	0.041	171.93 (112.5–217.4)	214.25 (101.1–926.33)	0.266	0.479
BTLA (pg/mL)	1870.44 (1359–2928)	685.26 (476.4–1475)	0.001	0.012	1067.53 (741.7–1813)	1926.12 (564.7–9545)	0.240	0.455
PD-1 (pg/mL)	53.23 (39.63–78.91)	28.58 (21.45–50.7)	0.004	0.036	41.63 (33.20–50.16)	79.48 (36.97–135.1)	0.135	0.303
PD-L2 (pg/mL)	6471.15 (4679–8458)	3995.95 (2885–5858)	0.011	0.044	5425.52 (4896–6693)	4904.76 (3830–10014)	0.576	0.771

Median with 95% CI or the absolute count with the percentage within the outcome group are displayed. After FDR correction (proposed by Benjamini and Hochberg) only patients who were newly diagnosed and high sICPs plasma concentrations showed a significant association with a durable complete remission in RTX-treated patients. In CYC/AZA arm, only the PR3- ANCA serotype showed a higher risk for relapse after complete remission. The concentrations of selected soluble immune checkpoints showed no predictive potential in CYC/AZA-treated patients.

ANCA, antineutrophil cytoplasm antibody; BMI, body mass index; BTLA, B- and T-lymphocyte attenuator; BVAS/WG, Birmingham Vasculitis Activity Score/Wegener's granulomatosis; CD, cluster of differentiation; CYC/AZA, cyclophosphamide/azathioprine; FDR, false discovery rate; IDO, indoleamine 2,3-dioxygenase; Lag-3, lymphocyte activation gene 3; MPO, myeloperoxidase; PD-1, programmed cell death protein 1; PD-L2, programmed cell death-ligand 2; PR3, proteinase 3; RTX, rituximab; sICP, soluble immune checkpoint; Tim-3, T-cell immunoglobulin and mucin domain 3.

sBTLA (r=0.160, p=0.028 and r=0.149, p=0.040); the latter in addition with sPD-1 (r=0.151, p=0.038). Next, we asked whether treatment with steroids before randomization as used in 166 (87.8%) of patients had an influence on sICP concentrations. The use of steroids was associated with higher baseline concentrations of sTim-3 (r=0.177, p=0.017), sCD27 (r=0.212, p=0.004) and sPD-L2 (r=0.172, p=0.02) (online supplemental table 3). Finally, we tested whether the above identified markers of relapse might act as measures of 'immunocompetence', defined as predictors of disease relapse but also infectious complications. Higher concentrations of sTim3, sCD27 and sBTLA were associated with more infections in RTX-treated patients (p=0.035, figure 3A), and this was the case when at least one of the markers of interest was above the predefined cut-off. Thus, sTim3, sCD27 and sBTLA are markers of immunocompetence in RTX-treated patients. Again, this could not be confirmed in CYC/AZA-treated patients (figure 3B).

Differences in treatment response based on baseline plasma concentrations of soluble immune checkpoint sICP concentrations predicted relapses in RTX-treated patients, but not in those receiving CYC/AZA. In detail, patients with high plasma sICP concentrations showed a borderline RFS benefit when treated with RTX (OR=0.273, p=0.05; online supplemental figure 4), whereas low plasma sICPs were associated with relapse. Again, these results need to be interpreted in the light of small sample size and a lack of confirmation in independent investigations.

Longitudinal assessment of soluble immune checkpoint concentrations

Follow-up measurements (6 and 18 months) were available for 143 and 87 patients. Although sICPs decreased over time, longitudinal assessment showed a high coherence of the baseline concentrations and follow-up measurements of sTim-3, sCD27 and sBTLA (online supplemental figure 5A–C). Most patients (n=83, 95.4%) with analysis at three time points had sustained remission.

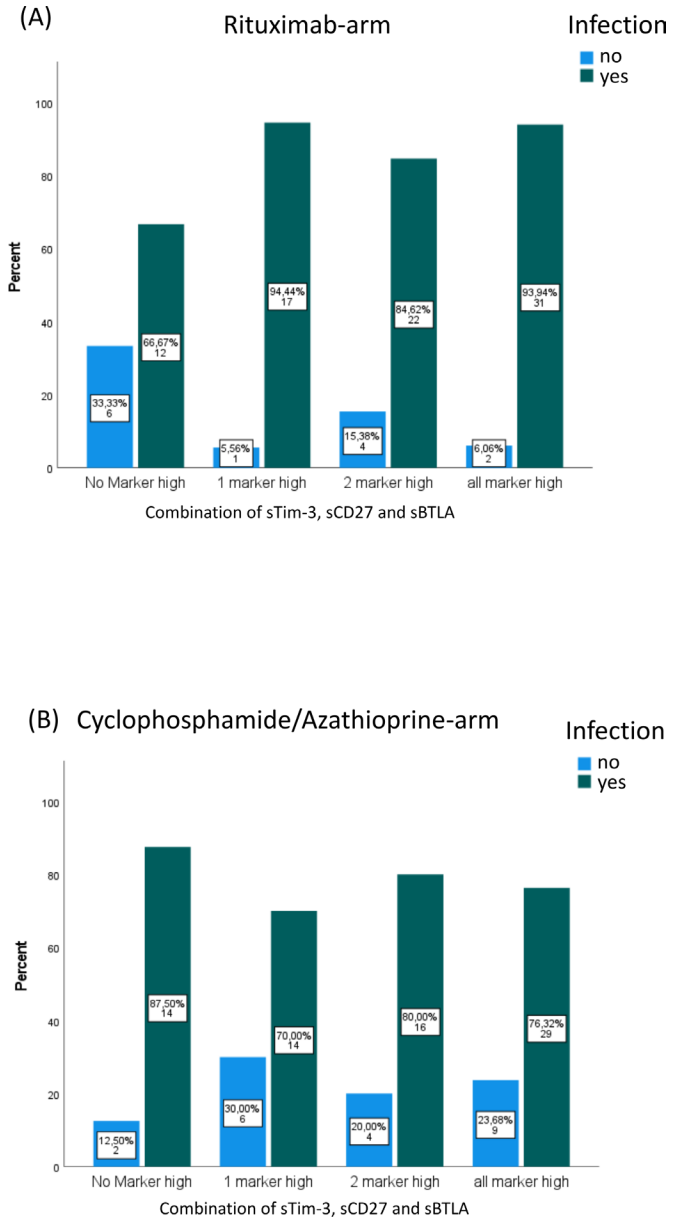

Figure 3 Occurrence of infections in the rituximab (A) and cyclophosphamide/azathioprine arms (B) grouped into the number of soluble markers (sCD27, sTim-3 and sBTLA). In the rituximab arm, fewer infections (p=0.035) were observed when no marker was high at baseline, in comparison to the groups with at least one highly expressed marker. In comparison, the expression of these three soluble immune checkpoints did not predict occurrence of infections in the cyclophosphamide/azathioprine arm (p=0.643). BTLA, B- and T-lymphocyte attenuator; CD, cluster of differentiation; Tim-3, T-cell immunoglobulin and mucin domain 3.

Relationship between soluble immune checkpoint concentrations at baseline, CD4$^+$/CD8$^+$ T-cell exhaustion/maturation and serum protein markers reflecting active disease

To understand the association of soluble immune checkpoints with CD4$^+$ and CD8$^+$ T-cell subsets, a major source of sICPs, available flow cytometry data at baseline of 87 RTX-treated patients were analysed. Overall, we observed a negative correlation with exhaustion phenotype in CD8 T cells (highlighted in red, gates 1, 3 and 5) and a positive correlation with regulatory CD4 T cells and follicular T helper cells (highlighted in blue, gates 9–11) with the measured sICPs (figure 4A; online supplemental table 4). These findings underline that the concentration of the sICPs might be influenced by T-cell exhaustion and warrants further investigation. A prior published set of serum proteins[6 14 15] comprising mainly chemokines/cytokines and other inflammation markers showed a positive correlation with several sICPs (figure 4B). Interestingly, the set of biomarkers best discriminating disease activity in AAV (active vs remission samples, B-cell-attracting chemokine 1, matrix metalloproteinase-3 and tissue inhibitor of metalloproteinase-1) showed only modest or no correlation with plasma concentrations of sICPs.[6] sICPs might therefore not be another tool to predict disease activity in AAV, but rather long-term outcomes.

DISCUSSION

Despite refinement of therapeutic approaches, primary resistance and relapse remains common in patients with AAV. As an example, approximately 5% of patients in observational studies have refractory disease,[16] 30%–40% do not achieve remission on a prednisolone dose lower than 10 mg/day by 6 months as shown in the RAVE trial, and 30% of those patients initially achieving remission in both treatment arms of RAVE subsequently relapsed within the follow-up period of 18 months.[7] Our study aimed to investigate the potential of baseline sICP concentrations as potential biomarkers for both, primary resistance and relapse, in patients receiving either RTX or CYC/AZA.

In RTX-treated patients, the combination of high levels of the activating immune checkpoint sCD27 together with low concentrations of inhibitory sLag-3 was linked to primary treatment failure. The biomarkers were not linked to response in CYC/AZA-treated patients. When focusing on patients achieving remission on RTX induction, those with high baseline sTim-3, sBTLA and sCD27 had a higher probability to maintain remission. In line with an increase immune-suppressive status (linked to response), these patients (even when only at least one of these markers was high) also suffered from higher infection rates during follow-up.

This report first highlights the potential of baseline quantification of sICP concentrations to predict RTX resistance and disease recurrence in AAV. As only data on the respective membrane-bound precursor forms are available, many aspects of the discussion below relate our data to the function of their membrane-bound counterparts.

Our results linking low sLag-3 and high sCD27 with primary RTX resistance, however, must be interpreted with caution, as the number of patients in the treatment failure groups were low (n=10 and 9 in the RTX and CYC/AZA subgroups, respectively). In addition, information about TNFSF13B (BAFF) single-nucleotide polymorphism, known to be associated with RTX failure in AAV,[17] was not available. sLag-3 is shed from mLag-3-expressing immune cells. Deficiency of mLag-3 induces increased T-cell reactivity which is supported by the observation that mLAg-3 deficiency on immune cells accelerates type 1 diabetes in preclinical murine models. mLag-3 is mainly expressed on exhausted cells, and blockade of mLag-3 synergized with PD-L1 blockade to improve CD8$^+$ T-cell responses against viral infections.[9 18] In contrast, mCD27 transmits co-stimulatory signals, inducing activation and proliferation of T and B lymphocytes, thereby promoting activation of antigen-experienced B cells.[19] Higher sCD27 concentrations are linked to increased disease activity in patients with systemic sclerosis.[20] Increased sCD27 concentrations might reflect a stronger immune activation in

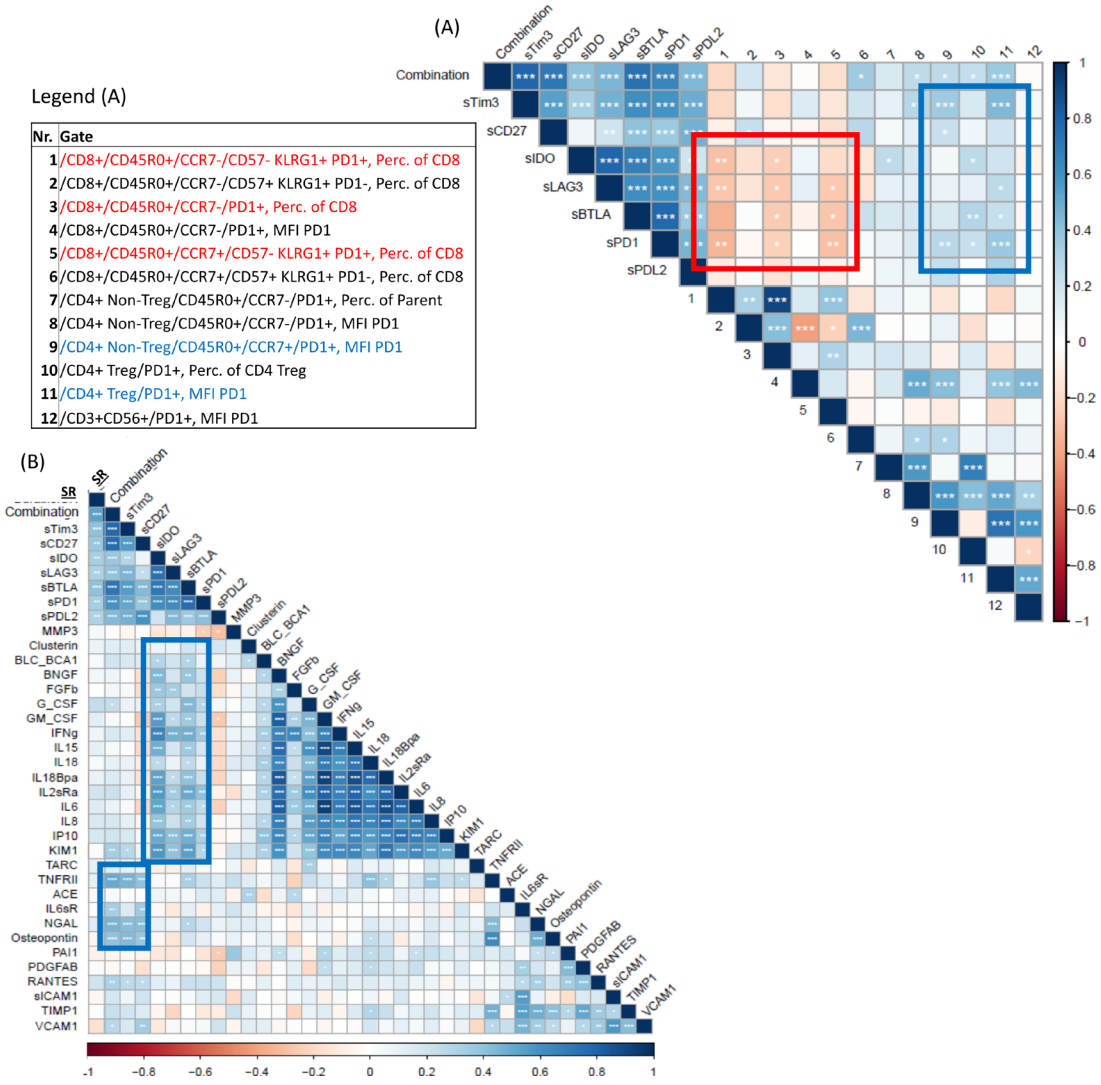

Figure 4 Spearman correlation matrix of soluble immune checkpoints and T-cell populations (A) (n=87) and of biomarker expressions (n=69) (B), each at baseline in rituximab-treated patients. Blue represents positive correlations and red represents negative correlations. The given flow cytometry legend shows the analysed and gated T-cell subpopulations, and numbers are used accordingly in the correlation matrix (A). 'Combination' includes the highly expressed soluble checkpoints (sCD27 (>1250 pg/mL), sTim-3 (>1200 pg/mL) and sBTLA (>1000 pg/mL)). Significant findings are flagged using * for p<0.05, ** for p<0.01 and *** for p<0.001. In matrix (A) red frames highlight significant negative correlations (CD8+ subpopulations) and blue frames the positive correlations (CD4+ subpopulations). In matrix (B), the blue frame highlights significant positive correlations with biomarker expressions. The combination correlated strongly with CD4+ Treg/PD1+ cells (A), as well as tumour necrosis factor receptor II (TNFRII), neutrophil gelatinase-associated lipocalin (NGAL) and osteopontin. None of the biomarkers correlate with sustained remission (SR). BCA, B-cell-attracting chemokine; BNGF, ß-nerve growth factor; BTLA, B- and T-lymphocyte attenuator; CD, cluster of differentiation; FGF, fibroblast growth factor; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte macrophage-colony-stimulating factor; ICAM, intracellular adhesion molecule; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; IP10, interferon-gamma-induced protein 10; KIM-1, kidney injury molecule-1; Lag-3, lymphocyte activation gene 3; NGAL, neutrophil gelatinase-associated lipoprotein; PAI-1, plasminogen activator inhibitor-1; PD-1, programmed cell death protein 1; PD-L2, programmed cell death-ligand 2; PDGF, platelet-derived growth factor; TARC, thymus- and activation-regulated chemokine; Tim-3, T-cell immunoglobulin and mucin domain 3; TIMP, tissue inhibitor of metalloproteinase; TNFR, tumour necrosis factor receptor; VCAM, vascular cell adhesion protein.

patients with AAV, ultimately resulting in reduced RTX efficacy. sCD27 concentrations also correlated with BVAS/WG at baseline, which underscores the more aggravated disease phenotype in these trial participants.

When focusing on patients in remission after induction with RTX, those with high baseline concentrations of sTim-3, sBTLA, but also sCD27 had a higher probability to achieve sustained remission even though these individuals also had a higher risk to develop infections. mTim-3 and mBTLA are known immune-suppressive regulators of T cells, whereas the role of mCD27 in this scenario is counterintuitive. mTim-3 negatively regulates various murine autoimmune diseases.[21 22] In AAV, reduced abundance of mTim-3 has been found on blood-derived dendritic cells from patients with active MPO-ANCA vasculitis.[23] Similarly, lower expression levels of mTim-3 have been associated with enhanced inflammation and thus correlated with an unfavourable clinical course in human autoimmune diseases.[24] In line with our observation as higher sBTLA concentrations are linked to sustained remission, relapse rate was lower in patients with AAV with mBTLA-positive double-negative T cells

($CD3^+CD4^-CD8^-$).[25] The immune-suppressive role of mBTLA is also supported by reduced rates of acute allograft rejection and prolonged graft survival in mBTLA high rates on organ transplantation.[26] In contrast to the inhibitory ICPs discussed above, mCD27 belongs to the family of activating ICPs and its role for sustained remission after RTX induction is difficult to explain. sCD27 has been shown to predict relapses in multiple sclerosis, but no information about treatment modalities of these patients was provided.[27] One explanation for the here presented counterintuitive findings of high sCD27 concentrations and relapse prediction might be the use of RTX as induction agent. Moreover, ongoing strong CD27 signalling may support CD4 and CD8 T-cell proliferation, terminal differentiation, exhaustion, and apoptosis—thus finally being not immune activating but rather immune-suppressive.[28]

Our study indicates that the presence of higher sICP concentrations in RTX-treated patients suggest protection from disease relapse. This group, however, exhibited a higher infection rate. We speculate that T-cell exhaustion might play a role to explain these findings, as T-cell exhaustion has been linked to a reduced clearance of viral infections and impaired T-cell function, but these patients were protected from disease relapse.[29] In line, a specific role for higher concentrations of sTim-3, sCD27 and sBLTA has recently been found in patients with severe infections.[30 31]

Of note, the combination of sTim-3, sCD27 and sBTLA did not predict outcome in the CYC/AZA-treated patients with AAV. This observation may point towards an important role of B-cell recovery following RTX induction implicated in disease relapse, as subsets such as $CD5^+$ B cells might be continuously suppressed in CYC/AZA-treated patients.[32] In tumour-specific $CD8^+$ T cells, the administration of CYC lowered the expression of mPD-1 and thus increased cytotoxicity,[33] which also indicates a specific role of CYC and potentially AZA to interfere with mICP expression and consequently also with sICP release. It is worth mentioning that participants in the RAVE trial received high cumulative CYC doses,[2 7] and there is no information on relapse risk in those receiving CYC intravenously.[34] In this respect, cumulative CYC doses correlated with mBTLA expression on double-negative T cells in AAV.[25] Follow-up investigations are urgently needed to investigate the potential of sICP concentrations to predict long-term remission following either RTX- or CYC-based induction regimens, including also patients receiving lower cumulative CYC doses.

When focusing on the different ANCA vasculitis subtypes, sICP concentrations were lower in PR3-ANCA vasculitis than in MPO-ANCA vasculitis. This might reflect differences in pathogenesis between both entities. Several independent investigations have found a higher relapse rate among individuals with PR3- as compared with MPO-ANCA vasculitis.[1] Serum PR3-ANCA positivity and increasing titres of PR3-ANCA during follow-up were each associated with relapse in several studies.[5 35] Notably, only few patients with MPO-ANCA vasculitis relapsed during the follow-up of the RAVE trial, and MPO-ANCA vasculitis usually presents at older age and with impaired kidney function, which may have been negative predictors of relapses.[1] Findings concerning baseline sICPs as predictors for relapse under RTX treatment in MPO-ANCA vasculitis were limited by the lower numbers, but in part followed the trend shown in the substantially larger PR3 ANCA vasculitis cohort.

We are well aware that this ancillary study of the RAVE trial has several limitations to consider: (1) physicians could have started glucocorticoids before randomization, which might have impacted sICP concentrations,[8] (2) sICP assays lack standardisation and validation and (3) the small sample sizes, especially in some of the subgroups (ie, MPO-ANCA vasculitis). Notably, AAV is considered a rare disease, and few biomarker studies have been described with comparable numbers of patients. However, we could not confirm our findings in an independent cohort, as most other trials have either not collected samples in a protocolised manner[36] or used CYC as an induction agent.[37]

In conclusion, we found easy to measure plasma-based baseline biomarkers predicting primary treatment failure and risk of relapse in RTX-treated patients with AAV, especially those with PR3-ANCA vasculitis. The immune phenotype defined by our sICP signatures (sTim-3, sCD27 and sBTLA) was linked to reduced rates of disease relapses, but increased rates of infection in the RTX group. If confirmed in another study, the panel of markers identified by our analysis might further aid in individualising treatment decisions about optimal induction therapy and the duration of maintenance therapy. Trials implementing baseline detection of these markers are required to validate and refine the proposed risk stratification.

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Acknowledgements We thank S. Alice Long, supervising Principal Investigator of the BRI Human Immunophenotyping Core along with HIP Core members Jerill Thorpe and Megan Maertz for their contributions in flow cytometry analyses.

Contributors All authors contributed to sample collection. GG, FM, US, JHS and AK designed the study. KH, LC, NL and FM performed statistical analyses. All authors contributed to drafting the manuscript, revised it and approved the final version. AK takes full responsibility for the work and/or the conduct of the study.

Funding This research was performed as a project of the Immune Tolerance Network (Protocol number ITN401).

Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Obtained.

Ethics approval This study involves human participants, but this study was exempted. Explanation for exemption: ITN021AI trial (www.itntrialshare.org), freely available anonymized patient data Ethics name: all contributing centers obtained ethics approval for participation in the RAVE trial. EC approval: obtained for the original trial (RAVE) Reason: ancillary studies (such as the submitted work) are covered by the original ethics committee vote. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request.

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ONLINE SUPPLEMENTAL APPENDIX

Association of baseline soluble immune checkpoints with risk of relapse in PR3-ANCA-vasculitis following induction of remission

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STUDY COHORT

The RAVE trial randomized 197 patients with severe *de novo* or relapsing GPA or MPA to receive remission induction with a standardised regimen of glucocorticoids plus either RTX, 4-times 375 mg/m^2 weekly, or oral CYC at a target dose of 2 mg/kg/day followed by AZA 2 mg/kg/day as maintenance therapy. The proportion of patients achieving remission at 6 months after induction therapy was similar in the two groups [1]. During follow-up of 18 months, the relapse rates were also indistinguishable between both treatment groups [2]. Patients with PR3-ANCA vasculitis treated with RTX achieved remission at 6 months more frequently than those assigned to CYC/AZA. Study participants with a relapsing disease course and PR3-ANCA vasculitis showed higher remission rates following rituximab induction therapy throughout the 18 months of the trial [3]. Clinical data were retrieved from https://www.itntrialshare.org/.

METHODS

Suppl. 1: protocols

Protocols for flow cytometry analyses including PBMCs thawing, staining and analyses:

Cryopreserved PBMC from all per-protocol participants that met complete remission criteria at 6 months in the RAVE trial were thawed and batch-analyzed after the 18 months timepoint. Samples included baseline (v-1), v8 (6 months), v10 (12 months), and v12 (18 months) or the relapse visit if available. Vials of cryopreserved cells were thawed in a water bath before immediately diluting drop-wise into 1 mL media with 10% serum and DNase. This 1 mL was diluted more quickly with an additional 9 mL of media prior to centrifugation, aspiration of media with DMSO, and resuspension in 2 mL fresh media. After resting for 5 minutes, cells were strained to remove clumps, counted, and distributed for staining.

Staining and flow cytometry:

Surface markers were stained using cocktails prior to eBio FOXP3 fix/perm for intracellular staining. Cells were stained using the antibody panel shown in Supplemental Table 1 and analyzed using an LSR-Fortessa (BD Biosciences) with FACS Diva software and analyzed with FlowJo Version 9 (TreeStar Inc.) at the Benaroya Research Institute (Seattle, Washington, USA). To permit direct comparisons between samples acquired across days, instrument standardization was performed using 8 peak rainbow calibration beads (Spherotech, Lake Forest, IL), adjusting PMT voltages so that 7th peak mean fluorescent intensities for each parameter were consistent. All samples from the same subject were run on the same day, and an internal control from the same subject was run each week to identify any machine or staining issues. An average of 3.24 x 10^5 live lymphocyte events were collected per sample and gated populations with <100 events were excluded from analysis. Frequencies for populations with counts under 25 and MFIs for populations with counts under 50 were not included.

Supplemental Tables

Supplemental Table 1: List of used antibodies to stain peripheral blood mononuclear cells including clone, fluorochrome and detection level

Marker	Clone	Fluorochrome	Detector
CD56	NCAM16.2	BUV395	355-379/28
CD45RA	HI100	BUV737	355-740/35
GrzmB	GB11	BV421	405-450/50
CCR7	G043H7	BV510	405-525/50
CD3	OKT3	ev605	405-605/12
PD1	J105	ev655	405-655/8
CD127	A019D5	BV711	405-710/50
CD45R0	UCHL1	BV786	405-780/60
CD4	RPA-T4	BB515	488-530/30
Eomes	WD1928	PE	561-582/15
FoxP3	259D	PE-CF594	561-610/20
KLRG1	REA261	PE-vio770	561-780/60
TIGIT	MBSA43	APC	640-660/20
CD8	RPA-T8	AF700	640-710/50
CD57	TB03	APC-vio770	640-780/60
Fixable Blue Live/Dead			355-450/50

Abbreviations: CCR (C-C chemokine receptor), CD (cluster of differentiation), Eomes (eomesodermin), Fox (forkhead-box-protein), GrzmB (granzyme B), KLRG (killer cell lectin like receptor), PD (programmed cell death protein), TIGIT (T cell immunoreceptor with Ig and ITIM domains)

Online supplemental table 2: Patient characteristics and soluble immune checkpoint concentrations.

	Rituximab-arm				**Cyclophosphamide/azathioprine-arm**			
Durable complete remission?	**Yes (n = 49)**	**No (n = 24)**	**p-value**	**FDR-adjusted p-value**	**Yes (n = 47)**	**No (n = 19)**	**p-value**	**FDR-adjusted p-value**
Age, years	55 (48-66)	57 (50-66)	0.986	1.0	53 (47-58)	49 (42-60)	0.346	0.582
Gender, female	29 (74.4)	10 (25.6)	0.159	0.337	19 (40.4)	8 (42.1)	0.900	0.982
BMI (kg/m^2)	25.7 (24.8-28.3)	26.8 (23.9-28.7)	1.0	1.0	28.8 (27.0-30.7)	29.3 (24.9-35.3)	0.772	0.879
Serotype PR3 MPO	 29 (59.2) 20 (40.8)	 20 (83.3) 4 (16.7)	0.039	0.108	 25 (53.2) 22 (46.8)	 17 (89.5) 2 (10.5)	0.006	0.036
Systemic at baseline	27 (55.1)	13 (54.2)	0.94	0.995	32 (68.1)	12 (63.2)	0.701	0.869
Newly diagnosed	30 (61.2)	7 (29.2)	0.01	0.044	28 (59.6)	8 (42.1)	0.197	0.394
Kidney failure	27 (55.1)	11 (45.8)	0.456	0.657	30 (63.8)	7 (36.8)	0.045	0.116
Alveolar hemorrhage	14 (28.6)	6 (25.0)	0.748	0.869	6 (12.8)	7 (36.8)	0.026	0.094
BVAS/WG	8 (7-10)	8 (6-9)	0.578	0.771	8 (7-9)	7 (5-10)	0.623	0.801
Creatinine clearance (mL/min/1.73 m^2)	59.1 (42.4-73.0)	82.2 (64.3-109)	0.033	0.105	73.8 (63.5-93.7)	103.3 (89.0-141.9)	0.035	0.105
Steroids prior randomization	42 (89.4)	20 (83.3)	0.766	0.869	43 (91.5)	16 (84.2)	0.385	0.582
Concentrations of sICP								
Tim-3 (pg/mL)	2417.58 (1620-3035)	821.97 (471.38-1453)	0.001	0.012	1698.06 (1226-2338)	1330.44 (528.4-2610)	0.388	0.582
CD2**7** (pg/mL)	2153.67 (1310-4961)	928.49 (572.2-1229)	0.001	0.012	2417.27 (1440-3305)	1659.20 (990.0-3027)	0.368	0.582
IDO (pg/mL)	96.58 (56.65-129.02)	26.97 (14.21-73.64)	0.005	0.036	41.16 (20.1-66.8)	57.22 (31.2-288.1)	0.102	0.245
Lag-3 (pg/mL)	275.9 (158.7-456.0)	120.51 (71.13-244.9)	0.008	0.041	171.93 (112.5-217.4)	214.25 (101.1-926.33)	0.266	0.479
BTLA (pg/mL)	1870.44 (1359-2928)	685.26 (476.4-1475)	0.001	0.012	1067.53 (741.7-1813)	1926.12 (564.7-9545)	0.240	0.455
PD-1 (pg/mL)	53.23 (39.63-78.91)	28.58 (21.45-50.7)	0.004	0.036	41.63 (33.20-50.16)	79.48 (36.97-135.1)	0.135	0.303
PD-L2 (pg/mL)	6471.15 (4679-8458)	3995.95 (2885-5858)	0.011	0.044	5425.52 (4896-6693)	4904.76 (3830-10014)	0.576	0.771

Baseline characteristics of patients according achieving sustained complete remission or relapse and their assigned treatment arm. Median with 95 % confidence interval or the absolute count with the percentage within the outcome group are displayed. After FDR-correction (proposed by Benjamini and Hochberg) only patients who were newly diagnosed and had high plasma concentrations of sICPs showed a significant association with sustained complete remission after rituximab induction therapy. In the CYC/AZA arm, only the PR3-ANCA serotype showed a higher risk of relapse after complete remission. Concentrations of selected sICPs showed no predictive potential in patients receiving CYC/AZA. Abbreviations: BMI (body mass index), BTLA (B-and T-

lymphocyte attenuator), BVAS/WG (Birmingham Vasculitis Activity Score/Wegener's granulomatosis), CD (cluster of differentiation), CR (complete remission), CYC/AZA (cyclophosphamide/ azathioprine), FDR (false discovery rate) GPA (granulomatosis with polyangiitis), IDO (indoleamine 2,3-dioxygenase), Lag-3 (lymphocyte-activation gene 3), MPA (microscopic polyangiitis), MPO (myeloperoxidase), PD-1 (programmed cell death protein 1), PD-L2 (programmed cell death-ligand 2), PR3 (proteinase 3), RTX (rituximab), sICP (soluble immune checkpoints), Tim-3 (T-cell immunoglobulin and mucin domain 3)

Supplemental Table 3: sICP concentrations in newly diagnosed patients and patients receiving steroids before randomization.

Newly diagnosed	**Yes**	**No**	**P-value**
sTIM-3 (ng/ml)	2297 (1698-2828)	1281 (950-1436)	< 0.001
sCD27 (ng/ml)	2670 (1734-4338)	1311 (989-1760)	< 0.001
sBTLA (ng/ml)	1616 (1200-2137)	1004 (774-1588)	0.04
sPD-1 (ng/ml)	49.6 (41.6-62.6)	37.5 (30.6-47.3)	0.038
Steroids before randomization			
sTIM-3 (ng/ml)	1698 (1394-2199)	842 (471-1452)	0.017
sCD27 (ng/ml)	1999 (1539-2670)	989 (536-1190)	0.004
sPD-L2 (ng/ml)	5615 (5064-6229)	3830 (2847-4722)	0.02

P-value is calculated using Mann-Whitney-U test. Values are presented as median with 95 % confidence interval. Abbreviations: BTLA (B-and T-lymphocyte attenuator), CD (cluster of differentiation), PD-1 (programmed cell death protein 1), PD-L2 (programmed cell death-ligand 2), Tim-3 (T-cell immunoglobulin and mucin domain 3)

Supplemental Table 4: Gating strategies and associated duration of sustained remission based on T cell subpopulations

FACS data Gating strategy	Sustained remission (months)			
	Yes (n = 32)	No (n = 14)	p-value	FDR-adjusted p-value
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7-/CD57- KLRG1+ PD1+ Freq. of CD8	5.28 (3.38 - 7.96)	8.484 (4.03 - 11.64)	0.133	0.684
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7-/CD57- KLRG1+ PD1+ Freq. of Parent	27.35 (22.7 - 36.5)	35.75 (24.7 - 44.0)	0.352	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7-/CD57+ KLRG1+ PD1- Freq. of CD8	1.33 (0.54 - 2.24)	0.75 (0.16 - 6.46)	0.377	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7-/CD57+ KLRG1+ PD1- Freq. of Parent	6.76 (3.39 - 10.10)	3.16 (0.77 - 18.70)	0.166	0.684
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7-/PD1+ Freq. of CD8	11.03 (5.89 - 14.40)	15.98 (8.20 - 23.81)	0.11	0.684
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7-/PD1+ Freq. of Parent	58.20 (45.0 - 69.2)	65.0 (49.2 - 80.8)	0.252	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7-/PD1+ MFI PD1	1017 (832 - 1155)	1055 (828 - 1455)	0.559	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7+/CD57- KLRG1+ PD1+ Freq. of CD8	2.29 (1.66 - 4.03)	3.34 (0.72 - 5.37)	0.535	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7+/CD57- KLRG1+ PD1+ Freq. of Parent	18.25 (14.7 - 24.6)	17.30 (10.1 - 22.7)	0.474	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7+/CD57+ KLRG1+ PD1- Freq. of CD8	0.18 (0.07 - 0.71)	0.25 (0.06 - 5.78)	0.615	0.747
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7+/PD1+ Freq. of CD8	4.19 (3.16 - 5.57)	5.82 (1.97 - 9.06)	0.519	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7+/PD1+ Freq. of Parent	35.8 (30.7 - 47.2)	34.9 (27.2 - 39.5)	0.445	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45R0+/CCR7+/PD1+ MFI PD1	800.5 (708 - 920)	789.5 (649 - 944)	0.943	0.943
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45RA+ CD45R0-/CCR7-/CD57- KLRG1+ PD1+ Freq. of CD8	1.60 (1.08 - 2.67)	1.94 (0.58 - 3.06)	0.769	0.817
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45RA+ CD45R0-/CCR7-/CD57- KLRG1+ PD1+ Freq. of Parent	11.7 (9.28 - 19.0)	18.5 (9.75 - 27.60)	0.371	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45RA+ CD45R0-/CCR7-/CD57+ KLRG1+ PD1- Freq. of CD8	3.58 (1.68 - 6.08)	2.67 (0.67 - 5.06)	0.445	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45RA+ CD45R0-/CCR7-/CD57+ KLRG1+ PD1- Freq. of Parent	27.9 (21.6 - 31.9)	24.6 (8.27 - 38.1)	0.39	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45RA+ CD45R0-/CCR7-/PD1+ Freq. of CD8	3.93 (2.19 - 7.70)	3.42 (1.52 - 8.71)	0.731	0.802
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45RA+ CD45R0-/CCR7-/PD1+ Freq. of Parent	38.0 (31.0 - 49.4)	48.7 (16.6 - 59.2)	0.508	0.704
/Live Lymphocyte/CD3+CD56-/CD4-CD8+/CD45RA+ CD45R0-/CCR7-/PD1+ MFI PD1	691 (639 - 813)	818 (709 - 968)	0.056	0.684
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Non-Treg/CD45R0+/CCR7-/CD57- KLRG1- PD1+ Freq. of CD4 Non-Treg	4.23 (2.36 - 7.50)	2.74 (2.16 - 7.52)	0.551	0.704
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Non-Treg/CD45R0+/CCR7-/CD57- KLRG1- PD1+ Freq. of Parent	36.1 (31.5 - 43.1)	34.2 (27.3 - 50.7)	0.685	0.802
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Non-Treg/CD45R0+/CCR7-/PD1+ Freq. of CD4 Non-Treg	6.44 (3.62 - 13.05)	5.01 (2.91 - 12-25)	0.474	0.704

/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Non-Treg/CD45R0+/CCR7-/PD1+ Freq. of Parent	67.55 (58.7 - 76.9)	57.30 (50.8 - 66.7)	0.015	0.51
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Non-Treg/CD45R0+/CCR7-/PD1+ MFI PD1	1171 (1076 - 1373)	1086 (852 - 1259)	0.316	0.704
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Non-Treg/CD45R0+/CCR7+/CD57- KLRG1- PD1+ Freq. of CD4 Non-Treg	14.81 (12.03 - 18.19)	11.63 (7.67 - 20. 46)	0.459	0.684
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Non-Treg/CD45R0+/CCR7+/CD57- KLRG1- PD1+ Freq. of Parent	28.15 (22.2 - 38.3)	24.6 (13.2 - 37.9)	0.145	0.704
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Non-Treg/CD45R0+/CCR7+/PD1+ Freq. of CD4 Non-Treg	19.68 (16.2 - 24.1)	16.64 (11.5 - 25.0)	0.252	0.684
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Non-Treg/CD45R0+/CCR7+/PD1+ Freq. of Parent	37.05 (27.6 - 46.8)	29.3 (19.3 - 39.6)	0.064	0.684
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Non-Treg/CD45R0+/CCR7+/PD1+ MFI PD1	722.5 (675 - 844)	716.5 (636 - 842)	0.914	0.942
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Treg/PD1+ Freq. of CD4 Treg	48.85 (42.8 - 61.9)	42.15 (32.7 57.7)	0.181	0.684
/Live Lymphocyte/CD3+CD56-/CD4+CD8-/Live Lymphocyte/CD3+CD56-/CD4+ Treg/PD1+ MFI PD1	775 (695 - 830)	706 (641 - 844)	0.454	0.704
/Live Lymphocyte/CD3+CD56+/PD-1+,Freq. of Parent	30.7 (20.3 - 33.3)	26.1 (16.6 - 42.7)	0.711	0.802
/Live Lymphocyte/CD3+CD56+/PD1+ MFI PD1	677 (625 - 766)	724 (669 - 934)	0.133	0.684

Differences between patients with sustained complete remission or relapse in PD-1 expression of T cell subpopulation evaluated Flow cytometry. The first column shows the gating strategy used (see supplemental figure 1). Values are given as medians with 95 % confidence interval. Statistics were done using Mann-Whitney U and false discovery corrections (FDR).

References:

1. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, et al. Rituximab versus cyclophosphamide for ANCA-associated vasculitis. N Engl J Med. 2010 Jul; 363(3):221-232.
2. Specks U, Merkel PA, Seo P, Spiera R, Langford CA, Hoffman GS, et al. Efficacy of remission-induction regimens for ANCA-associated vasculitis. N Engl J Med. 2013 Aug; 369(5):417-427.
3. Unizony S, Villarreal M, Miloslavsky EM, Lu N, Merkel PA, Spiera R, et al. Clinical outcomes of treatment of anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis based on ANCA type. Ann Rheum Dis. 2016 Jun; 75(6):1166-1169.

Supplemental figure 1: Gating strategy of flow cytometry measurements

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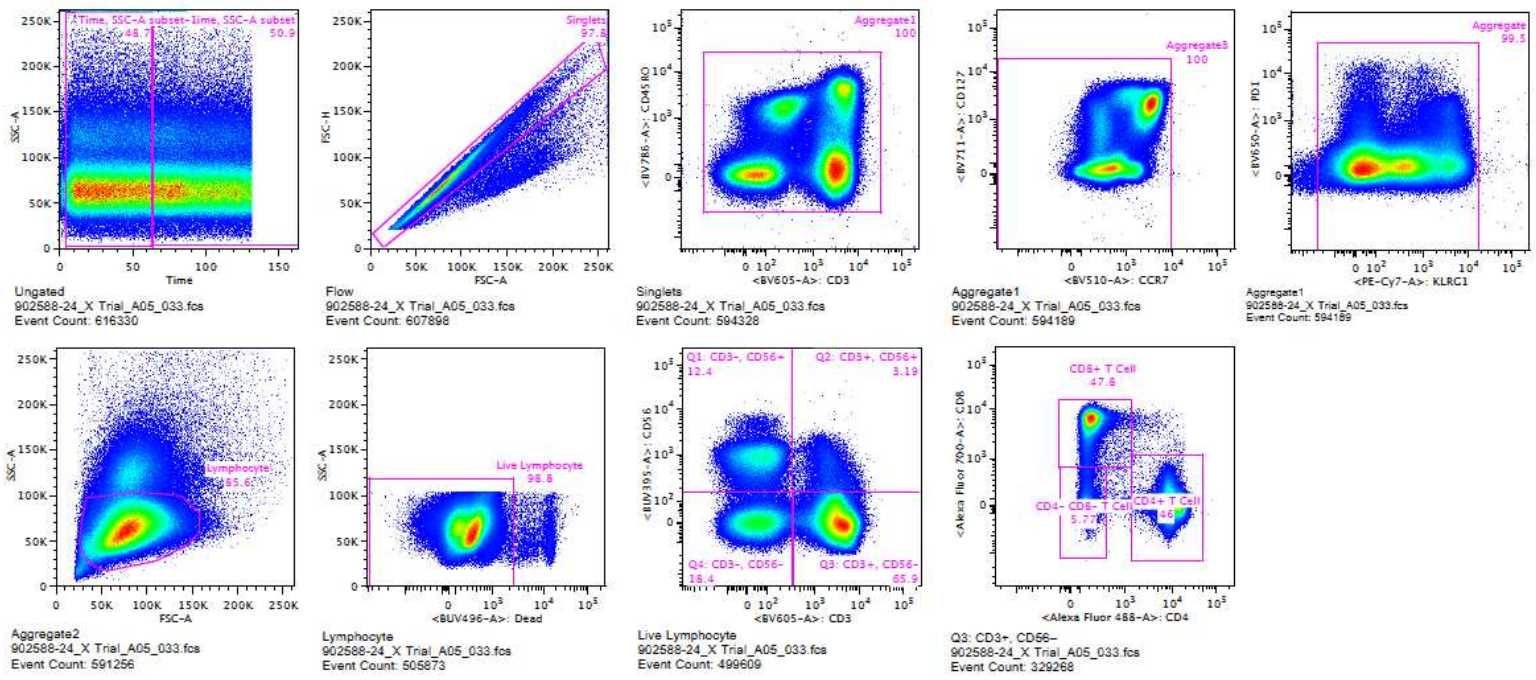
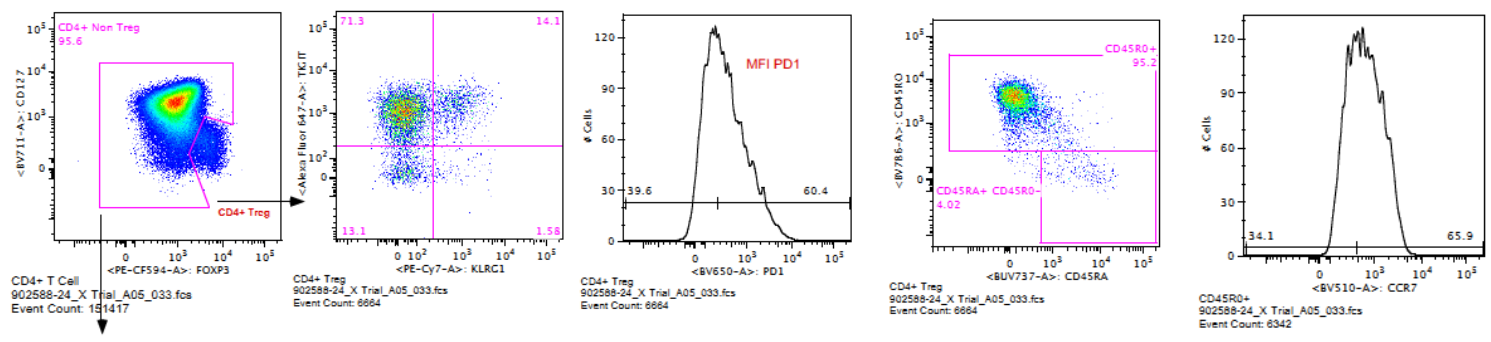
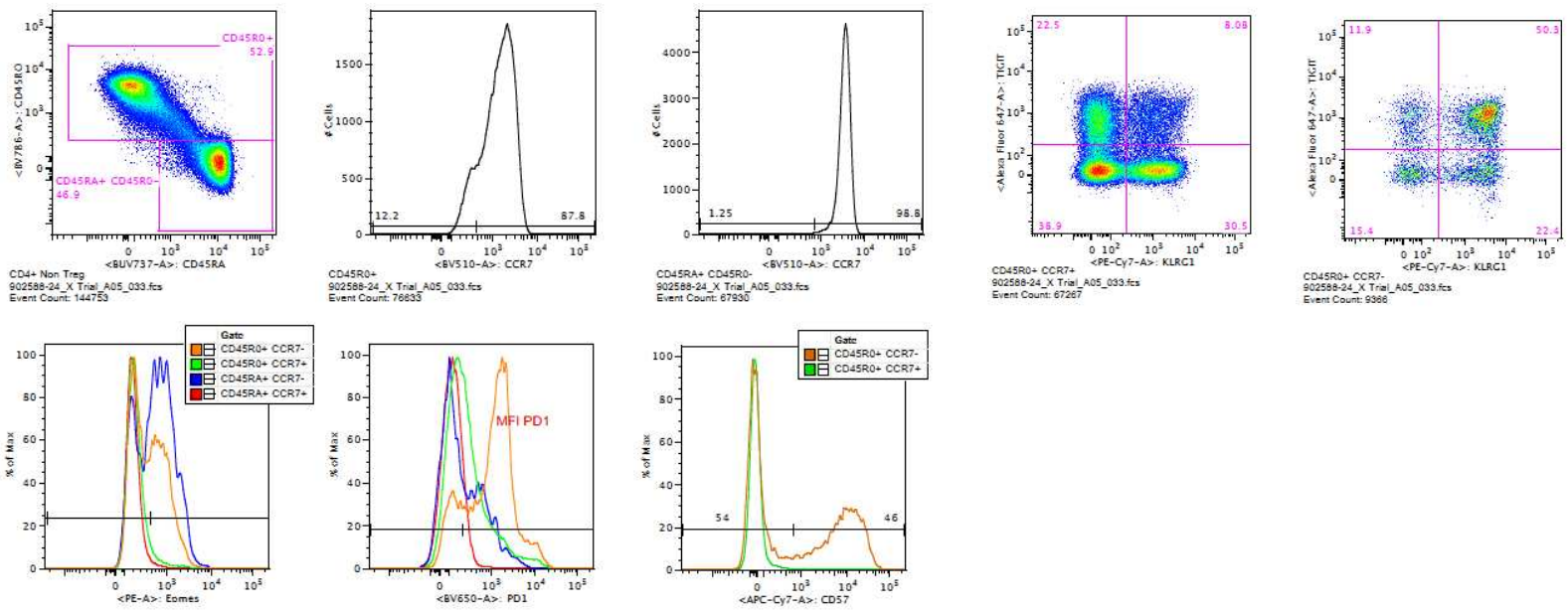


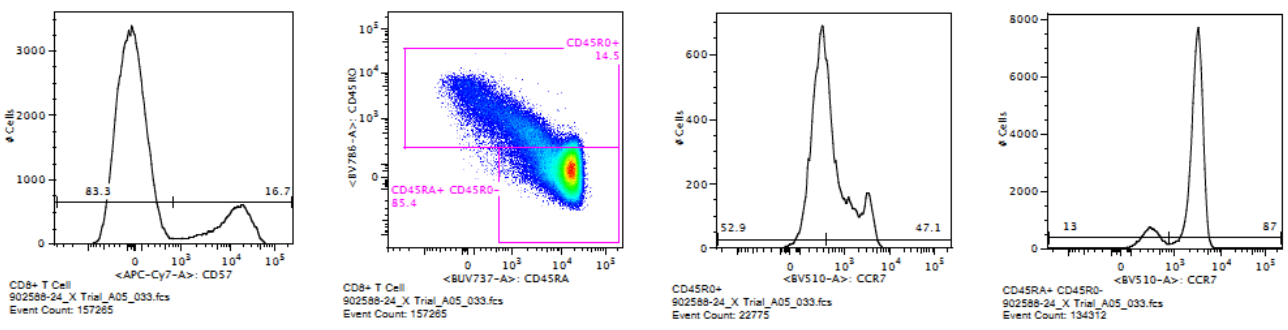
CD4+ T Cell Gating



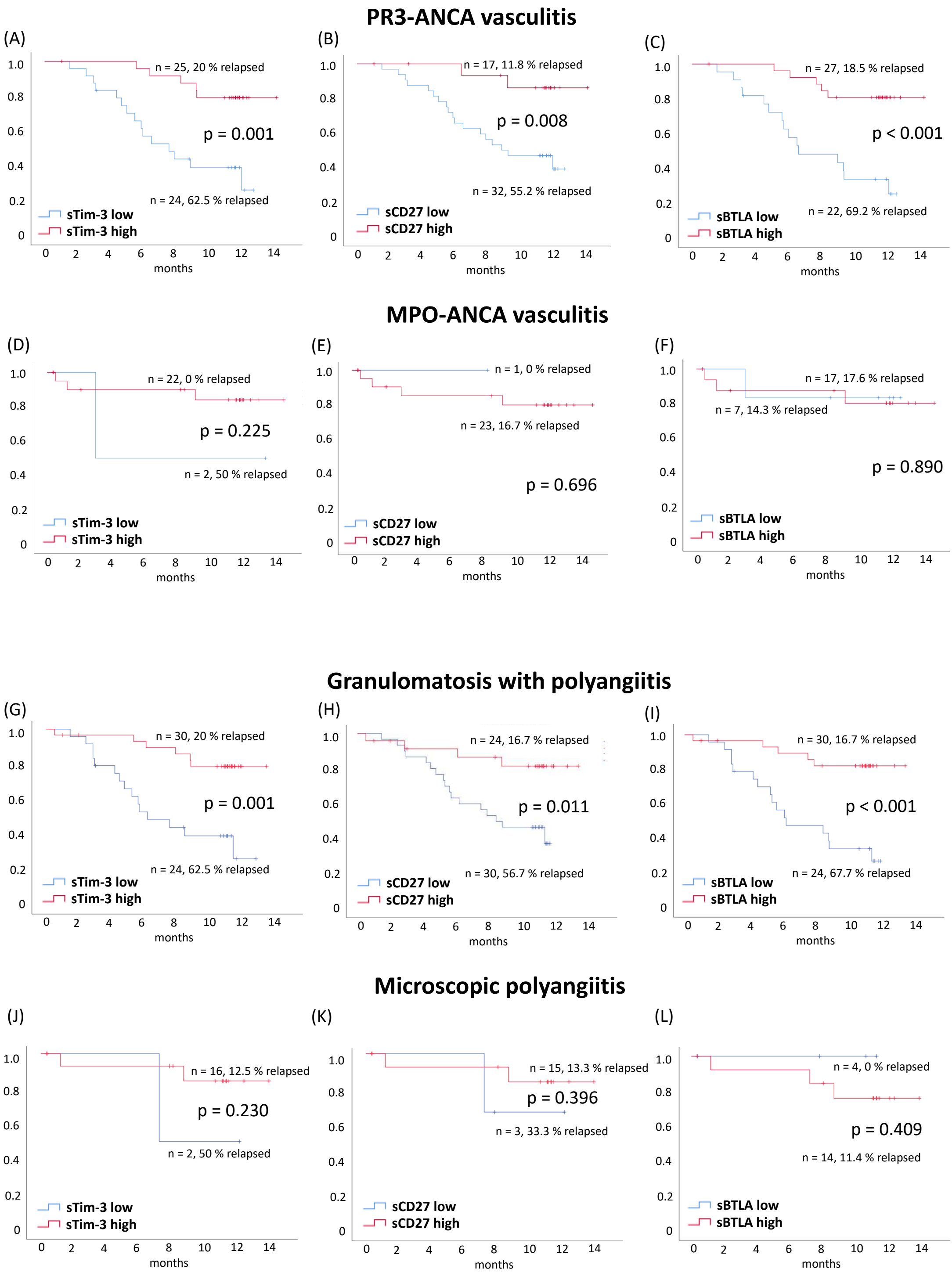
CD4+ Non-Treg



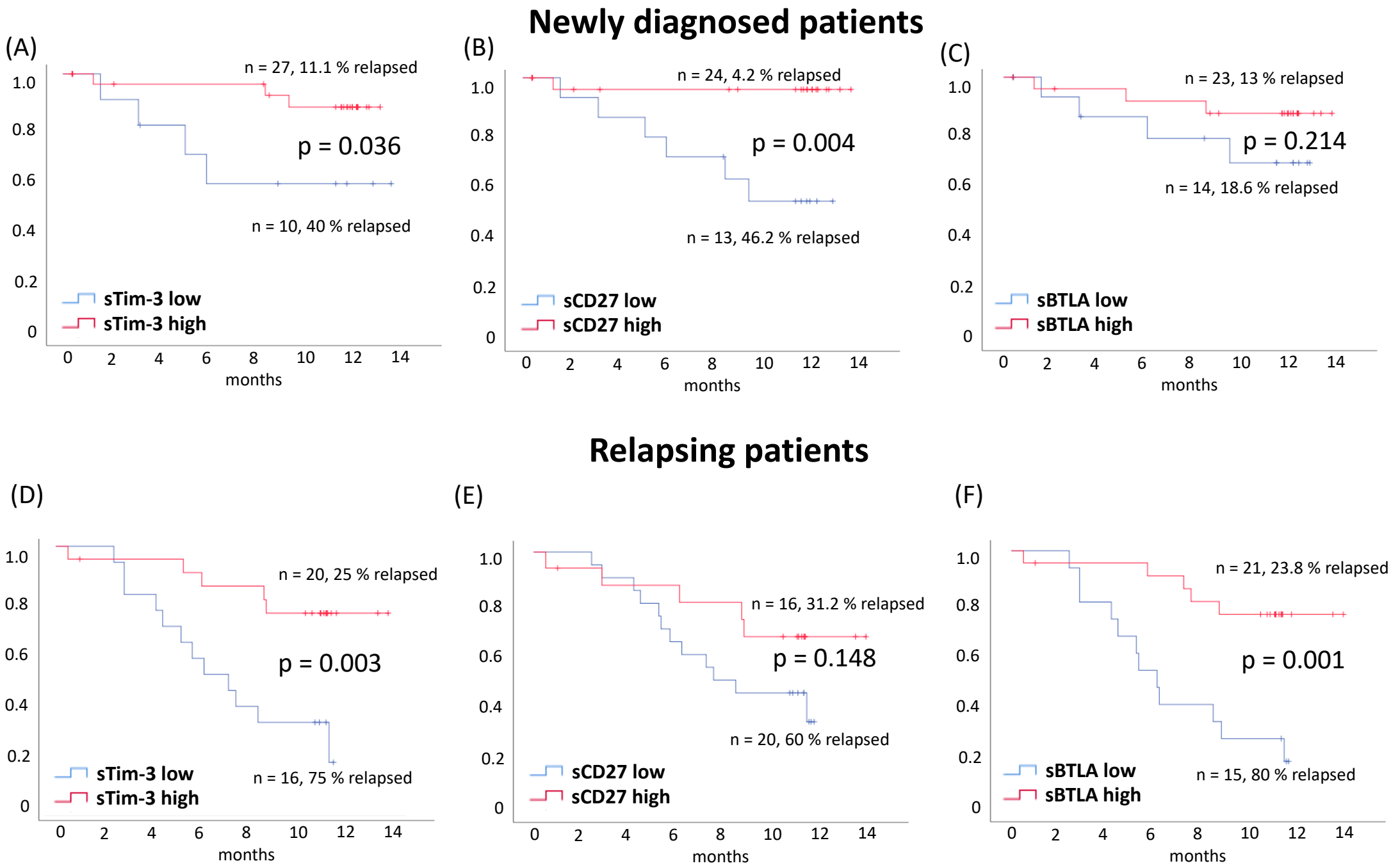
CD8+ T Cells



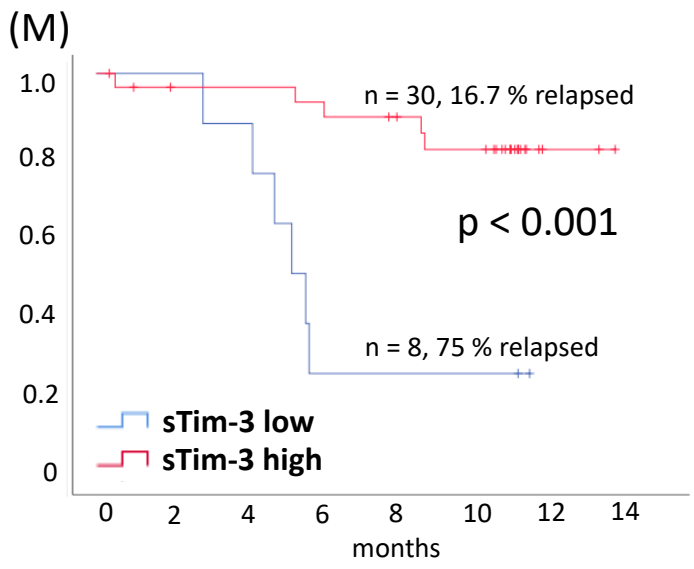
Supplemental figure 2: Sustained remission in rituximab treated patients based on vasculitis subtypes for predefined soluble immune checkpoint expressions

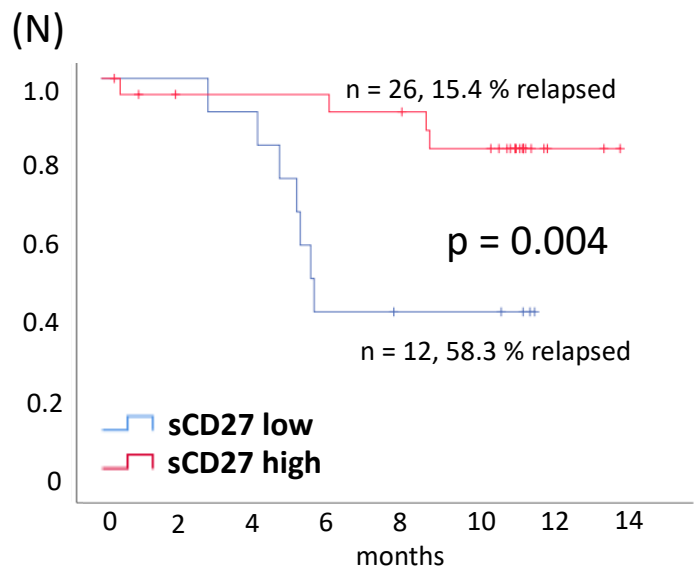


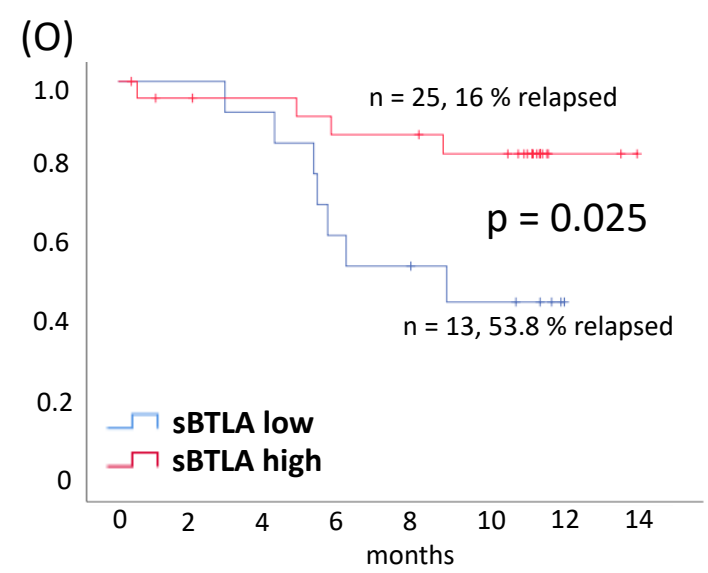
Supplemental figure 3: Subcohort analyses of sustained remission in rituximab treated patients for predefined soluble immune checkpoint expressions



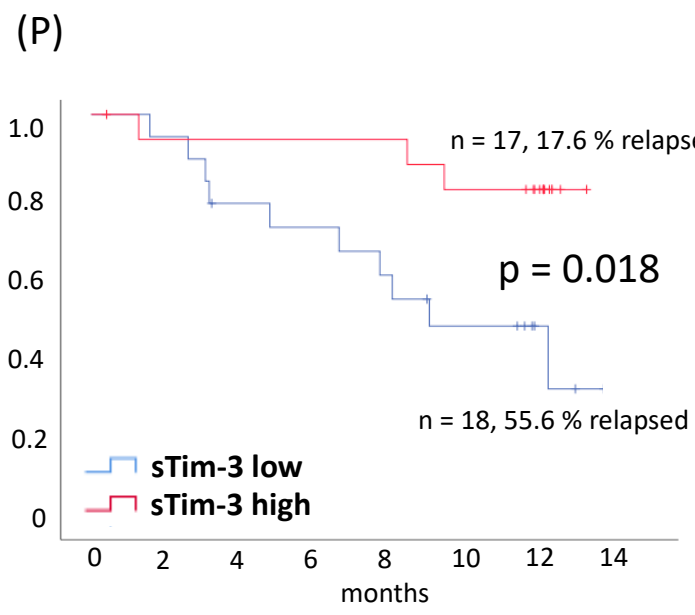
Acute kidney failure

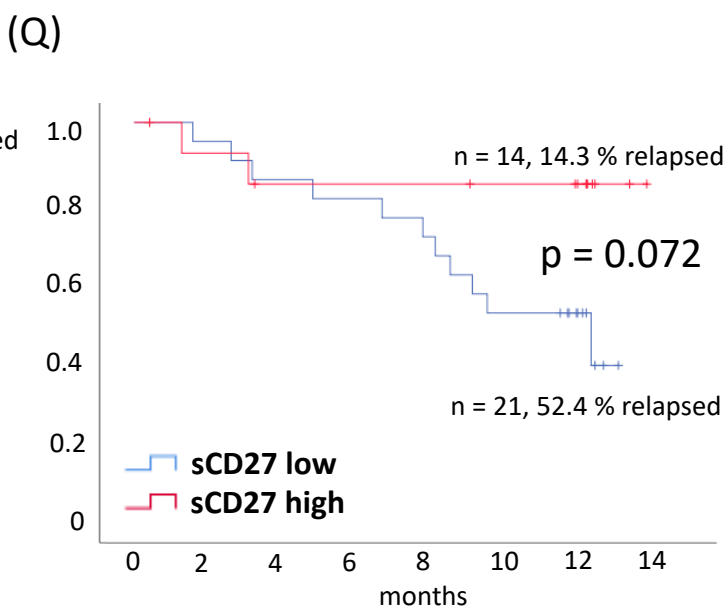


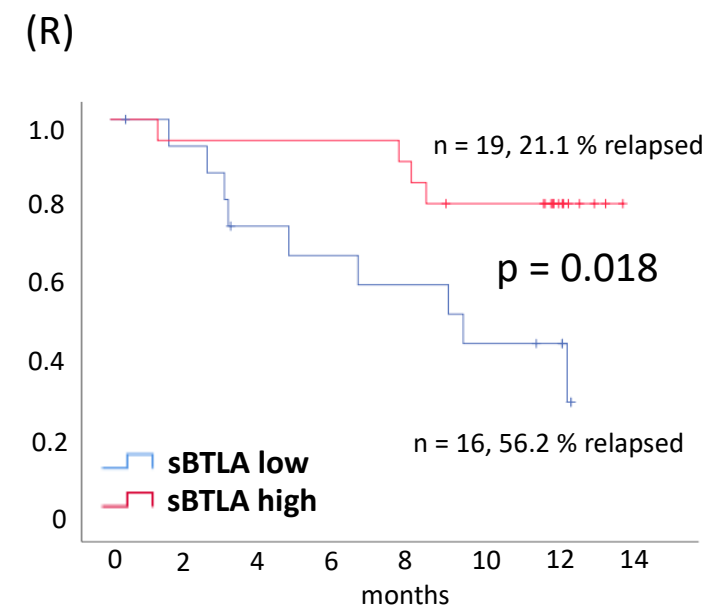




No kidney failure







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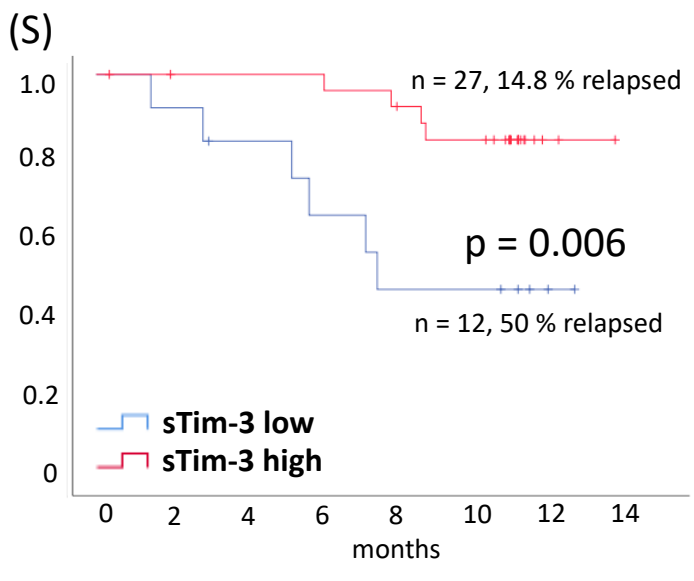
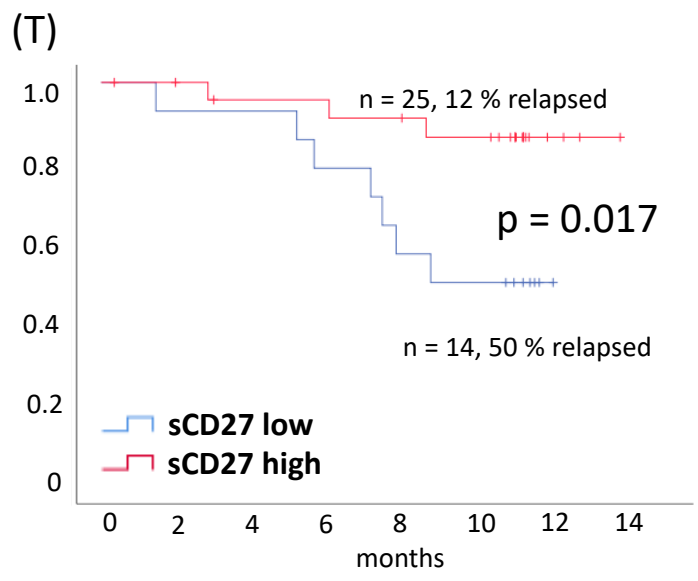
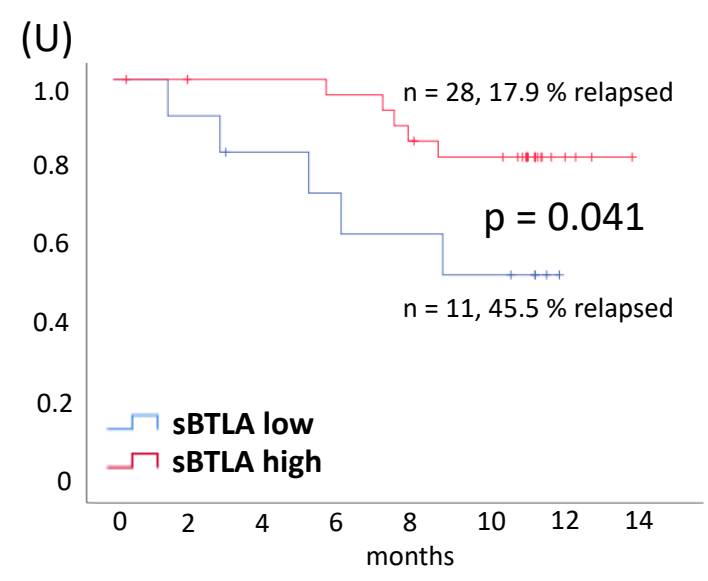
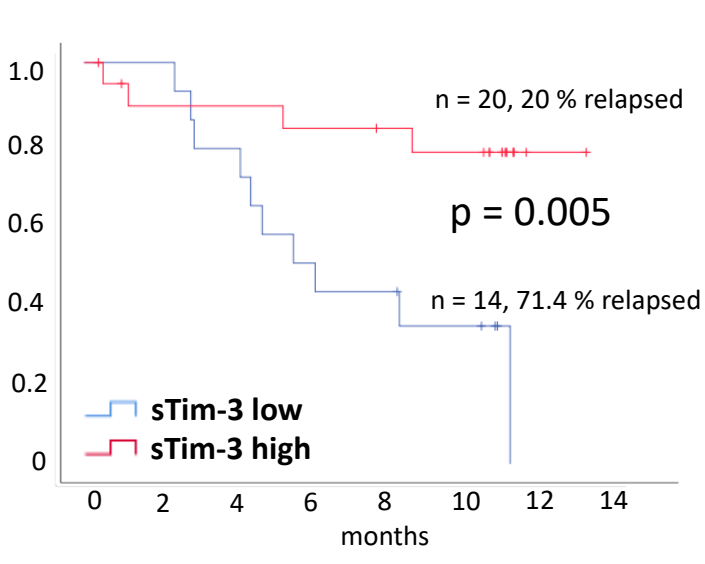
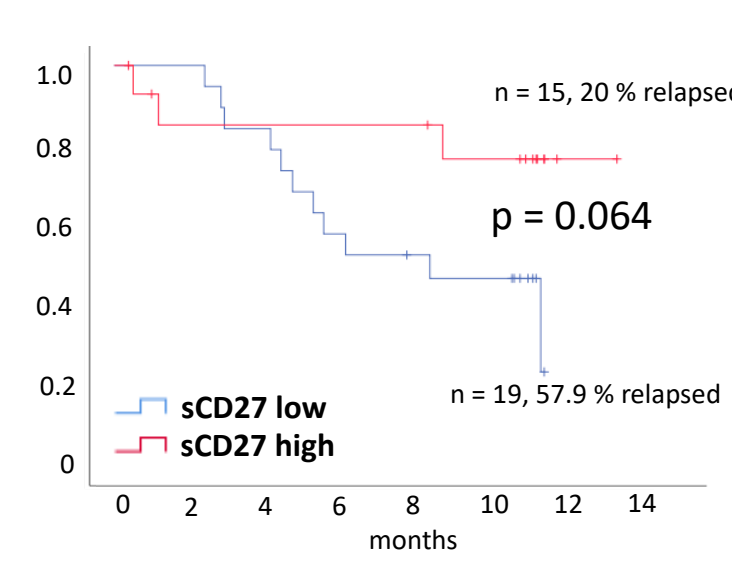


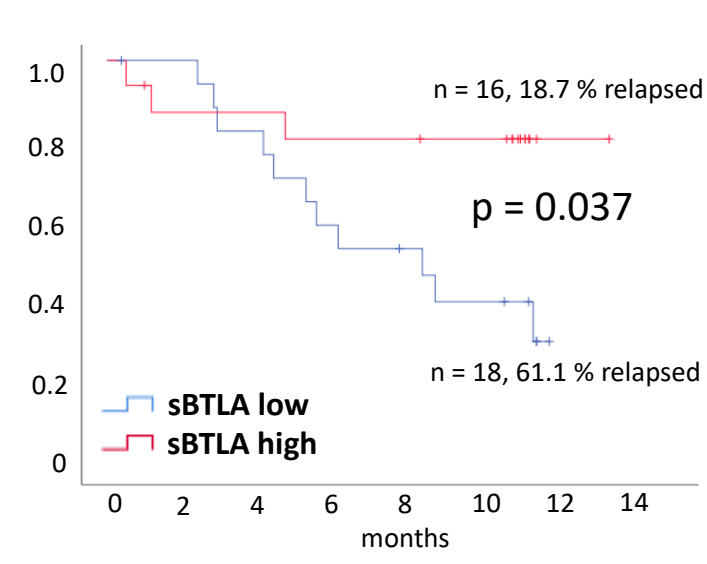




Male

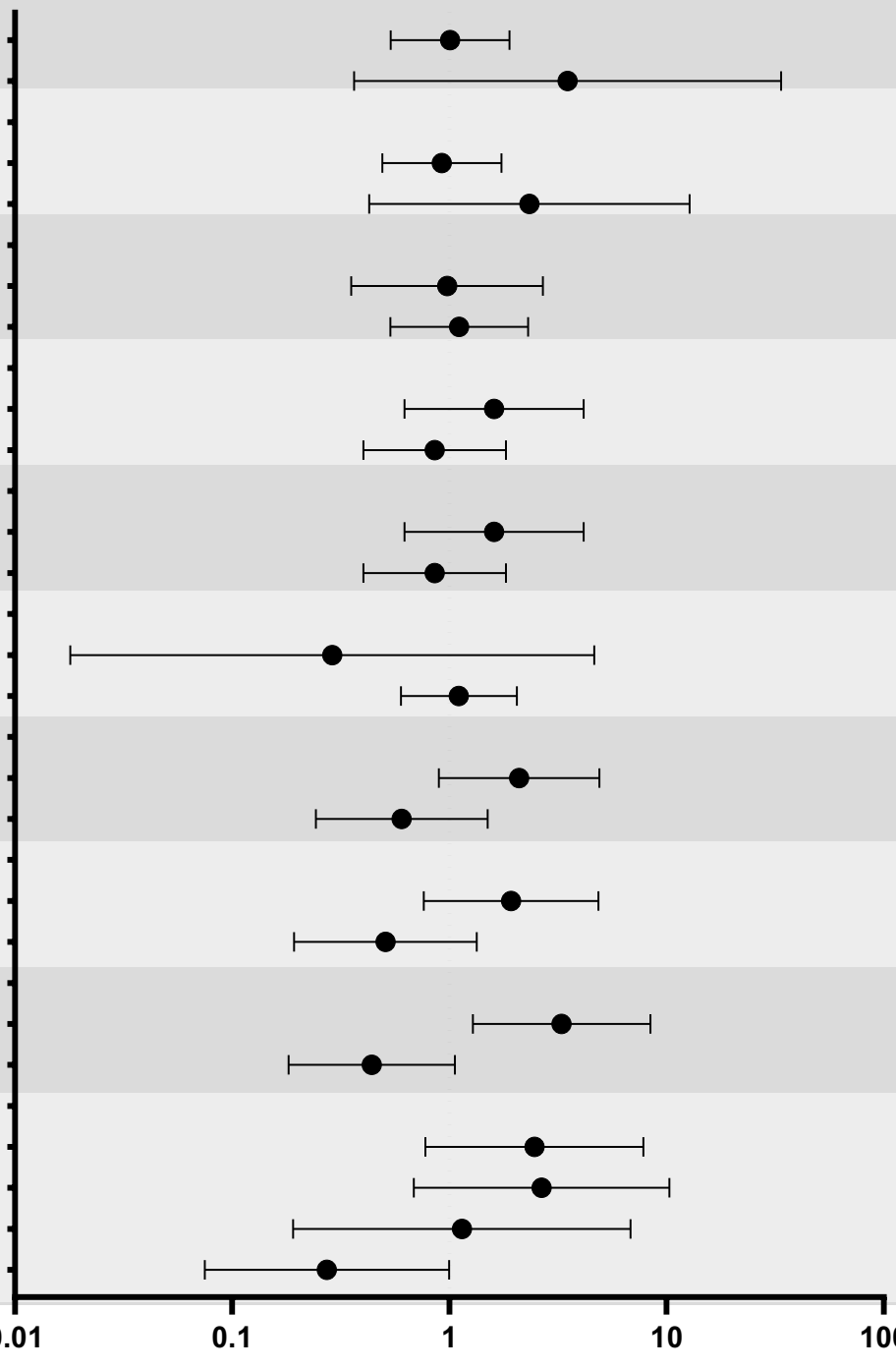






Supplemental figure 4: Risk stratification for sustained remission by treatment group

	n=	OR	Lower	Upper	p- value
AAV-Type					
GPA	108	1,010	0,538	1,896	0,975
MPA	37	3,505	0,364	33,705	0,278
ANCA					
PR3	95	0,924	0,492	1,736	0,806
MPO	51	2,339	0,428	12,775	0,327
NewlyDiagnosed					
Yes	77	0,977	0,354	2,695	0,964
No	69	1,110	0,535	2,306	0,779
Acute kidney failure					
Yes	77	1,609	0,623	4,151	0,326
No	69	0,856	0,402	1,822	0,687
Alveolar haemorrhage					
Yes	36	1,609	0,623	4,151	0,326
No	110	0,607	0,204	1,807	0,370
Malignancy					
Yes	5	0,289	0,018	4,648	0,381
No	141	1,107	0,599	2,046	0,745
sTim-3					
Low (< 1200 pg/ mL)	50	2,098	0,896	4,911	0,088
High (< 1200 pg/ mL)	89	0,604	0,243	1,503	0,279
sCD27					
Low (< 1250 pg/ mL)	54	1,926	0,764	4,860	0,165
High (> 1250 pg/ mL)	85	0,509	0,193	1,339	0,171
sBTLA					
Low (< 1000 pg/ mL)	58	3,290	1,284	8,431	0,013
High (> 1000 pg/ mL)	81	0,440	0,182	1,063	0,068
sTim-3, sCD27, sBTLA					
All marker low	22	2,465	0,777	7,822	0,126
2 marker low	29	2,661	0,687	10,306	0,157
1 marker low	38	1,142	0,191	6,837	0,885
No marker low	50	0,273	0,075	0,997	0,050

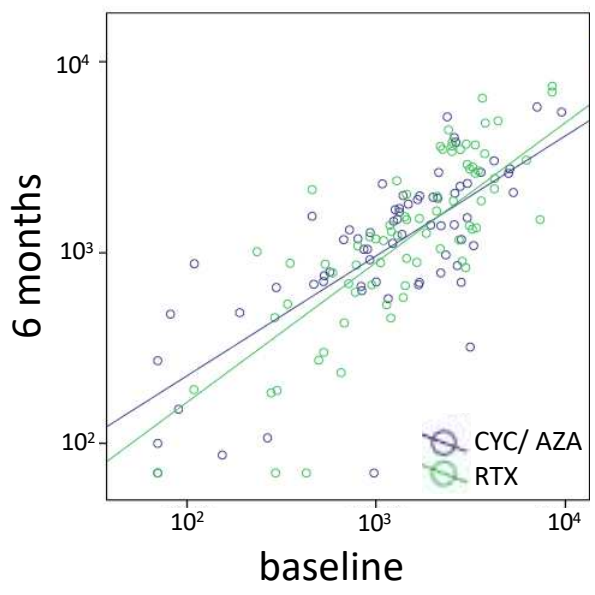


Favours RTX **Favours CYC/AZA**

Supplemental figure 5: Longitudinal expression: Correlation of expression levels of sTim-3, sCD27 and sBTLA between baseline and 6 months or 18 months.

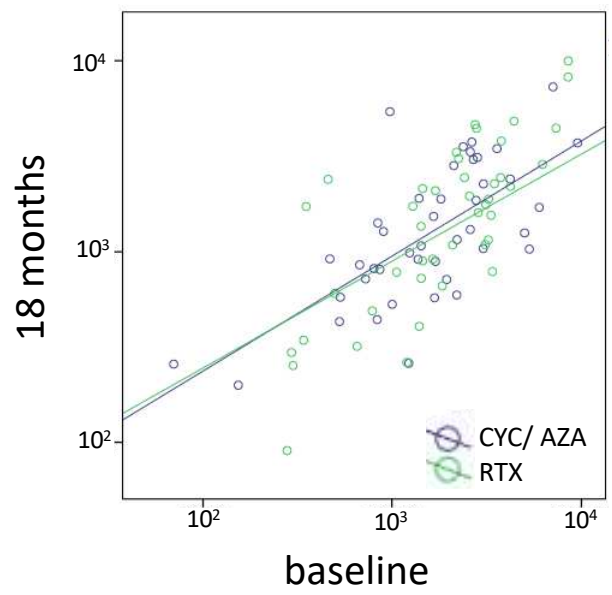
(A) Levels of sTim-3 over time

n = 145



CYC / AZA (n = 68):
Correlation = 0.686
p-value < 0.001
RTX (n = 77):
Correlation = 0.741
p-value < 0.001

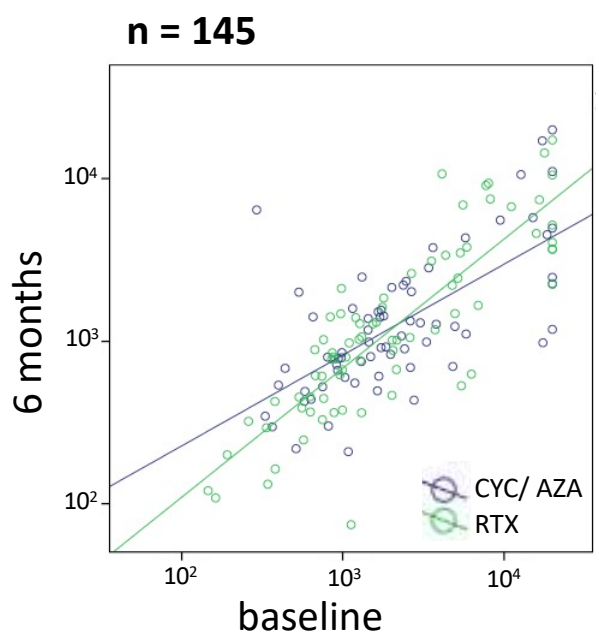
n = 87



CYC / AZA (n = 43):
Correlation = 0.670
p-value < 0.001
RTX (n = 44):
Correlation = 0.621
p-value < 0.001

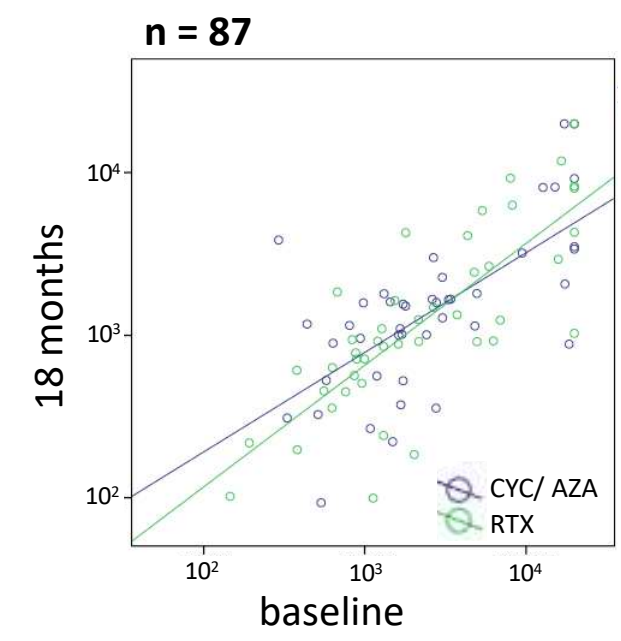
(B) Levels of sCD27 over time

n = 145



CYC / AZA (n = 68):
Correlation = 0.637
p-value < 0.001
RTX (n = 77):
Correlation = 0.836
p-value < 0.001

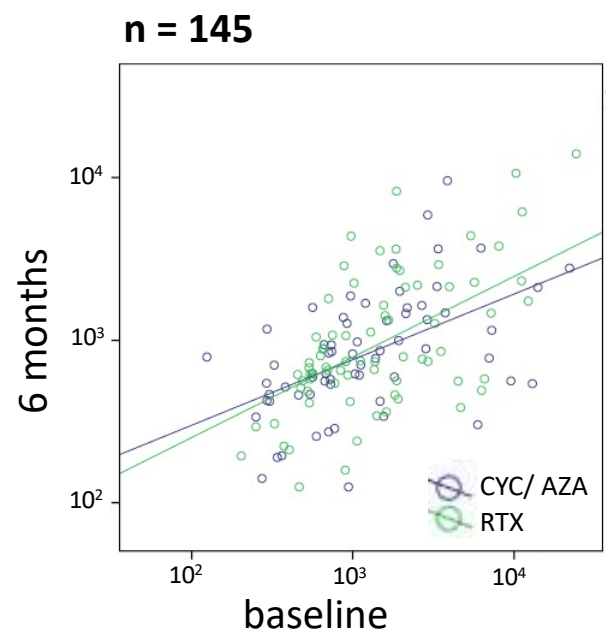
n = 87



CYC / AZA (n = 43):
Correlation = 0.637
p-value < 0.001
RTX (n = 44):
Correlation = 0.791
p-value < 0.001

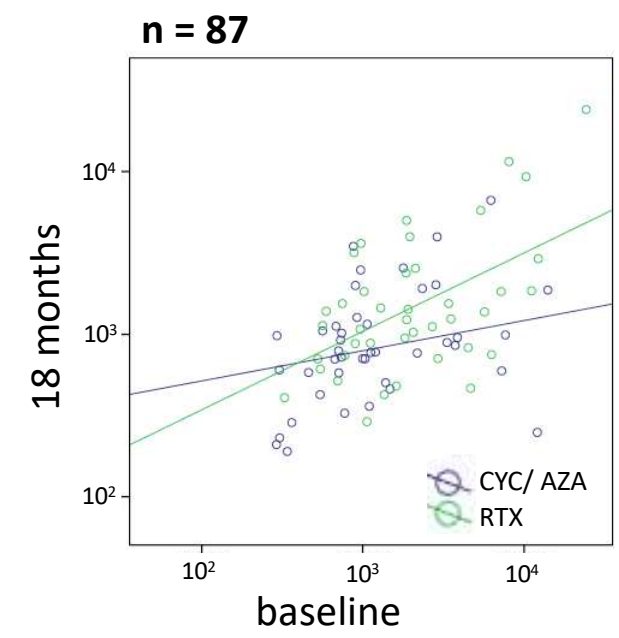
(C) Levels of sBTLA over time

n = 145



CYC / AZA (n = 68):
Correlation = 0.564
p-value < 0.001
RTX (n = 77):
Correlation: 0.534
p-value < 0.001

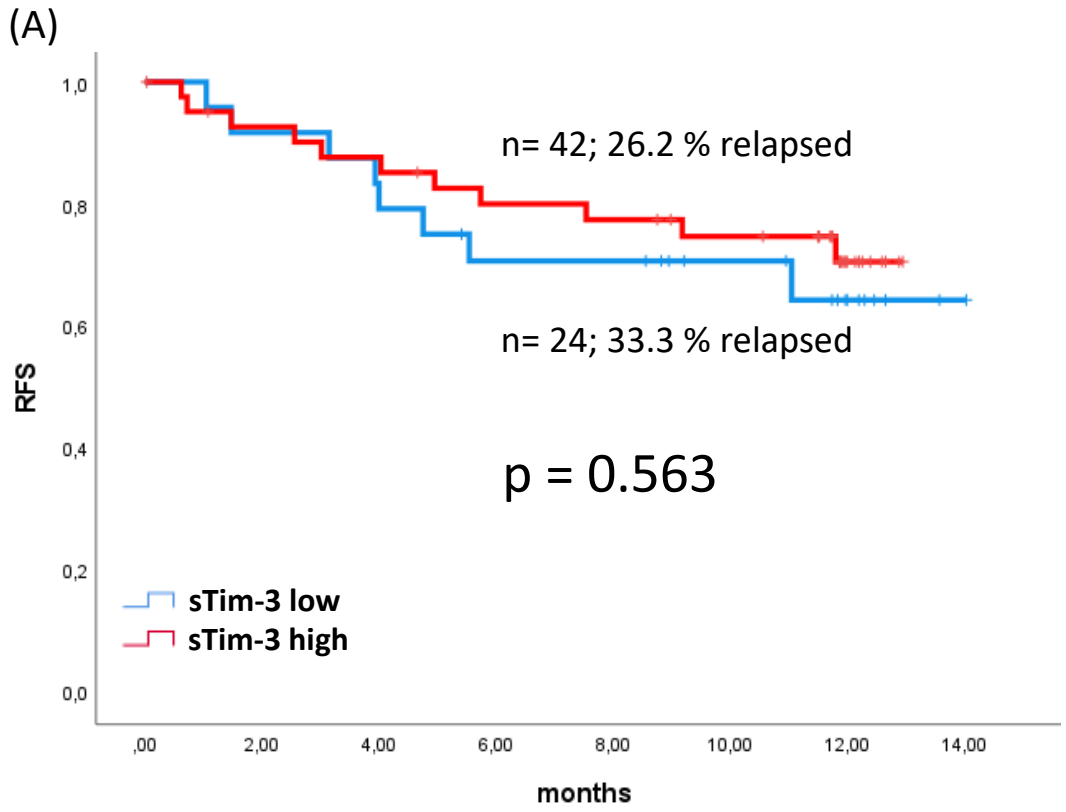
n = 87



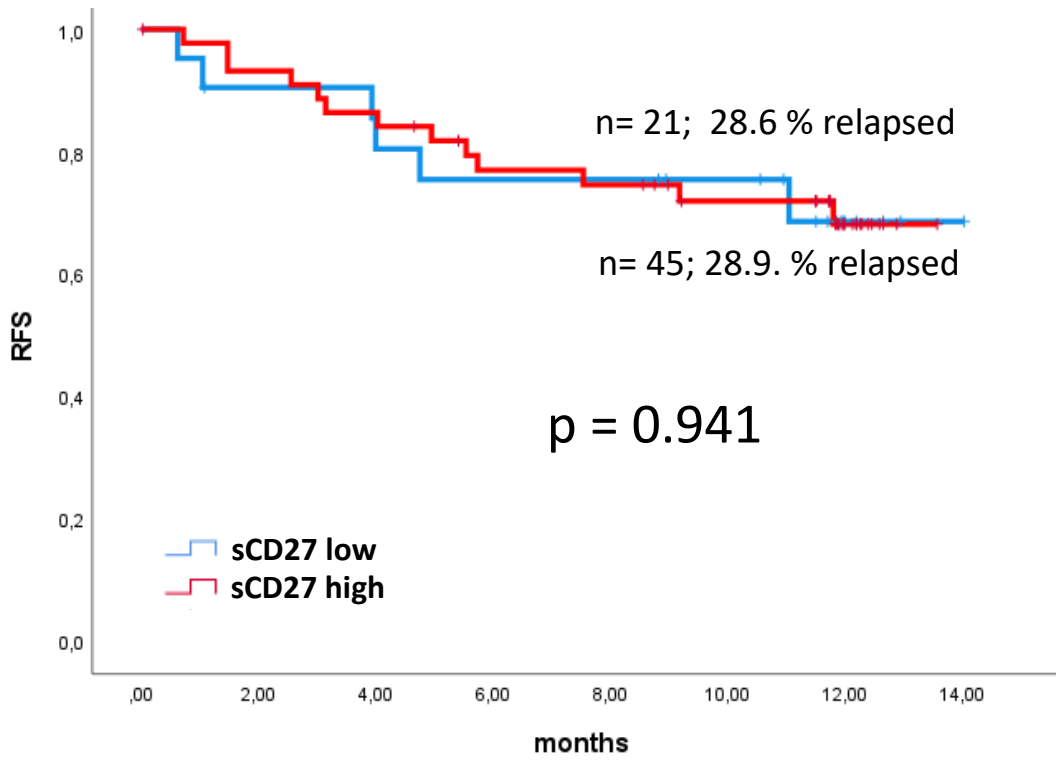
CYC / AZA (n = 43):
Correlation = 0.343
p-value = 0.024
RTX (n = 44):
Correlation = 0.429
p-value < 0.001

Supplemental figure 6: Analysis of sustained remission in CYC/AZA treated patients for predefined soluble immune checkpoint expressions.

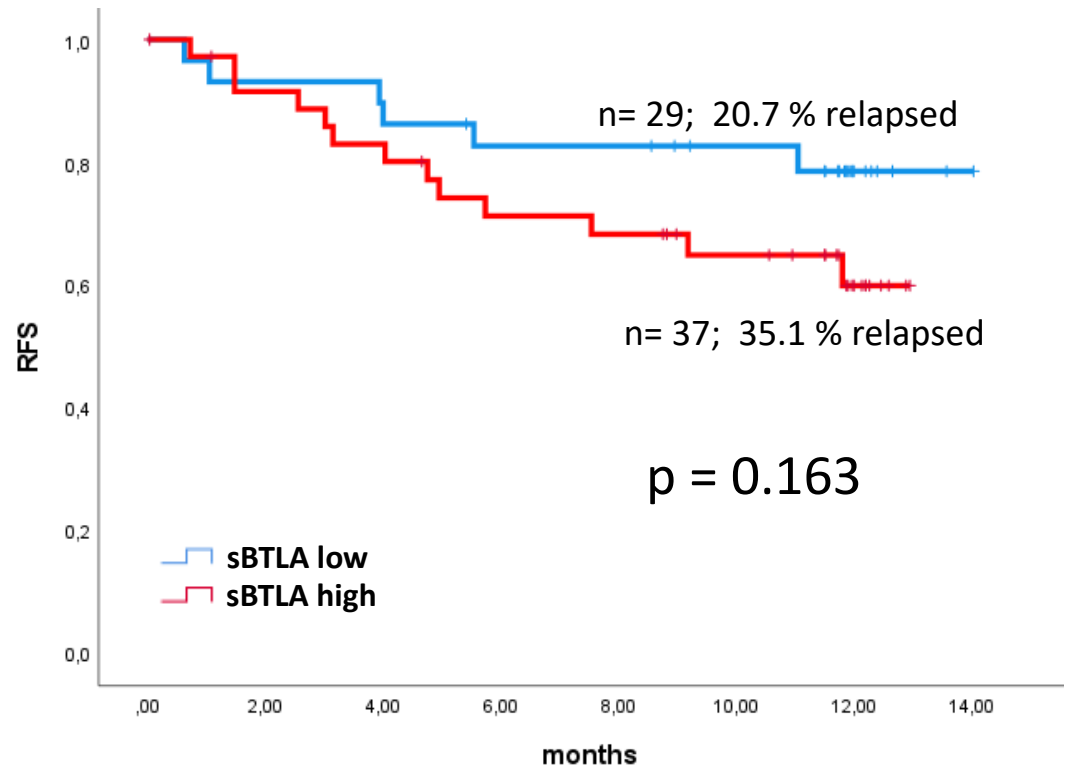
(A)



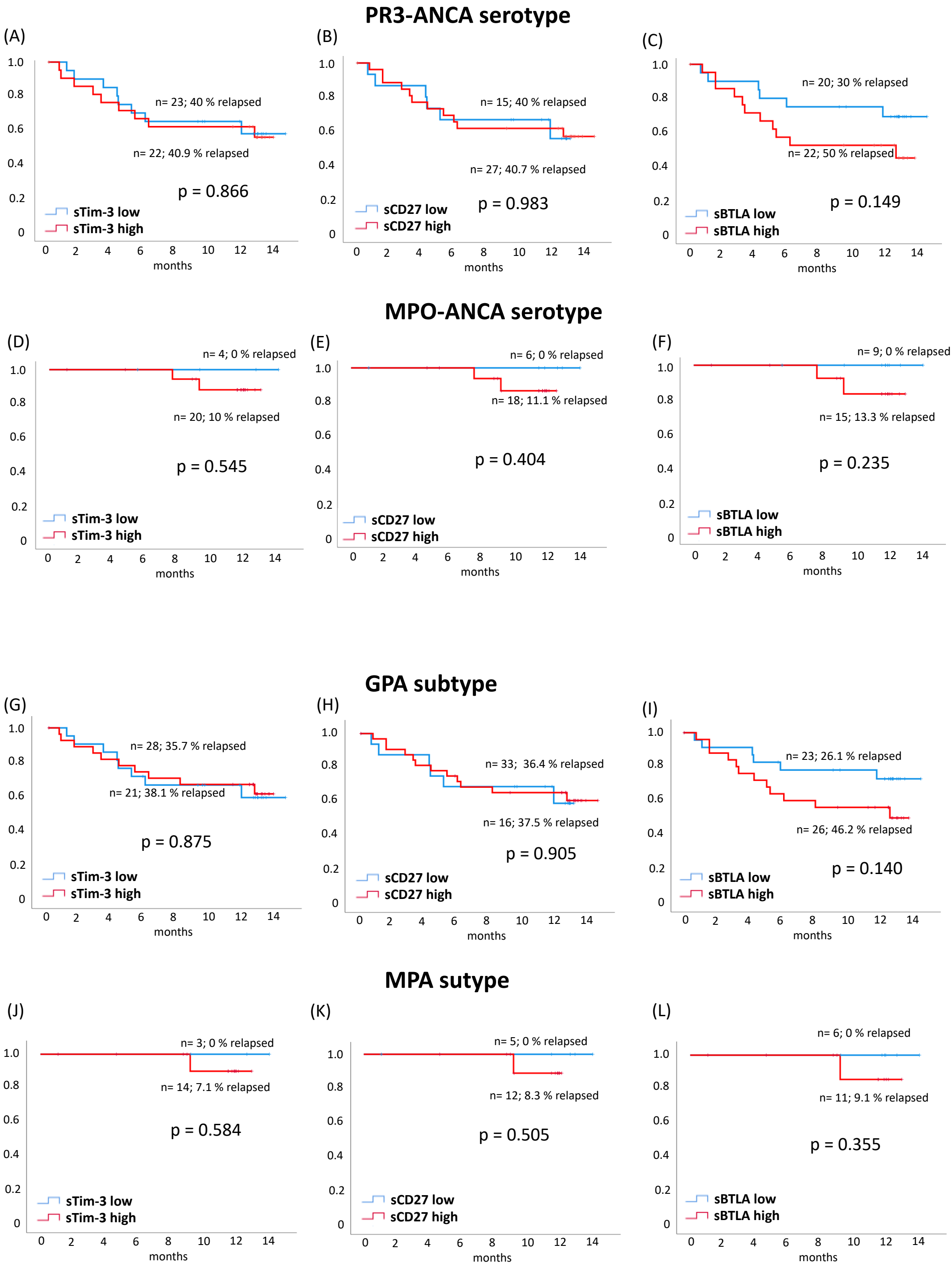
(B)



(C)



Supplemental figure 7: Sustained remission in CYC/AZA treated patients based on vasculitis subtypes for predefined soluble immune checkpoint expressions.



Supplemental figure 8: Subcohort analyses of sustained remission in CYC/AZA treated patients for predefined soluble immune checkpoint expressions.

Supplemental figure 9: Subcohort analyses of sustained remission in CYC/AZA treated patients for predefined soluble immune checkpoint expressions.