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A Phase I Study of the Combination of Rituximab and Ipilimumab in Patients with Relapsed/Refractory B-Cell Lymphoma

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Abstract

Purpose: Based on the potential for Ipilimumab (I) to augment T cell activation, we hypothesize that ipilimumab would augment the efficacy of rituximab (R) in patients with relapsed/refractory (R/R) CD20+ NHL. This phase 1 study aimed to identify a recommended phase 2 dose (RP2D), document toxicities, and preliminarily assess efficacy and potential predictive biomarkers.

Patients and methods: Thirty-three patients with R/R CD20+ B-cell lymphoma received R at 375mg/m^2 weekly for 4 weeks and I at 3mg/kg on day 1 and every 3 weeks for 4 doses. Responding patients went on to maintenance with each agent given every 12 weeks. To facilitate correlative analysis, the expansion phase randomized patients to simultaneous R+I vs. R with I delayed 2 weeks.

Results: Toxicity was manageable; no dose limiting toxicity was observed at the doses studied. When considering the entire cohort, efficacy was modest, with an objective response rate (ORR) of 24% and median progression-free survival (PFS) of 2.6 months. However, in follicular lymphoma (FL) patients, the ORR was 58% with a median PFS of 5.6 months. The randomized

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comparison of R to R+I demonstrated that R+I resulted in more effective B cell depletion (BCD). Both B cell depletion and the ratio of CD45RA- Treg to Treg were associated with response at all time points.

Conclusion: The combination of R+I has manageable toxicity and encouraging efficacy in R/R FL. The ratio of CD45RA$^-$ Tregs to total Tregs, and peripheral BCD should be studied further as potential predictors of response.

INTRODUCTION

Rituximab as a single agent produces an ORR in relapsed/refractory indolent lymphoma of approximately 20–50%[1]. While the specific mechanism of action of rituximab is likely multifactorial and incompletely understood, most agree that host immune effector mechanisms are critical. To this end, there have been many attempts to utilize agents in combination with rituximab that augment these host immune effector mechanisms, including IL-2, IL-12, IFN, and cpg [2–5], which have produced modest improvements in efficacy, often with considerable toxicity. Lenalidomide is an immune modulator that is a potent NK and T cell stimulant and has demonstrated efficacy in NHL and CLL [6]. The combination of rituximab and lenalidomide has produced considerable activity in patients with both treatment naive and previously untreated mantle cell and follicular lymphoma; ORR ranging from 57% in rituximab refractory patients to over 90% in previously untreated FL [7,8.]

Immunotherapeutic approaches to cancer therapy, including immune checkpoint inhibition (ICI), have produced exciting results in both solid tumors and hematologic malignancies, reversing T cell anergy and facilitating an effective T cell-mediated anti-tumor response. Cytotoxic T lymphocyte associated protein 4 (CTLA4) is a major negative regulator of the immune system. CTLA4-blocking monoclonal antibodies, like ipilimumab activate anti-tumor T cells by obstructing their negative regulation, allowing for unopposed T cell activation [9–11]. Ipilumumab may also affect the tumor microenvironment by varied mechanisms, including the depletion of intratumoral CTLA4-expressing Treg cells,[12,13] which has been correlated with response in melanoma and colon cancer patients. Based on these observations, we hypothesize that ipilimumab may enhance host immune effector mechanisms and thereby augment the efficacy of rituximab.

The primary objective of this study was to evaluate the toxicity associated with adding ipilimumab to rituxumab for the treatment of patients with recurrent/refractory histologically confirmed CD20+ B cell lymphoma, and to establish a maximum tolerated dose (MTD) and/or recommended Phase II dose (RP2D). Secondary objectives were to conduct mechanistic studies to understand the effect of this combination on the immune system, and to collect clinical data on anti-tumor response/overall response rates (ORR: complete + partial), and on PFS.

METHODS

Patients

Patients ≥18 years of age with relapsed or refractory CD20 positive NHL that were ineligible for high-dose chemotherapy and/or hematologic stem cell transplantation (SCT) or

any other established curative therapy were eligible for this California Cancer Consortium study. Further inclusion criteria were Karnofsky performance status ≥ 70 and signed informed consent. Patients were excluded from the study in case of central nervous system involvement, prior allogeneic SCT, known HIV or hepatitis B or C virus infection, treatment with steroids or another investigational agent within 4 weeks, previous anti-PD-1 antibody, CD137 agonist or other immune activating therapy unless 5 half-lives have intervened (minimum 8 weeks). Patients on steroids or other immune suppressants or patients with autoimmune disease were excluded.

Protocol Treatment

During the 12-week induction, ipilimumab was administered every 3 weeks for 4 doses and rituximab was given every week for 4 weeks. In responding patients with acceptable toxicity, induction was followed by maintenance, during which ipilimumab and rituximab were given together every 12 weeks for 1 year, until unacceptable toxicity or disease progression. On days when both drugs were administered, rituximab was given before ipilimumab.

Study design

All relevant institutional review boards or ethics committees approved the research methods used in these studies, and all patients provided written informed consent prior to enrolling. The studies were conducted in accordance with general ethical principles outlined in the Declaration of Helsinki, the International Conference on Harmonization guidelines, and Title 21 of the US Code of Federal Regulations and registered at www.clinicaltrials.gov as NCT01729806.

This trial began with a dose escalation, followed by an expansion at the RP2D. Initially, two dose-levels of the ipilimumab were planned (3 and 10 mg/kg) during escalation; additional dose-levels of 1 and 5 mg/kg were to be included if 3 or 10 mg/kg, respectively, exceeded the MTD. (Supplementary Table 1); the dose of rituximab was fixed at 375 mg/m^2 per dose. Three-plus-three (3+3) rules for dose escalation were used to decide whether to escalate, expand, or de-escalate the dose of ipilimumab. The dose-limiting toxicity (DLT) observation period was defined as the first 6 weeks of induction which included the first 2 doses of ipilimumab and first 4 doses of rituximab. The MTD was based on toxicities observed during the DLT observation period and was defined as the highest dose tested in which only 0 or 1 patient of 6 patients evaluable for toxicity at that dose experienced DLT attributable to the study drugs. To be evaluable for toxicity, a patient must have received at least two doses of ipilimumab and 4 doses of rituximab and be observed for at least 3 weeks after the second dose of ipilimumab or have experienced a DLT. All patients enrolled were fully followed for toxicity for the duration of the study, but patients who were not evaluable for dose-escalation decisions were replaced.

In the expansion cohort, an additional 20 patients were to be treated at the RP2D to further evaluate safety/toxicity, to obtain preliminary estimates of the objective response/remission rate and PFS, and to compare the immune response to rituximab with and without concurrent ipilimumab, as measured by immune subset analysis, antibody dependent cell mediated cytotoxicity (ADCC) based on the kinetics and magnitude of B cell depletion

(BCD). These additional patients were randomized 1:1 to one of two schedules: on Arm A, ipilimumab was given on Day 1 together with the first dose of rituximab, and on Arm B, ipilimumab was first given on Day 15 together with the third dose of rituximab (Supplementary Figure 1). This permitted the assessment of whether or not I enhanced R-mediated ADCC during the first 15 days of treatment.. The sample size of 20 (10 per schedule) evaluable patients was selected to ensure at least 80% power, using a one-sided 0.10-level two-sample t-test, when the true difference, when comparing R+I vs. R, in the change (increase in activated T cells and ADCC, or decrease in B-cells) exceeded one standard deviation – where the standard deviation is intrinsic variability between patients in terms of the change. Blood for correlative analysis was drawn pretreatment on days 1, 8, 15, 60 and 90. During the expansion, safety boundaries using a modified sequential probability ratio test were used to flag an excessive number of DLTs.

Response Criteria

Objective response was assessed according to the revised response criteria for malignant lymphoma [14]. To be evaluable for response, a patient must have received at least two doses of ipilimumab and 4 doses of rituximab. Imaging to assess tumor burden and response to treatment was done prior to treatment and every 8 weeks after start of treatment.

Safety

Toxicities were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE, Version 4.0, http://ctep.cancer.gov). Hematologic DLT was defined as any of the following adverse events (AEs) considered by the investigator to be related or possibly related to one of the study drugs: grade 3 thrombocytopenia with hemorrhage and/or requiring transfusion, grade 4 thrombocytopenia regardless of duration, grade 3 thrombocytopenia without hemorrhage lasting ≥ 28 days, grade 3 thrombocytopenia with potentially life-threatening morbidity, grade 3 or 4 neutropenia lasting ≥ 28 days (despite the use of growth factors), or grade 3 or 4 neutropenia with potentially life-threatening morbidity. Non-hematologic DLT was defined as any grade 3 or greater non-hematologic toxicity, except immune-related adverse events (irAEs). Grade 3 irAEs, which included inflammatory autoimmune events involving the gastrointestinal, skin and nervous systems, were not DLT if they resolved to grade 1 or baseline with adequate steroid treatment tapered to maintenance or replacement doses (typically ≤ 10 mg/day) within 28 days and did not involve severe events such as bowel perforation or events requiring life-saving interventions. The inability to complete at least 2 doses of Ipilimumab and 4 doses of rituximab for reasons of toxicity or lack of tolerability was a DLT unless caused by the 28-day resolution period for irAE's that eventually resolved.

Statistical Methods: Clinical Data

Standard descriptive statistics were used to summarize clinical results. PFS was calculated as the time from start of treatment to the date of progression or death, whichever came first; patients who were alive and free of progression were censored at the date that their status was last documented; patients who started another therapy prior to progression were censored at that time. The Kaplan-Meier product limit method was used to display the PFS

pattern over time. Median PFS and PFS probabilities were based on Kaplan-Meier plots; associated standard errors for the probabilities were based on Greenwood's formula[15].

Statistical Methods: Correlative studies

Two analyses were undertaken: (1) to compare the two schedules in terms of increase in activated T cell subsets and ADCC, (assessed based on BCD), and (2) to explore whether any immune measures had the potential to identify patients who were more or less likely to response to R+I. Establishing an association between a biomarker and response to treatment is the first step in identifying potential predictive biomarkers; further studies will be necessary to determine whether this association translates into a useful predictive biomarker of response.

Graphical methods were used to display the results: means or medians, as appropriate, were plotted along with confidence intervals or ranges or interquartile ranges; boxplots were also plotted. Samples for analysis of plasma cytokine levels and subset populations of T cells, were collected repeatedly over time. To quantitatively evaluate the patterns over time, a mixed effects linear regression model was constructed using log cytokine concentration (pg/ml) and log T cell subset counts as the dependent variables. Time (as a categorical variable) and response to therapy were set as fixed effects, as was the interaction term, and patient was set as random effect. In these mixed effect linear regression models, none of the interaction terms were statistically significant, indicating that differences between responders and non-responders, if they existed, tended to be constant over time. These interaction terms were dropped from the final models. To control for multiple testing, the Bonferroni method adjustment was used.

RESULTS

Participant characteristics

Between November of 2012 and June of 2016 33 patients with relapsed or refractory CD20 positive NHL were enrolled on this multi-institutional Phase I trial. The data cut-off for this manuscript was 5/1/2018. The median age was 62 and the median number of prior treatment regimens was 4. The majority (60%) had follicular lymphoma (FL) or DLBCL and 33% had failed an autologous SCT. Overall 87% were considered refractory to anti-CD20 therapy including 92% of FL patients. Patients were considered refractory to anti-CD20 therapy if they had progressed on or within 6 months of being treated with an anti-CD20 therapy-containing regimen. A full summary of patient demographics and characteristics can be found in Table 1.

Safety

The first 3 patients treated at Dose Level 1 (3 mg/kg of ipilimumab) were evaluable for DLT and one patient experienced DLT (prolonged diarrhea not successfully managed by steroid treatment); per the 3+3 rules this dose level was expanded to enroll 3 more evaluable patients. Five more patients were treated with 3 evaluable for DLT and 2 inevaluable for DLT (one patient died in less than 6 weeks due to disease progression, and another patient did not receive the 2nd dose of ipilimumab which was held for non-DLT Grade 2 diarrhea);

none of these 5 patients experienced DLT. With only 1 of the 6 evaluable patients experiencing DLT, consideration was given to escalating the dose of ipilimumab. After review of all toxicities during induction and maintenance, as well as the results of other trials comparing 3 mg to 10 mg/kg in melanoma patients and discussion with the NCI/Cancer Therapy Evaluation Program, a decision was made to consider 3 mg/kg the RP2D and to use this dose for the expansion cohort.

In the expansion cohort, 25 patients were enrolled and randomized to either Arm A or Arm B. Of the 13 patients randomized to Arm A, 10 patients were confirmed evaluable for DLT and none of these patients experienced any DLT's; one patient experienced an immune related adverse event (irAE) – grade 3 diarrhea. Of the 3 inevaluable patients, 2 went off early for disease progression and a 3rd patient received steroids in the absence of irAE's.

Of the 12 patients randomized to Arm B, 2 went off treatment early (one of whom experienced toxicities prior to receiving any ipilimumab and one who went to hospice) and thus 10 patients on Arm B were confirmed evaluable for DLT; 2 of these 10 experienced DLT including prolonged neutropenia and a grade 3 skin infection. In addition, 2 patients experienced Grade 3+ irAEs: two patients experienced grade 3 diarrhea and another patient experienced grade 3 hypoxia (Table 2). Hematologic toxicity was modest with 4 patients (12%) having grade 3 anemia and 1 patient with febrile neutropenia (comprehensive toxicity assessment, Supplementary Table 2)

Efficacy

Eight of the 33 treated patients (24%) achieved a response, with 2 achieving a CR (6%); in addition, 6 (18%) patients had SD, corresponding to a disease control rate of 42%, (Table 3). Eleven (33%) had disease progression as the best response and 8 came off too early for disease evaluation. When considering the entire cohort of 33 patients, the median (95% CI) PFS was 2.6 months (1.6, 4.6 months) (Figure 1A), with a median follow-up time of 5.5 months among the 9 who were censored (range: 0.5 – 18.5 months). Of the 13 patients with FL, 7 responded (54%), 2 with CR (15%); the median (95% CI) PFS was 5.6 months (1.6, 18.4+ months), (Figure 1B). Considering the entire cohort, 27 of 33 patients (87%), and 12 of the 13 patients (92%) with FL were considered refractory to anti-CD20 therapy

Correlative Analyses

Blood for correlative analysis was drawn pretreatment and on days 1, 8, 15, 60 and 90. This permitted the measurement of plasma cytokine levels (17 cytokines) by the Multiplex Bead-based Luminex® platform and lymphocyte subsets (T cells, B-cells), as well as T cell subset populations by flow cytometry. For these analyses, blood was available for 18 patients – 5 responders and 13 nonresponders.

Comparison of the Two Schedules—Randomization during the expansion cohort allowed for testing the hypothesis that the magnitude of B cell depletion (BCD) would be increased when I and R were administered simultaneously. B cell levels were compared in patients randomized to initial treatment with the combination of R + I (group A) versus R and delayed I (group B) (Figure 2A). Although group A had fewer B cells on D8 and D15

when compared to group B (Figure 2A), this reduction did not reach statistical significance [p-value = 0.15 (linear mixed effects model) and p-value = 0.08 (D8 student's *t*-test)]. However, the increase in B cell reduction in group A (R + I) is consistent with enhanced R-mediated ADCC in group A (Figure 2A). Also of interest is that the initial effect of simultaneous R+I persisted through Weeks 10 and 12 after patients on both arms were receiving ipilimumab, although this difference was attenuated. This ultimately will need to be explored further in future studies given that this trial was not sufficiently powered to detect this difference.

Exploratory Analyses to Identify Potential Predictive Biomarkers—Exploratory and descriptive analyses were also planned to identify patterns which would explain the treatment effects, or which would merit further study with the goal of identifying a potential predictive biomarker.

Plasma cytokine analysis—For the 17 plasma cytokines measured, mean values (+/− SD) were graphed against time according to response and based on the arm with the expansion phase (Supplementary Figure 2). Visually, the plasma concentrations of IL-2 and TNF stood out as being consistently higher in non-responders, when compared to responders (Supplementary Figure 2A). This difference was most pronounced on day 70 (p=0.044 and 0.050, TNF and IL-2, respectively; Supplementary Figure 2B). In these analyses none of the cytokine associations (Supplementary Figure 2 and Supplementary Table 3) remained significant after adjustment for multiple testing using the Bonferroni method.

Immune subset analysis—For each of subset populations of T cells, the log transformed counts (+/− SD) were graphed against time for responders and non-responders (Figure 2B and C; gating strategy shown in Figure 2B and Supplementary Figure 3). This revealed that increased percentages of naïve CD8+ T cells and IFN-γ-secreting CD8+ T cells were associated with response to therapy at the first 2 timepoints (Figure 2C, D, and E). In contrast, effector CD4+ T cells or effector memory CD8+ T cells were increased in non-responders (Figure 2C and E). NK T cells, invariant NK T cells, and NK cells did not appear to be associated with response to therapy (Supplementary Figure 4).

The linear mixed effect model revealed that B cells and T cells (both quantified as log percent of PBMC) were changed over time (p = 0.005 and 0.015, respectively). B cells were calculated as the percentage of live lymphocytes that were CD19 positive and HLADR positive. The gating strategy used to identify live lymphocytes is presented in Supplementary Figure 3 and the gating strategies to identify T cells and B cells are presented in Figures 2B and 3B, respectively. In terms of response to therapy, the percentage of B cells ($p \leq 0.001$) and CD4+ effector cells (p = 0.047) were associated with treatment outcome and naïve CD8+ T cells and IFN-γ-secreting cytotoxic T cells trended towards significance (p = 0.064 and 0.094, respectively) (Supplementary Table 4). In terms of actual lymphocyte percentages, pre-treatment B cell percentages were 21.5 (SD 16.1) and 9.0 (SD 6.7) for non-responders and responders, respectively. These levels fell to a post-therapy low of 11.19 (SD 11.5) and 1.4 (SD 0.5), respectively. Of note, responders could be successfully classified from non-responders by their percentages of B cells, even before initiation of therapy (Figure 3D).

In a follow-up analysis, we examined whether the ratio of suppressive Tregs (CTLA4+ CD4+ CD25+ Foxp3+ cells) or the ratio of CD45RA$^-$ Tregs (CD4+ CD25+ Foxp3+ CD45RA$^-$) to total Tregs could better separate responders from non-responders (Figure 4A, B, and C). While when assessed independently no Treg sub-population (Treg, CD45RA$^-$ Treg, suppressive Treg) was strongly associated with response to therapy (Figure 4A), the ratio of CD45RA$^-$ Treg to Treg was significantly elevated in responders when compared to non-responders at all time points (Figure 4A & C) including at baseline (Figure 4D). When normalized to total Treg, no other cellular subset was significantly associated with response to therapy (Supplementary Figure 5) and no other possible paired combinations among all cell populations was clearly associated with response. To assess for the potential role of I-mediated Treg depletion we assessed the predictive potential of the ratio in group A versus group B and no predictive difference was observed (data not shown).

DISCUSSION

While the majority of patients with NHL initially respond to Immuno-chemotherapy, most eventually relapse. Resistance and intolerance to chemotherapy increases over time, making the development of non-chemotherapeutic strategies for NHL of clinical relevance. The use of CD20-targted approaches remains the standard of care both as initial therapy as well as in relapsed disease. Many attempts to improve the effectiveness of antibody-based CD20-targeted therapeutics have focused on strategies to enhance host immune effector mechanisms particularly in patients that are considered refractory to anti-CD20-based therapy.

In the planned dose-escalation phase of this trial, 1 of 6 patients evaluable for toxicity at the ipilumumab dose of 3 mg/kg experienced dose-limiting toxicity. Due to non-DLT adverse events in this trial as well as adverse events experienced at higher doses in other trials, in collaboration with CTEP a Phase 2 dose of 3 mg/kg was recommended without establishing an MTD. An additional 25 patients were enrolled onto the randomized expansion cohort at 3 mg/kg of ipilimumab. The combination had moderate but manageable toxicity. Eight patients came off study due to treatment-related toxicities, the most common being grade 3–4 diarrhea (12%) (Table 2). When considering the entire cohort, efficacy was modest with an overall response rate of 24% and a CR rate of 6%, with a median PFS of 2.6 (1.6, 4.6) months. However, patients with FL, 92% of whom were refractory to prior anti-CD20-based therapy, had an ORR of 54% and a CR rate of 15% and a median PFS of 5.6 months (1.6, 18.4+) months (Figure 1, Table 3). Although the numbers were small, this compares favorably with a similar group of rituximab refractory FL patients treated with ofatumumab[16], IL-2/rituximab[17], lenalidomide/rituximab[7] and bendamustine/obinutuzumab[18], ibrutinib[19] and idelalisib[20]

With regard to our ability to identify predictors of response, flow cytometry proved superior to serum cytokine detection. This result is likely due to the large variation in cytokine levels at baseline among individual patients and the small sample size. Of the cytokines that were elevated in non-responders, only IL-2 and TNF reached significance at several time points, but neither could predict response to therapy or remain significant if adjusted for multiple testing (Supplementary Figure 2). However, the possibility that IL-2 and TNF might be

elevated in patients with progressive disease is not surprising. With regards to IL-2, malignant B cells can secrete IL-2, which in this setting would likely function as a pro-survival mitogen. Furthermore, when used as an immunotherapy agent, IL-2 has the potential to expand Tregs, which when expanded predict treatment non-responsiveness in other tumor types[21]. Like IL-2, TNF can also be secreted by malignant B cells, and therefore, increased levels of TNF over time might represent continued growth of the B cell lymphoma. Importantly, in experimental systems it has been demonstrated that TNF-blockade can overcome resistance to anti-PD1 therapy[22].Thus, the TNF elevation seen in our non-responders may also be involved in cancer circumvention of immune recognition.

The randomized component of this study was designed to test the hypothesis that ipilumumab will enhance rituximab-mediated ADCC. BCD data for the two groups (Rwith simulateous I versus R + delayed I) was consistent with the possibility that the addition of ipilumumab to rituximab augmented BCD (Figure 2A); however, the difference in BCD was not significant ($p = 0.08$, D8 Student's *t*-test) and thus requires further studies to establish if the effect was real.

As expected, in this trial, B cell and T cell levels were dependent on time [$p = 0.005$ and 0.015, respectively (Supplementary Table 4)], which was an *a priori* prediction given that the patients received rituximab, which depletes B cells, and ipilimumab, which allows for T cell activation. ADCC is a well-described lymphomacidal mechanism of rituximab and peripheral blood BCD is a known consequence. Based on this and the dependence on host immune effector function, we hypothesized that peripheral blood BCD could be used as a surrogate marker for the ADCC-mediated anti-lymphoma response. Indeed, B cell depletion was not equal between non-responders and responders [21.5 (SD 16.1) and 9.0 (SD 6.7) to a post-treatment low of 11.19 (SD 11.5) and 1.4 (SD 0.5), respectively]. The linear mixed effects model also determined that B cell percentage was significantly altered by responder status ($p < 0.0006$, and there was no remaining variability in effect size that was unexplained $p = 0.72$). The difference between B cell percentages in responders versus non responders was also established for individual time points using the Wilcoxon rank sum test (Figure 3C). Finally, the ability of peripheral blood BCD to serve as a surrogate for the anti-lymphoma response was demonstrated by the area under the receiver operator curves for B cell percentages at each individual time point. Thus, the percentage of live B cells within the lymphocyte gate can predict response to therapy, i.e. identify responders from non-responders (Figure 3D Supplementary Figure 6). In the future it will be important to investigate the ability of BCD to predict response in other anti-CD20-based therapies.

While there was an overall increase in T cells over time, not all T cell populations behaved similarly. For example, after an initial expansion on Day 8, regulatory T cells generally decreased over time (Figure 4A). This Treg-time association was clearly demonstrated by the linear effects model ($p = 0.021$, 0.047 and 0.010, for Tregs, $CD45RA^-$ Tregs and suppressive Tregs, respectively) (supplementary Table 4). The Treg expansion followed by contraction is likely due in part to the expression of CTLA-4 by the suppressive Tregs. CTLA-4 is the target of ipilimumab which has been demonstrated to deplete Tregs, possibly by ADCC.[23,24] While our study examined Treg subsets in the peripheral blood, intra-

tumoral depletion of suppressive Tregs has been associated with response in other tumor types.

Other studies have suggested that the ratio of effector cells to Tregs may be increased in patients who respond to immunotherapy[25] but this phenomenon is likely treatment-specific as the ratio was not informative in our study. However, over the past decade Tregs (historically CD4+ CD25+ Foxp3+ T cells) have been further subdivided into more defined subpopulations depending on their surface and functional phenotypes. Of these, the memory Treg subpopulation has the capacity to secrete pro-inflammatory cytokines and may even play a pathogenic role in the setting of autoimmunity[26]. The ability of specific Treg subpopulation ratios to predict response to cancer immunotherapy, specifically the $CD45RA^-$ Treg to total Treg ratio has not been previously studied. We hypothesized here that the potential inflammatory nature of some Tregs make them more desirable in patients receiving immunotherapy. Indeed, the ratio of $CD45RA^-$ Tregs to conventional Tregs differed between responders and non-responders at every time point in our study including baseline (Figure 4A, 4C and 4D and Supplementary Figure 7), which highlights the reproducibility of this finding. The exact mechanism that explains the strength of the association of this ratio to response, compared to the degree of association of the absolute levels of each component is unclear and needs further study but may suggest that there is a complex interplay within the Treg compartment. Moreover the observation that the ratio is predictive at baseline as well as the lack of predictive potential in group A versus group B suggests that ipilimumab-induced suppressive Treg depletion (in the peripheral blood) alone is not solely responsible for enhancing immune-mediated tumor responses.

Given the relatively small sample size and the heterogeneity of the NHL subtypes, future studies are needed to determine the reproducibility of these findings. Nevertheless, the identification of a biomarker that can predict response is potentially clinically significant in patients receiving immunotherapy for lymphoma as well as other malignancies. Our study did not include validation sample sets and thus this finding would be considered hypothesis generating and further evaluation of the CD45RA-/Treg ratio to predict response to therapy is warranted.

In summary, this clinical study in relapsed and refractory B cell lymphoma demonstrated that the combination of ipilumumab and rituximab was moderately tolerated at the dose studied. Although efficacy was modest in the entire cohort, encouraging efficacy was observed in patients with mostly anti-CD20 refractory FL. Moreover, the results of this trial suggest that $CD45RA^-$ Treg to Treg ratio has the potential to identify patients who are likely to respond to this regimen, even prior to initiation of therapy. The efficacy of this combination and the predictive potential of this biomarker need validation in larger studies of patients with follicular lymphoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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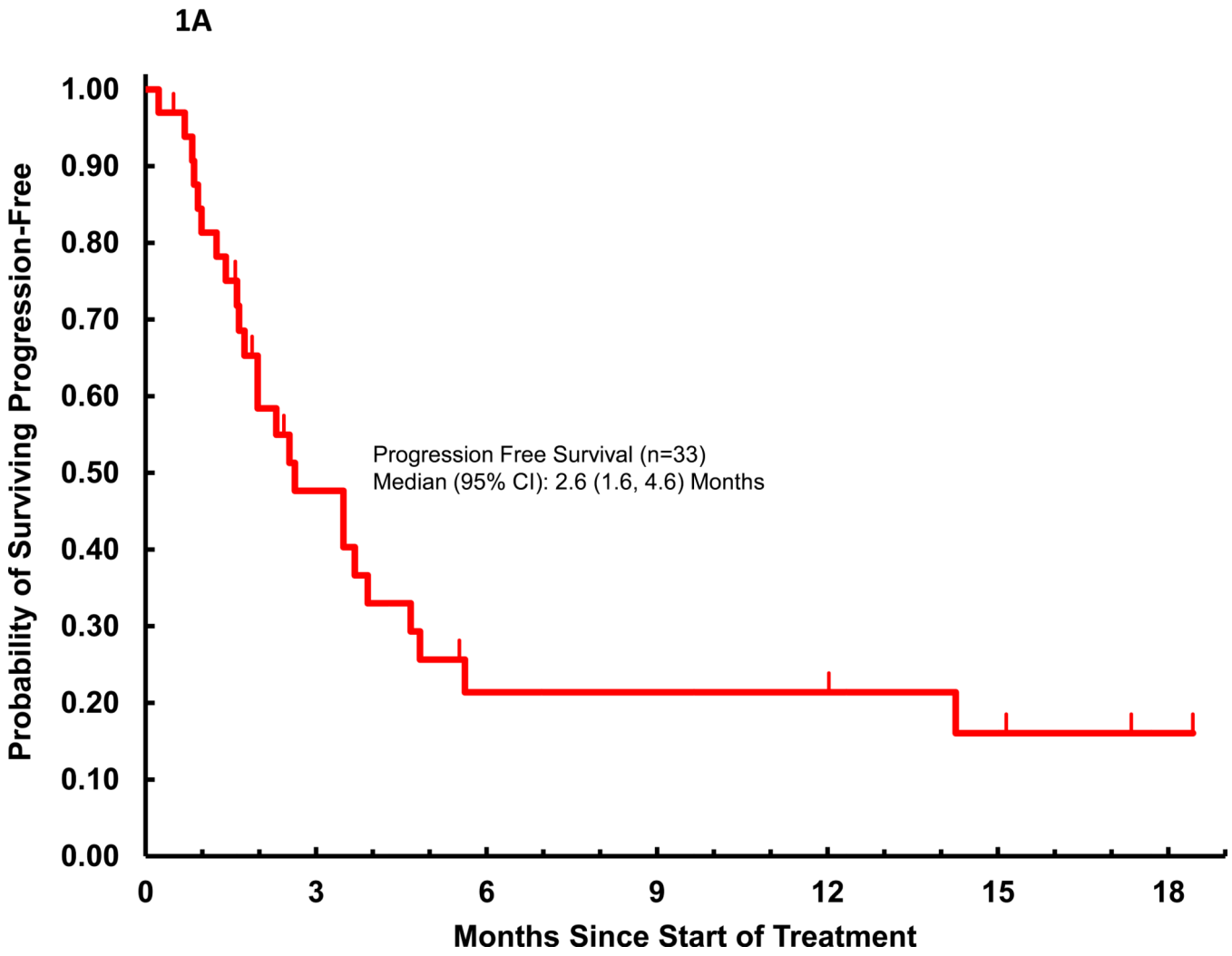
Translational Relevance

Based on our hypothesis that ipilimumab would augment rutiximab-mediated efficacy we used B cell depletion (BCD) as a surrogate biomarker of rituximab-mediated antibody-dependent cell mediated cytotoxicity (ADCC). A randomized expansion phase that delayed ipilimumab administration allowed for assessment of the effects of ipilimumab on rituximab-mediated ADCC. To better understand the immune effects of ipilimumab in this disease and to explore other biomarkers, we examined a broad array of immune correlatives. Based on the known effects of ipilimumab on regulatory T cells we examined regulatory T cells and their subsets focusing on the predictive potential of their relative frequencies. Our findings demonstrated the combination has a manageable safety profile and in a mostly rituximab refractory population, is associated with encouraging efficacy in follicular lymphoma. Moreover, several biomarkers were identified that are potentially associated with response to this combination which should be further studied and validated in larger clinical trials.



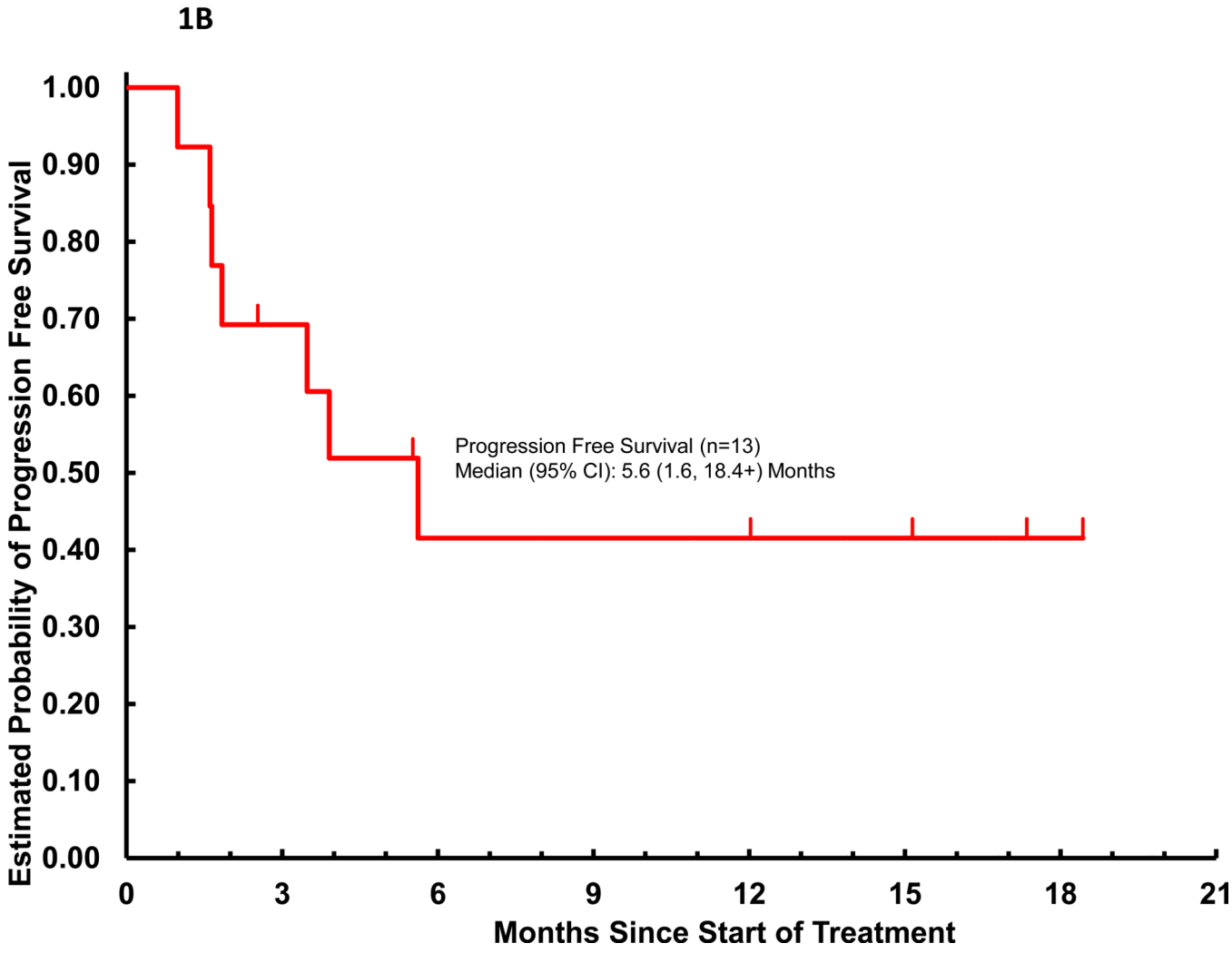


Figure 1:

Kaplan-Meier estimates of PFS. (**A**) When considering the entire cohort the median (95% CI) progression free survival (PFS) was 2.6 months (1.6, 4.6 mo) (**B**) Of the 12 follicular lymphoma patients the median PFS (95% CI) was 5.6 months (1.6, 18.4+).

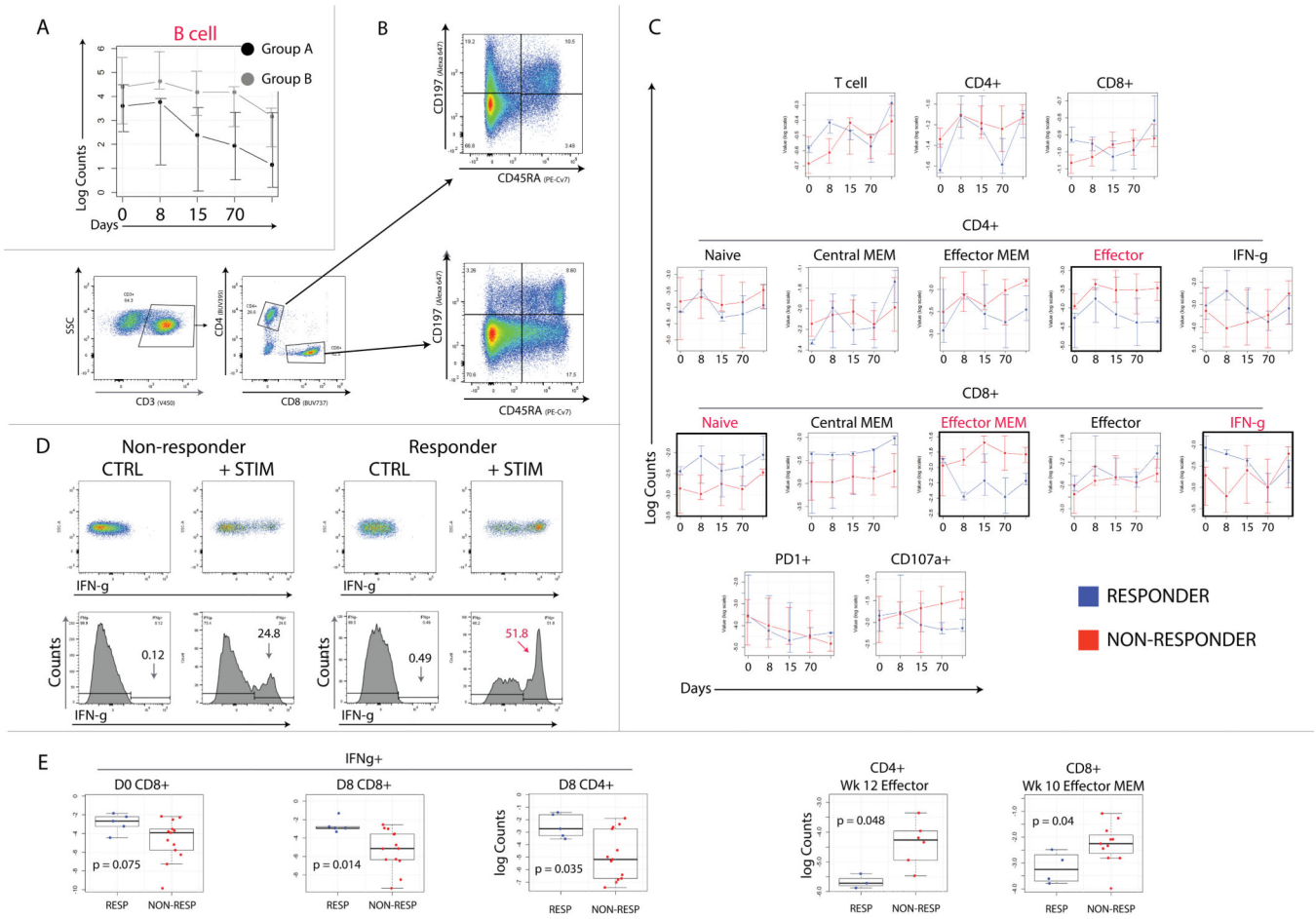


Figure 2:

Flow cytometric analysis of T cell subpopulations in responders and non-responders. (A) Flow cytometric analysis of B cells in arm A (R+I simultaneous) versus arm B (R alone, R+I delayed). Log of median values with error bars indicating 75th and 25th percentiles. (B) Flow cytometry gating strategy used to quantify T cell subpopulations. (C) T cell subset analysis. Small differences were detected in CD4+ effector, CD8+ naïve, CD8+ effector memory, and CD8+ IFN-g+ T cells between responders and non-responders were detected. (D) Intracellular staining for IFN-g in CD8+ T cells. Responders tendended to have more IFN-g-secreting CD8+ T cells at baseline and on day 8 of therapy. (E) Box whisker plots of IFN-g-secreting T cells. Responders tended to have more CD8+ IFN-g-secreting T cells on days 0 and 8 when compared to non-responders. Most significant days are also shown for differential expression of CD4+ effector T cells and CD8+ effector memory T cells.

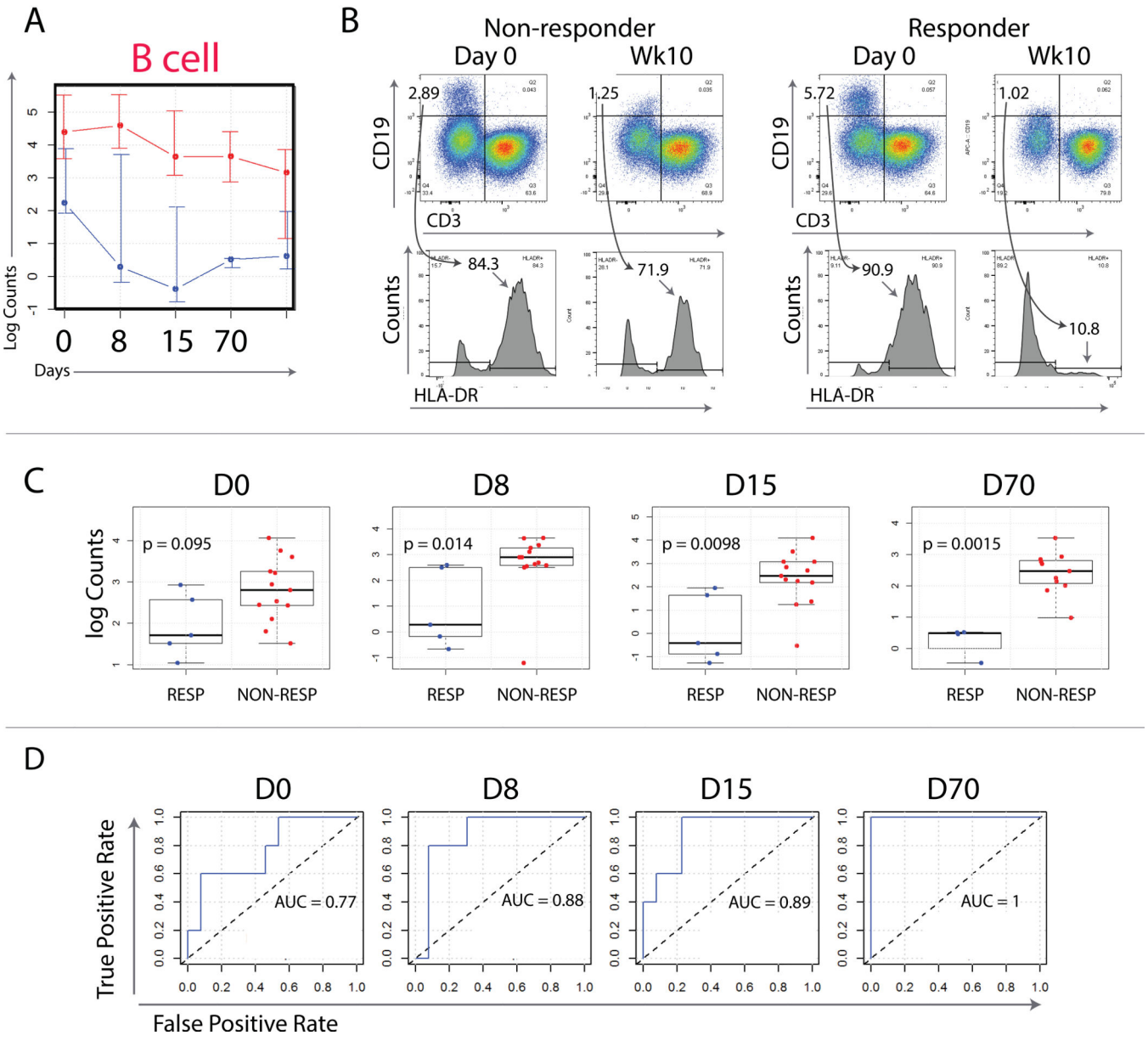


Figure 3:

Flow cytometric analysis of B cells in responders and non-responders. (A) Log of median values with error bars indicating 75th and 25th percentiles. Non-responders (red) tended to have both more B cells than responders (blue) as well as less of a reduction in B cells following rituxan (B) Flow cytometry gating strategy used to quantify B cells. (C) Box whisker plots of quantified B cells across the entire study duration. Responders tended to have fewer B cells and less of a reduction in B cell numbers following administration of rituximab. (D) Receiver operator characteristic curves constructed for B cells number as a classifier to distinguish responders from non-responders. Area under the curve (AUC) values are calculated for each time point.

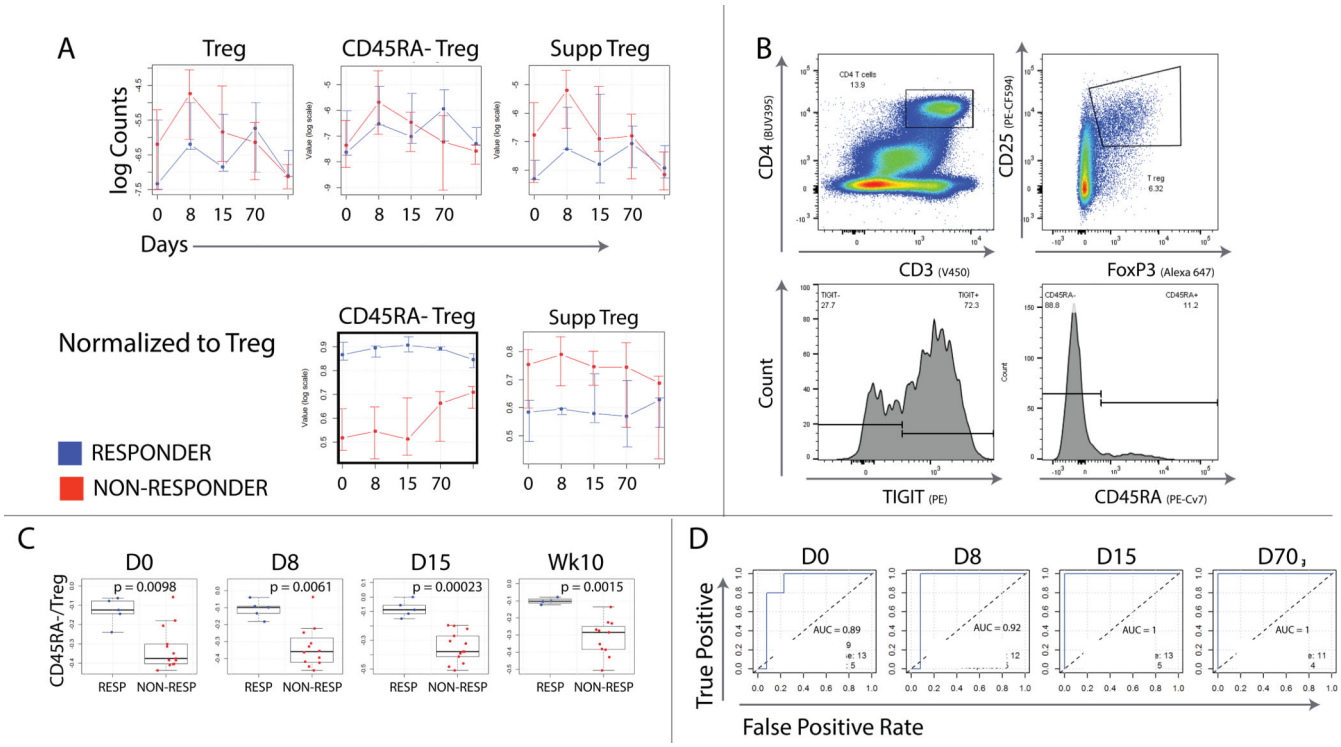


Figure 4:

Flow cytometric analysis of Treg subpopulations. (A) Upper panel- Line graphs of log median values (Tregs, CD45RA- Tregs, and Suppressive T regs) are graphed across the study duration. Error bars correspond to 25 and 75 percentiles. Lower panel represent CD45-Treg and Supp Treg populations normalized to total Treg numbers. This analysis reveals that the ratio of CD45RA- Tregs to total Tregs is able to clearly separate responders from non-responders. (B) Flow cytometry gating strategy used to quantify Treg subsets (C) Box whisker plots of normalized CD45RA- Tregs (CD45RA- Treg/Treg) across the entire study duration. Responders tended to have a higher ratio of CD45RA- Tregs to total Tregs. (D) ROC curves were constructed and AUCs calculated to illustrate CD45RA- Treg/Treg ratio ability to perform as a classifier to predict response to therapy.

Table 1.

Patient Demographic, Clinical, and Treatment Characteristics

Characteristic	Overall (n=33) n (%)
Age, years	
Median (range)	62 (33 – 78)
Karnofsky Performance Status	
100	4 (12%)
90	11 (33%)
80	12 (36%)
70	6 (18%)
Diagnosis	
Follicular lymphoma	13 (39%)
Diffuse large B-cell lymphoma	7 (21%)
Mantle cell lymphoma	2 (6%)
Small lymphocytic lymphoma	2 (6%)
Mediastinal large B-cell lymphoma	1 (3%)
Non-Hodgkin lymphoma, NOS	8 (24%)
Number of Prior Regimens	
Median (range)	4 (1–7)
Prior Stem Cell Transplant	
Yes	11 (36%)
Refractory to last treatment	22 (67%)
Refractory to anti-CD20-based therapy	27 (87%) *
Gender	
Female	9 (27%)
Male	24 (73%)
Race/Ethnicity	
African-American	2 (6%)
Caucasian	26 (79%)
Hispanic	5 (15%)

* defined as progression during or within 6 months of treatment with any anti-CD20-containing therapy. 31 patients assessed

Table 2:

Non-Hematologic Toxicities with at Least Grade 3 Toxicity*

CTCAE v4 System of AE's	Toxicity	Maximum Grade				Number of Patients with Any Grade of Toxicity
		1	2	3	4	
Gastrointestinal disorders	Colonic perforation	0	0	0	**1**	1
Immune system disorders	Serum sickness	0	0	0	**1**	1
Renal and urinary disorders	Acute kidney injury	0	0	0	**1**	1
Gastrointestinal disorders	Diarrhea	4	2	4	0	10*
Skin and subcutaneous tissue disorders	Rash maculo-papular	3	4	**1**	0	8
Gastrointestinal disorders	Abdominal pain	2	2	**1**	0	5
Respiratory, thoracic and mediastina	Dyspnea	1	1	**1**	0	3
Gastrointestinal disorders	Colitis	0	0	**1**	0	1
General disorders and administration	Non-cardiac chest pain	0	0	**1**	0	1
Musculoskeletal and connective tissue	Arthralgia	0	0	**1**	0	1
Psychiatric disorders	Agitation	0	0	**1**	0	1
Renal and urinary disorders	Urinary tract obstruction	0	0	**1**	0	1
Respiratory, thoracic and mediastina	Hypoxia	0	0	**1**	0	1
Respiratory, thoracic and mediastina	Pleural effusion	0	0	**1**	0	1

* possibly or definitely related to treatment, 2 other patients experienced Grade 2 diarrhea that was classified as unlikely or unrelated to treatment

Table 3.

Disease Response and Duration of Treatment

Characteristic	Overall (n=33) n (%)
Treatment Received	
Did not Complete 6 Weeks	7 (21%)
Completed 6 Weeks of Induction Only	10 (30%)
Started 2nd 6 Weeks of Induction	16 (48%)
Completed 12 Weeks of Induction	10 (30%)
Started of Maintenance	6 (18%)
Completed 4 Doses During Maintenance	4 (12%)
Reason Off Treatment	
Completed Treatment	4 (12%)
Progression	17 (52%)
Early Death (due to disease)	1 (3%)
Toxicity (includes 1 patient treated with steroids but no irAE – was a protocol deviation)	8 (24%)
Patient Decision (declined treatment, to hospice, found BMT donor)	3 (9%)
Progression-Free Survival (months)	
Median (95% confidence interval)	2.6 (1.6, 4.6) months
Follicular Lymphoma: Progression-Free Survival (months) (n = 13)	
Median (95% confidence interval)	5.6 (1.6, 18.4+) months
Tumor Response (n = 33)	
Evaluated (n = 25)	
Complete Response	2 (6%)
Partial Response	6 (18%)
Stable Disease	6 (18%)
Progressive Disease	11 (33%)
Not Evaluated – Off too early	8 (24%)
Observed Response Rate (n = 8/33)	
% (Exact 95% CI*)	24% (11%, 42%)
Tumor Response-Follicular n = 13	
Complete Response	2 (15%)
Partial Response	5 (38%)
Stable Disease	2(15%)
Progressive Disease	3(23%)
Not Evaluated	1 (8%)
Observed Response Rate (N=7/13)	
% (Exact 95% CI*)	54% (25%, 81%)

*Pearson-Clopper confidence interval