CD47 Expression Defines Efficacy of Rituximab with CHOP in Non-Germinal Center B-cell (Non-GCB) Diffuse Large B-cell Lymphoma Patients (DLBCL), but Not in GCB DLBCL

Bouwstra, Renee; He, Yuan; de Boer, Janneke Willemien; Kooistra, Hilde; Cendrowicz, Ewa; Fehrmann, Rudolf S N; Ammatuna, Emanuele; Eulenburg, Christine; Nijland, Marcel; Huls, Gerwin

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Renée Bouwstra[1], Yuan He[1#,] Janneke de Boer[1#,] Hilde Kooistra[1], Ewa Cendrowicz[1], Rudolf SN Fehrmann[2], Emanuele Ammatuna[1], Christine zu Eulenburg[3], Marcel Nijland[1], Gerwin Huls[1], Edwin Bremer[1‡*], and Tom van Meerten[1‡*]

Affiliations: [1]University of Groningen, University Medical Center Groningen, Department of Hematology, Groningen, the Netherlands. [2]University of Groningen, University Medical Center Groningen, Department of Medical Oncology, Groningen, the Netherlands. [3]University of Groningen, University Medical Center Groningen, Department of Epidemiology, Groningen, the Netherlands

YH and JdB contributed equally to this work, and EB and TVM contributed equally to this work

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CD47 impacts response of non-GCB DLBCL patients upon R-CHOP

***Corresponding Authors:** Dr. T. van Meerten, University of Groningen, University Medical Center Groningen, Department of Hematology, E-mail: t.van.meerten@umcg.nl or Prof Dr. E. Bremer, University of Groningen, University Medical Center Groningen, Department of Hematology, E-mail: e.bremer@umcg.nl

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Abstract

Addition of rituximab (R) to "CHOP" (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy improved outcome for diffuse large B-cell lymphoma (DLBCL) patients. Approximately 40% of patients that received R-CHOP still succumb to disease due to intrinsic resistance or relapse. A potential negative regulator of DLBCL treatment outcome is the CD47 "don't eat me" immune checkpoint. To delineate the impact of CD47, we used a clinically and molecularly well-annotated cohort of 939 DLBCL patients, comprising both germinal center B cell (GCB) and non-GCB DLBCL subtypes, treated with either CHOP or R-CHOP. High (above median) CD47 mRNA expression correlated with a detrimental effect on overall survival (OS) when DLBCL patients received R-CHOP therapy (p=0.001), but not when receiving CHOP therapy (p=0.645). Accordingly, patients with low CD47 expression benefited most from addition of rituximab to CHOP (HR, 0.32; CI, 0.21-0.50; P < 0.001). This negative impact of high CD47 expression on OS after R-CHOP treatment was only evident in cancers of non-germinal center B-cell (GCB) origin (HR, 2.09; CI, 1.26-3.47; P = 0.004) and not in the GCB subtype (HR, 1.16; CI, 0.68-1.99; P = 0.58). This differential impact of CD47 in non-GCB and GCB was confirmed in vitro, as macrophage-mediated phagocytosis stimulated by rituximab was augmented by CD47-blocking antibody only in non-GCB cell lines. Thus, high expression of CD47 mRNA limited the benefit of addition of rituximab to CHOP in non-GCB patients and CD47-blockade only augmented rituximab-mediated phagocytosis in non-GCB cell-lines. Patients with non-GCB DLBCL may benefit from CD47-targeted therapy in addition to rituximab.

Introduction:

Diffuse Large B-cell Lymphoma (DLBCL) is the most common form of lymphoma. DLBCL is an aggressive and heterogeneous disease that can be classified into germinal center B-cell–like (GCB), activated B cell–like (ABC) and unclassifiable DLBCL, with the latter two often being grouped together as non-germinal center B cell–like (non-GCB) [1]–[3]. The standard therapy for all DLBCL subtypes is chemotherapy consisting of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) combined with the rituximab monoclonal antibody to CD20. Addition of rituximab to CHOP (R-CHOP) has improved progression free survival (PFS) and overall survival (OS) of DLBCL [4]. Nevertheless, ~40% of DLBCL patients will develop resistance to R-CHOP and these patients have poor outcomes [5], [6]. Further, efficacy of R-CHOP differs between subclasses, with ABC DLBCL having a lower 5-year progression free survival (PFS) than GCB DLBCL (40% vs. 74%) [7].

Attempts to improve outcome of R-CHOP treatment with dose-intensified chemotherapy or new CD20 antibodies have so far not been successful [8], [9] and the mechanisms that underlie resistance to rituximab treatment remain unclear. Rituximab has multiple modes of action that include

induction of antibody-dependent cellular phagocytosis (ADCP) [10], [11]. Resistance to ADCP has been attributed to aberrant activation of the innate immune checkpoint CD47/signal regulatory protein alpha (SIRPα). CD47 is a so-called "don't eat me" signal that, upon binding to SIRPα expressed on phagocytes, triggers inhibitory signaling that limits phagocyte activity [12]. Correspondingly, overexpression of CD47 associates with poor prognosis in various cancers [13]–[16]. Blocking of the CD47-SIRPα interaction in preclinical murine xenograft models augments the anti-tumor activity of monoclonal antibodies, including that of rituximab [13].

Based on these findings, CD47 blocking has emerged as an immunomodulatory therapy that is being evaluated in early clinical trials, among others in combination with rituximab (NCT02953509). Indeed, high expression of CD47 mRNA was associated with poor survival in DLBCL, positioning DLBCL as a candidate for CD47-targeted therapy. Combination therapy with CD47 mAb (5HuF9-G4 clone) and rituximab resulted in 40% OR (overall response) and 33% CR (complete response) of relapsed or refractory DLBCL patients [17]. However, for CD47 blocking to be useful in clinical treatment of DLBCL, DLBCL patients who might benefit from CD47 blocking therapy must be identified.

In this study, we assembled a DLBCL transcriptome dataset comprising 939 clinically annotated DLBCL patients to delineate the impact of CD47 mRNA expression on CHOP and R-CHOP treatment in GCB and non-GCB DLBCL patients. Further, we defined in a preclinical setting whether these DLBCL subtypes differentially responded to combination therapy with rituximab and a CD47 blocking antibody.

Methods

Data acquisition, sample processing, quality control, probe selection and patient characteristics

Publicly available raw microarray expression data of DLBCL samples from various platforms (Affymetrix HG-U133A (GPL96) and Affymetrix HG-U133 Plus 2.0 (GPL570)) were extracted from the Gene Expression Omnibus (GEO) as previously described [18]–[20] (Supplementary Table S1). Probe 213857_s_at was used in the analyses. For patient characteristics see Supplementary Table S2.

Statistics

High CD47 mRNA expression was defined by expression above median (10.00 $\log_2$ mRNA expression) as determined on the total 939 patient DLBCL cohort. Clinical parameters analyzed were overall survival (OS), defined as the time from primary diagnosis to death from any cause. Survivors were censored on the last date known to be alive or at 5 years of follow up. Univariate group comparisons were performed with the χ^2 test for categorical data, the independent *t* test for continuous data and Kaplan-Meier method and the log-rank test for survival data. The Cox proportional hazard model was used to determine relevance of clinical and pathological characteristics [age and international prognostic index (IPI)-score] for OS expressed as hazard ratios with 95% confidence intervals (CI). Multivariate Cox analysis with interactions was used to determine the effect of CD47 expression on survival after CHOP or R-CHOP treatment. These analyses were performed in all patients who were CHOP/R-CHOP–treated. To analyze impact of CD47 high or low mRNA expression in distinct DLBCL subtypes, GCB and non-GCB patient populations were analyzed. All analyses were tested two-sided and p-values < 0.05 were considered statistically significant. Analyses were performed using SPSS (version 25.0 Armonk, NY, IBM Corp.) or STATA 14 (StataCorp LP, College Station, TX).

Cell lines and culture conditions

DLBCL cell lines OCI-ly3 (non-GCB DLBCL), U-2932 (non-GCB DLBCL), SUDHL4 (GCB DLBCL), SUDHL6 (GCB DLBCL), SUDHL10 (GCB DLBCL) were obtained from Deutsche Sammlung from Microorganism und Zellculturen, Braunschweig, Germany, and SUDHL2 (DLBCL non-GCB) was obtained from American Type Culture collection, Manassus, Virginia, US. All cell lines were cultured in RPMI-1640 supplemented with 10% Fetal Calf Serum (FCS) at 37°C with 5% CO2 in a humidified atmosphere and in 1% Penicillin-Streptomycin (Lonza BioWhittaker) and 1% Glutamine (Lonza BioWhittaker). The cell line identity was checked periodically (~ each 6 months) by STR profiling. All cell lines were tested mycoplasm free on 12/23/2017). Experiments were performed within 4 months after start of culture and mycoplasm testing. The cells were tested with a PCR assay that detects 25 mycoplasma and acholeplasma species that include those that most commonly contaminate cell cultures. The following primers were used in the mycoplasm PCR; forward primer sequences (cgc ctg agt agt acg ttc gc, cgc ctg agt agt acg tac gc, tgc ctg agt agt aca ttc gc, tgc ctg ggt agt aca ttc gc, cgc ctg ggt agt aca ttc gc, cgc ctg agt agt atg ctc gc) Reverse primer sequences (gcg gtg tgt aca aga ccc ga ,gcg gtg tgt aca aaa ccc ga, gcg gtg tgt aca aac ccc ga)

Generation and differentiation of human macrophages

Peripheral blood mononuclear cells were isolated from blood of healthy donors by density gradient centrifugation after informed consent. Monocytes were enriched by MACS sorting with CD14 magnetic beads (Miltenyi Biotec). In brief, CD14+ cells were magnetically labeled with CD14 microbeads and the suspension was loaded onto MACS column in a magnetic field. Only CD14+ cells were retained within the column and were subsequently eluted from the column. Next, CD14+ monocytes were differentiated to macrophages (M0) in RPMI1640-10% FBS supplemented with GM-CSF (50 ng/mL) and M-CSF (50 ng/mL) for 7 days. Type 1 macrophages were generated by priming with LPS (100 ng/mL) and IFNγ (20 ng/mL) on day 8. Type 2 macrophage were generated by priming with IL-10 (20 ng/mL) for an additional 48 h. All cells were cultured with 5% CO2, 37°C. For each experiment, macrophages were harvested and the phenotype was verified by flow cytometry using cell surface markers CD14, CD68, and CD80.

Macrophage phagocytosis assays

Macrophages were pre-seeded in 96-well plates at a density of 1.5x10^4 cells/well and cultured for 24h. DBLCL cells were labeled with cell proliferation dye V450 (Thermofisher) or CFSE (Thermofisher) according to manufacturer's instructions. Subsequently, tumor cells were incubated with rituximab alone or rituximab in combination with human CD47 IgG4 antibody (Inhibrix) (both at 5 µg/ml) on ice for 1h. Labeled DLBCL cells were washed twice (with PBS) and added to pre-seeded macrophages at an effector-to-target ratio of 1 to 5. Mixed cultures were incubated for 3h at 37°C, after which non-adherent DLBCL cells were removed by washing twice with PBS. Subsequently, phagocytosis was assessed by fluorescent microscopy (Leica, DM6000) by counting the number of adherent/stretched macrophages containing V450-labeled tumor cells per 100 macrophages, yielding the percentage phagocytosis. For phagocytosis of M1 macrophages, counting was performed based on stretched morphology of macrophages, whereas for M2 macrophages cells were counterstained with CD11b-PE antibody (clone, MEM-174, Immunotools) at room temperature for 45 minutes. In addition, the phagocytic index was calculated using the formula (number of tumor cells per macrophage/ total number of $V450^{+}$ macrophages). Each condition was quantified by evaluating three randomly chosen fields of view. Statistical significance was evaluated using two-tailed paired Student *t* test. P-value <0.05 was considered statistically significant.

Anti-human CD47 IgG4 (Inhibrix)

The sequence of the inhibrix antibody (clone Ab6.12) was obtained from US patent US_2014_0140989. The human IgG4-containing antibody was produced by the company Genscript (USA).

Results

High expression of CD47 predicts survival in R-CHOP, but not CHOP-treated DLBCL patients

In CHOP/R-CHOP–treated DLBCL patients, high expression of CD47 (i.e. above median) was associated with decreased overall survival (OS) compared to patients with low CD47 expression (i.e. below median) (HR, 1.63; CI, 1.20-2.00; p=0.0003), Supplementary Fig. S1A and for patient characteristics see Supplementary Table S2). However, when analyzing the CHOP-treated DLBCL patients separately, no significant difference in OS was observed between patients with high and low CD47 expression (Fig. 1A, p=0.645). In contrast, separate analysis of R-CHOP–treated patients identified that OS was significantly worse in patients with high expression of CD47 (Fig. 1B p=0.001). In a Cox proportional hazard model with interaction, high CD47 expression (compared to low) was associated with decreased OS, independent of IPI-score and age in R-CHOP–treated patients. The 5-year OS in patients with high CD47 expression after R-CHOP treatment was 2-fold reduced compared to patients with low CD47 expression (Table 1, (HR, 2.1; CI, 1.39-3.25; p=0.001)). In contrast, expression of CD47 did not significantly impact the 5-year OS in DLBCL patients treated with CHOP (HR, 0.93; CI, 0.62-1.40, p=0.741). In multivariate analysis of R-CHOP–treated patients, IPI, – DLBCL cell-of-origin subtype and CD47 expression significantly affected the 5-year OS (Supplementary Table S3). Thus, CD47 expression predicts the OS in R-CHOP–treated but not CHOP-treated patients.

Addition of rituximab to CHOP has resulted in a significant increase in survival of DLBCL patients (see also Supplementary Fig. S1B, (HR, 0.46; CI, 0.37-0.58; p<0.001)). We identified that, in patients with low CD47 expression, addition of rituximab to CHOP improved the 5-year OS 4-fold (Table 1, Fig. 1C. (HR,0.25; CI, 0.15-0.43; p<0.001). However, in patients with high CD47 expression, addition of rituximab to CHOP improved OS only 2-fold. (Table 1, Fig. 1D, (HR, 0.57; CI, 0.43-0.77; p<0.001). Taken together, these data suggest that high expression of CD47 is associated with a limited therapeutic effect of rituximab upon treatment of DLBCL patients with CHOP.

High expression of CD47 associated with poor response to R-CHOP only in non-GCB subgroup

In a subsequent analysis of CD47 expression within GCB and non-GCB DLBCL subtypes, mean expression of CD47 mRNA in non-GCB DLBCL patients was significantly higher than the mean in GCB DLBCL patients (Supplementary Fig. S2A,B $p<0.0001$). Further, and in line with literature, non-GCB DLBCL patients had a two-fold higher risk of death after R-CHOP treatment compared to GCB DLBCL patients ((HR, 1.9; CI, 1.27-2.90; p=0.002), Supplementary Fig. S3A, Supplementary Table S4 for multivariate analysis). In line with our analyses in the complete DLBCL study cohort, addition of rituximab to CHOP significantly improved survival in both GCB and non-GCB DLBCL patients (Supplementary Fig. S3B, C, $p<0.0001$).

To assess the impact of CD47 expression on the outcome in non-GCB and GCB patients the OS in the cohort of R-CHOP–treated patients was studied. These analyses indicated that within the R-CHOP-treated non-GCB DLBCL subgroup, patients with high CD47 expression had an inferior survival compared to patients with low CD47 expression (Fig. 2A, (HR, 1.9; CI, 1.44-3.26; p=0.015). Within this non-GCB subtype no difference of age or IPI score was observed between patients with high or low CD47 expression using univariate analysis (Supplementary Table S5, Age p=0.550, IPI p=0.594). In the GCB-DLBCL patient subgroup no difference was observed in OS between patients with high or low expression of CD47 (Fig. 2B, p=0.584). Taken together, these data indicate that high expression of CD47 predicts OS after R-CHOP treatment in the non-GCB DLBCL patient population and not in the GCB DLBCL patient population.

To further assess the impact of DLBCL subtype on outcome in patients with low and high CD47 expression next the full cohort of R-CHOP–treated patients was studied. In patients defined as having low CD47 expression (e.g. below median), survival did not differ between GCB and non-GCB patients (Fig. 2C, p=0.7662). In contrast, in patients with high CD47 expression, non-GCB patients had worse OS compared to GCB patients (Fig. 2D, p=0.0006). Within the CD47-high population, the age and IPI-score were also significantly different between GCB and non-GCB population (Table 2). However, in multivariate analysis, correcting for age and IPI-score, non-GCB patients with high CD47 expression still had a 2-fold increased risk of death compared to GCB patients with high CD47 expression (Table 2, (HR, 2.09; CI, 1.26-3.47; p=0.004). Thus, only the non-GCB DLBCL subtype negatively impacts survival of patients with high CD47 expression after R-CHOP treatment (Supplementary Fig. S3D).

CD47 blockade promotes rituximab-mediated ADCP of non-GCB but not GCB DLBCL cells

As high expression of CD47 predicted survival only in the non-GCB subtype, we wondered whether CD47 blockade might also selectively facilitate rituximab-mediated phagocytosis in the non-GCB subtype of DLBCL only. To evaluate this hypothesis, non-GCB and GCB cell lines were mixed with allogeneic macrophages differentiated towards M1 or M2 phenotype and treated *in vitro* with the previously reported CD47 antibody Ab6.12 comprising human IgG4 (termed inhibrix) (for representative pictures see Fig. 3A). Human IgG4 does not trigger ADCP, thus allowing direct evaluation of the impact of CD47-blocking [21]. Treatment with inhibrix dose-dependently enhanced rituximab-mediated phagocytosis of non-GCB cell line U2932, but not of the GCB cell line SUDHL4 by M1-differentiated macrophages (Fig. 3B,C). Treatment with human IgG4 isotype control did not significantly ($p>0.05$) induce phagocytosis (Supplementary Fig. S4A, see Methods for statistics). This differential impact of CD47-blocking between GCB and non-GCB cell lines by M1 macrophages was detected in a larger cell panel, with rituximab-mediated phagocytosis being significantly augmented by inhibrix in 3 out of 3 non-GCB cell lines and in none of the 3 GCB cell lines (Fig. 3D). Inhibrix co-treatment also increased the number of tumor cells ingested per macrophage, with increased numbers of phagocytosed cells per macrophage (i.e. phagocytic index, see method section for statistics) in non-GCB cells, but not in GCB cells (Fig. 3E). Thus, CD47 blockade using the human IgG4 containing antibody inhibrix significantly ($p<0.05$) augmented rituximab-mediated phagocytosis by M1-differentiated macrophages in non-GCB cells, but not in GCB cells. In analogous experiments with M2-differentiated macrophages, co-treatment with inhibrix again only significantly augmented rituximab-mediated macrophage-mediated phagocytosis of non-GCB cell lines and not GCB cell lines (Fig. 3F), although in this case the impact on phagocytic index was minimal (Fig. 3G). Neither expression of CD20 nor expression of CD47 on the respective cell lines strongly correlated with experimental induction of phagocytosis (Supplementary Fig. S4B, C; r2 0.29 and 0.14 respectively). These *in vitro* data indicate that the therapeutic effect of rituximab may be increased by CD47 blocking antibody in the non-GCB subtype of DLBCL only.

Discussion

Although addition of R to CHOP chemotherapy improves the treatment outcome in DLBCL patients, the data presented here demonstrate that patients with high expression of the "don't eat me" signal CD47 benefited less from the addition of rituximab to CHOP than patients with low expression of CD47. CD47 expression only associated with poor survival after R-CHOP treatment in non-GCB DLBCL. Indeed, in multivariate analysis CD47 expression was an independent risk factor for outcome of non-GCB but not for outcome of GCB DLBCL patients treated with R-CHOP. In line with these observations, macrophage-mediated phagocytosis of DLBCL cells upon rituximab treatment *in vitro* is

augmented by a CD47-blocking antibody in non-GCB cell lines, but not in GCB cell lines. The findings presented here suggest that only CD47 high expressing patients of the non-GCB subtype and not the GCB subtype will benefit from addition of CD47-antibody therapy to rituximab treatment.

R-CHOP is the standard therapeutic regimen for DLBCL patients and resistance to R-CHOP associates with a dismal prognosis [22]. Resistance to rituximab has been attributed to inhibitory signals that limit its effector functions. Examples of resistance mechanisms include polymorphisms of the Fcγ receptor III on cytotoxic cells that limit antibody dependent cellular cytotoxicity (ADCC) [23], expression of complement inhibitory proteins [24], and upregulation of anti-apoptotic proteins (such as BCL-2) [25]. Here, we showed that expression of CD47 may be another contributor to resistance, specifically to induction of phagocytosis by rituximab. Attempts to enhance the efficacy of CD20-targeting have included development of second-generation antibodies such as obinutuzumab. Compared to rituximab, obinutuzumab more effectively triggers direct cell death and induces phagocytosis [26]. However, obinutuzumab-CHOP failed to increase progression free survival compared to R-CHOP in untreated DLBCL patients [8]. Ofatumumab, a second generation anti-CD20 with enhanced capacity to activate complement-mediated cytotoxicity, also failed to improve survival compared to rituximab in refractory DLBCL patients in the setting of salvage therapy [6]. Thus, for certain DLBCL patients, combination of rituximab or obinutuzumab treatment with CD47-blocking therapy might be useful.

In addition to the selective negative association of CD47 expression with non-GCB DLBCL survival in the clinical analysis, *in vitro* macrophage-mediated phagocytic removal of DLBCL cells upon rituximab treatment was only augmented by CD47-blocking antibody in non-GCB cell lines. In contrast, phagocytosis of GCB cell lines was not augmented by CD47 blocking antibody. This effect was observed in a panel of 3 GCB and 3 non-GCB cell lines. In a clinical study [17], treatment with anti-CD47 (Hu5F9-G4) in combination with rituximab yielded a higher objective response rate in patients with ABC-DLBCL than in GCB-DLBCL patients (67% for ABC-DLBCL vs 17% for GCB-DLBCL). These data fit with our conclusions here, although follow-up studies in a larger cohort of patients will be required to address the impact of ABC and GCB subtype on CD47 therapy.

The reason underlying this differential response to CD47-blocking in GCB versus non-GCB cell lines has yet to be defined, but may be related to differences in the balance of “don’t eat me” signals such as CD47 and “eat me” signals such as phosphatidyl serine on GCB and non-GCB cells. A candidate pro-phagocytic protein reported in this respect was SLAMF7, an eat-me signal initially described as a requisite for CD47 antibody-induced phagocytosis [27]. However, we have shown that DLBCL expression of SLAMF7 is not required for CD47-mediated phagocytosis [19]. An alternate candidate

pro-phagocytic protein is calreticulin, an ER protein that on stressed (cancerous) cells can be detected on the cell surface, where the balance between CD47 and calreticulin expression determines phagocytosis [28]. On the other hand, differential expression of don't eat me signals, such as LILRB1, on macrophages and cognate receptors, such as MHC class I, on DBLCL cells may underlie GCB/non-GCB differences. In this respect, disruption of either MHC class I or LILRB1 potentiated phagocytosis of tumor cells upon CD47 mAb treatment both in vivo and in vitro [29]. Non-GCB tumor cells are characterized by loss of tumor major histocompatibility complex class I [30], thus removal of the CD47 axis in this cell type may suffice to trigger phagocytosis.

The clinical findings reported here on the negative association of CD47 expression upon R-CHOP treatment are in line with preclinical data on the potentiation of therapeutic antibodies by CD47 blockade [13], [31], [32], where high CD47 expression inhibits antibody dependent cellular phagocytosis (ADCP) [33], [34]. These findings partly contrast with a report by Chao et al, in which high mRNA expression of CD47 in DLBCL was predictive of survival in both CHOP and R-CHOP-treated patients [13], with the same CD47 probe used in both studies. A likely explanation for this difference is the fact that Chao et al. used an optimal CD47 expression cut off to measure survival differences after CHOP treatment. Although giving the most significant difference in survival between high and low CD47 expressing patients, this method resulted in disparate groups, with only 27 patients with high CD47 expression and 203 patients with low expression. In contrast, the median CD47 expression used as cutoff in the current study (as determined based on the complete study cohort; R-CHOP and CHOP-treated) guaranteed that high expression of CD47 was defined with the same threshold in all analyses (i.e., analyses of the total DLBCL cohort (939 patients), R-CHOP/CHOP analyses as in the GCB/non-GCB analyses). General survival outcomes for the subgroups GCB and non-GCB DLBCL and for CHOP versus R-CHOP–treated DLBCL patients were comparable to published data, suggesting that our selection procedure did not result in a selection bias.

As referred to above, combination therapy including both CD47 targeting and rituximab has been reported for CD47 mAb 5HuF9-G4 and is being evaluated in a phase 1 trial with mAb CC-90002 in patients with advanced/refractory $CD20^+$ B-cell Non-Hodgkin's Lymphoma patients (NCT02367196). Based on the data presented, survival after treatment with rituximab in combination with CHOP chemotherapy is only impacted by high expression of CD47 in non-GCB DLBCL patients. In line with these data, only cell lines of the non-GCB subtype benefitted from combining rituximab with CD47 blocking treatment *in vitro*. These results have implications for the interpretation and design of clinical trials for DLBCL. For example, we suggest that clinical trials with CD47-targeting therapeutics and rituximab in DLBCL should be stratified and specifically include the non-GCB subtype. Clinical trials are increasingly designed to evaluate subtype-specific DLBCL therapy, e.g. a clinical trial with

ibrutinib (NCT01855750) is only recruiting non-GCB DLBCL patients. Moreover, clinical trials with lenalidomide and bortezomib in combination with R-CHOP have demonstrated that non-GCB DLBCL patients especially benefit from these therapeutic improvements [35].

In summary, the data presented here support the implementation of anti-CD47 as a co-treatment with rituximab for DLBCL patients. Our data analysis as well as preclinical functional macrophage phagocytosis data indicate that non-GCB patients are likely to benefit from combined treatment of rituximab with CD47-blocking antibodies.

Contributions:

RB, performed research, analyzed data, wrote the paper, YH performed research analyzed data, wrote the paper, JdB analyzed the data, HK analyzed the data, RF collected the data, analyzed the data, EA designed research, CzE analyzed the data, MN performed research, GH designed the research, analyzed the data and wrote the paper, EB designed the research, analyzed the data and wrote the paper, TVM collected the data, analyzed the data, wrote the paper

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References

[1] A. A. Alizadeh *et al.*, "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling.," *Nature*, vol. 403, no. 6769, pp. 503–511, Feb. 2000.

[2] A. Rosenwald *et al.*, "The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma.," *N. Engl. J. Med.*, vol. 346, no. 25, pp. 1937–1947, Jun. 2002.

[3] G. Lenz *et al.*, "Molecular subtypes of diffuse large B-cell lymphoma arise by distinct genetic pathways.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 36, pp. 13520–13525, Sep. 2008.

[4] L. H. Sehn *et al.*, "Introduction of combined CHOP plus rituximab therapy dramatically improved outcome of diffuse large B-cell lymphoma in British Columbia.," *J. Clin. Oncol.*, vol. 23, no. 22, pp. 5027–5033, Aug. 2005.

[5] G. W. van Imhoff *et al.*, "Ofatumumab Versus Rituximab Salvage Chemoimmunotherapy in Relapsed or Refractory Diffuse Large B-Cell Lymphoma: The ORCHARRD Study.," *J. Clin. Oncol.*, vol. 35, no. 5, pp. 544–551, Feb. 2017.

[6] E. Van Den Neste *et al.*, "Outcome of patients with relapsed diffuse large B-cell lymphoma who fail second-line salvage regimens in the International CORAL study.," *Bone Marrow Transplant.*, vol. 51, no. 1, pp. 51–57, Jan. 2016.

[7] M. Roschewski, L. M. Staudt, and W. H. Wilson, "Diffuse large B-cell lymphoma-treatment approaches in the molecular era.," *Nat. Rev. Clin. Oncol.*, vol. 11, no. 1, pp. 12–23, Jan. 2014.

[8] U. Vitolo *et al.*, "Obinutuzumab or Rituximab Plus Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone in Previously Untreated Diffuse Large B-Cell Lymphoma.," *J. Clin. Oncol.*, vol. 35, no. 31, pp. 3529–3537, Nov. 2017.

[9] A. Chiappella *et al.*, "Rituximab-dose-dense chemotherapy with or without high-dose chemotherapy plus autologous stem-cell transplantation in high-risk diffuse large B-cell lymphoma (DLCL04): final results of a multicentre, open-label, randomised, controlled, phase 3 study.," *Lancet. Oncol.*, vol. 18, no. 8, pp. 1076–1088, Aug. 2017.

[10] T. van Meerten, R. S. van Rijn, S. Hol, A. Hagenbeek, and S. B. Ebeling, "Complement-induced cell death by rituximab depends on CD20 expression level and acts complementary to antibody-dependent cellular cytotoxicity.," *Clin. Cancer Res.*, vol. 12, no. 13, pp. 4027–4035, Jul. 2006.

[11] M. J. Glennie, R. R. French, M. S. Cragg, and R. P. Taylor, "Mechanisms of killing by anti-CD20 monoclonal antibodies.," *Mol. Immunol.*, vol. 44, no. 16, pp. 3823–3837, Sep. 2007.

[12] T. Matozaki, Y. Murata, H. Okazawa, and H. Ohnishi, "Functions and molecular mechanisms of the CD47-SIRPalpha signalling pathway.," *Trends Cell Biol.*, vol. 19, no. 2, pp. 72–80, Feb. 2009.

[13] M. P. Chao *et al.*, "Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma.," *Cell*, vol. 142, no. 5, pp. 699–713, Sep. 2010.

[14] L. Barrera *et al.*, "CD47 overexpression is associated with decreased neutrophil apoptosis/phagocytosis and poor prognosis in non-small-cell lung cancer patients.," *Br. J. Cancer*, vol. 117, no. 3, pp. 385–397, Jul. 2017.

[15] Y. Li *et al.*, "Overexpression of CD47 predicts poor prognosis and promotes cancer cell invasion in high-grade serous ovarian carcinoma.," *Am. J. Transl. Res.*, vol. 9, no. 6, pp. 2901–2910, 2017.

[16] R. Majeti *et al.*, "CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells.," *Cell*, vol. 138, no. 2, pp. 286–299, Jul. 2009.

[17] R. Advani *et al.*, "CD47 Blockade by Hu5F9-G4 and Rituximab in Non-Hodgkin's Lymphoma.," *N. Engl. J. Med.*, vol. 379, no. 18, pp. 1711–1721, Nov. 2018.

[18] R. S. N. Fehrmann *et al.*, "Gene expression analysis identifies global gene dosage sensitivity in cancer.," *Nat. Genet.*, vol. 47, no. 2, pp. 115–125, Feb. 2015.

[19] M. R. W. de Jong *et al.*, "Identification of relevant drugable targets in diffuse large B-cell lymphoma using a genome-wide unbiased CD20 guilt-by association approach.," *PLoS One*, vol. 13, no. 2, p. e0193098, 2018.

[20] Y. He *et al.*, "Cancer cell-expressed SLAMF7 is not required for CD47-mediated phagocytosis.," *Nat. Commun.*, vol. 10, no. 1, p. 533, Feb. 2019.

[21] X.-R. Jiang *et al.*, "Advances in the assessment and control of the effector functions of therapeutic antibodies.," *Nature reviews. Drug discovery*, vol. 10, no. 2. England, pp. 101–111, Feb-2011.

[22] M. R. Smith, "Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance.," *Oncogene*, vol. 22, no. 47, pp. 7359–7368, Oct. 2003.

[23] G. Cartron *et al.*, "Therapeutic activity of humanized anti-CD20 monoclonal antibody and

polymorphism in IgG Fc receptor FcgammaRIIIa gene.," *Blood*, vol. 99, no. 3, pp. 754–758, Feb. 2002.

[24] W. K. Weng and R. Levy, "Expression of complement inhibitors CD46, CD55, and CD59 on tumor cells does not predict clinical outcome after rituximab treatment in follicular non-Hodgkin lymphoma.," *Blood*, vol. 98, no. 5, pp. 1352–1357, Sep. 2001.

[25] B. Bonavida, "Rituximab-induced inhibition of antiapoptotic cell survival pathways: implications in chemo/immunoresistance, rituximab unresponsiveness, prognostic and novel therapeutic interventions.," *Oncogene*, vol. 26, no. 25, pp. 3629–3636, May 2007.

[26] J. Golay *et al.*, "Glycoengineered CD20 antibody obinutuzumab activates neutrophils and mediates phagocytosis through CD16B more efficiently than rituximab.," *Blood*, vol. 122, no. 20, pp. 3482–3491, Nov. 2013.

[27] J. Chen *et al.*, "SLAMF7 is critical for phagocytosis of haematopoietic tumour cells via Mac-1 integrin.," *Nature*, vol. 544, no. 7651, pp. 493–497, Apr. 2017.

[28] M. P. Chao *et al.*, "Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47.," *Sci. Transl. Med.*, vol. 2, no. 63, p. 63ra94, Dec. 2010.

[29] A. A. Barkal *et al.*, "Engagement of MHC class I by the inhibitory receptor LILRB1 suppresses macrophages and is a target of cancer immunotherapy.," *Nat. Immunol.*, vol. 19, no. 1, pp. 76–84, Jan. 2018.

[30] L. E. van der Meeren, L. Visser, A. Diepstra, M. Nijland, A. van den Berg, and P. M. Kluin, "Combined loss of HLA I and HLA II expression is more common in the non-GCB type of diffuse large B cell lymphoma.," *Histopathology*, vol. 72, no. 5. England, pp. 886–888, Apr-2018.

[31] S. Gholamin *et al.*, "Disrupting the CD47-SIRPalpha anti-phagocytic axis by a humanized anti-CD47 antibody is an efficacious treatment for malignant pediatric brain tumors.," *Sci. Transl. Med.*, vol. 9, no. 381, Mar. 2017.

[32] L. Liu *et al.*, "Anti-CD47 Antibody As a Targeted Therapeutic Agent for Human Lung Cancer and Cancer Stem Cells.," *Front. Immunol.*, vol. 8, p. 404, 2017.

[33] N. Gul and M. van Egmond, "Antibody-Dependent Phagocytosis of Tumor Cells by Macrophages: A Potent Effector Mechanism of Monoclonal Antibody Therapy of Cancer.," *Cancer Res.*, vol. 75, no. 23, pp. 5008–5013, Dec. 2015.

[34] S. T. Wilkinson *et al.*, "Partial plasma cell differentiation as a mechanism of lost major histocompatibility complex class II expression in diffuse large B-cell lymphoma.," *Blood*, vol. 119, no. 6, pp. 1459–1467, Feb. 2012.

[35] M. S. Czuczman *et al.*, "A Phase 2/3 Multicenter, Randomized, Open-Label Study to Compare the Efficacy and Safety of Lenalidomide Versus Investigator's Choice in Patients with Relapsed or Refractory Diffuse Large B-Cell Lymphoma.," *Clin. Cancer Res.*, vol. 23, no. 15, pp. 4127–4137, Aug. 2017.

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Tables

Table 1. Uni- and multivariate analysis with interaction to determine the effect of CHOP and R-CHOP treatment of DLBCL patients with high or low CD47 expression.

*p-value of two-way interaction analysis between CD47 expression and treatment on overall survival of DLBCL patients. Abbreviations: OS, overall survival, IPI, international prognostic index, R-CHOP = rituximab, cyclophosphamide, doxorubicin, vincristine and prednisolone.

	Univariate analysis		**Multivariate analysis**		
	Hazard ratio	***P* value**	**Hazard ratio**	***P* value**	**95% CI**
Age categorized					
<60 yr					
≥ 60 yr	1.759	0.000			
IPI categorized					
Low risk (0,1)					
Intermediate risk (2,3)	2.405	0.000			
High risk (4,5)	4.698	0.000			
high compared to low CD47 when treated with CHOP	1.093	0.645	0.934	0.741	[0.623-1.400]
high compared to low CD47 when treated with R-CHOP	1.826	0.001	2.129	0.000	[1.394-3.252]
Treatment effect of R-CHOP compared to CHOP in high CD47	0.541	0.000	0.573	0.000	[0.429-0.766]
Treatment effect of R-CHOP compared to CHOP in low CD47	0.324	0.000	0.251	0.000	[0.148-0.425]

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Table 2. Uni- and multivariate analysis of GCB and non-GCB patients with high CD47 expression. Comparison of GCB and non-GCB in high CD47 expressing patients treated with R-CHOP. Multivariate analysis were used to analyze the association of these prognostic markers with overall survival. Abbreviations: OS, overall survival, (non)GCB, (non) Germinal Center B-Cell, IPI, international prognostic index.

	Univariate analysis		***P* value**	**Multivariate analysis**		
	$CD47^{high}$	**$CD47^{high}$**				
	GCB	**non-GCB**		**Hazard ratio**	***P* value**	**95% CI**
Age categorized			0.002			
<60 yr	72	93				
≥ 60 yr	68	171		1.011	0.961	[0.652-1.568]
IPI categorized			0.031			
Low risk (0,1)	59	87				
Intermediate risk (2,3)	56	130		2.158	0.004	[1.288-3.617]
High risk (4,5)	10	36		5.975	0.000	[3023411.039]
risk of non-GCB vs. GCB				2.088	0.004	[1.258-3.467]

Figure legends

Figure 1. Association between CD47 expression and overall survival of DLBCL patients. 254 patients were treated with CHOP and 680 were treated with R-CHOP. The CHOP and R-CHOP–treated populations were sorted based on their CD47 expression (high CD47 is above median and low CD47 is below median expression of the whole cohort) and were used in the Kaplan Meijer curves (A, B). Comparison of CHOP with R-CHOP treatment effect in patients with low CD47 expression (C) and in high CD47 expression (D).

Figure 2. CD47 expression is associated with survival only in non-GCB DLBCL patients. The R-CHOP–treated patient population was divided into GCB and non-GCB groups, then sorted into high CD47 expressing and low CD47 expressing groups. (A) Kaplan Meijer curves of R-CHOP–treated high and low CD47 expressing non-GCB DLBCL patients. (B) Kaplan Meijer curves of R-CHOP–treated high and low CD47 expressing GCB DLBCL patients. (C) Kaplan Meijer curves of R-CHOP–treated non-GCB and GCB DLBCL patients with low CD47 expression or (D) high CD47 expression.

Figure 3. Rituximab-mediated phagocytosis of non-GCB, but not GCB, cell lines is augmented by CD47 mAb. (A) U2932 (non-GCB) tumor cells were fluorescent labelled and mixed with M1 or M2 macrophages. In the presence of CD47mAb (Inhibrix) (10 μg/mL) or rituximab (2.5 μg/mL), U2932 cells were phagocytosed by macrophages. Phagocytosis of (V450-labelled) cancer cells (white arrows) as well as adherent and non-phagocytosed cancer cells (yellow arrows) was visualized. (B) SUDHL4 (GCB cell line) and (C) U2932 (non-GCB cell line) was treated with a dose increase of rituximab in the presence or absence of CD47mAb (10 μg/mL). (%) phagocytosis was defined as the number of macrophages that phagocytosed tumor cells divided by the total amount of macrophages. (D) M1-mediated phagocytosis of a panel of GCB and non-GCB cell-lines after rituximab treatment alone or in combination with CD47mAb (inhibrix) (both 5 μg/mL). (E) Phagocytic index as determined for the GCB and non-GCB cell line experiments in D. (F) The same panel of cell-lines was used to evaluate phagocytosis of non-GCB and GCB cell-lines by M2 macrophages. (G) Phagocytic index determined for M2 phagocytosis experiment in panel F. Experiments were performed in triplicates with macrophages obtained from independent healthy donors. Abbreviations: R; rituximab, GCB; Germinal Center B-cell Like, INH; inhibrix.

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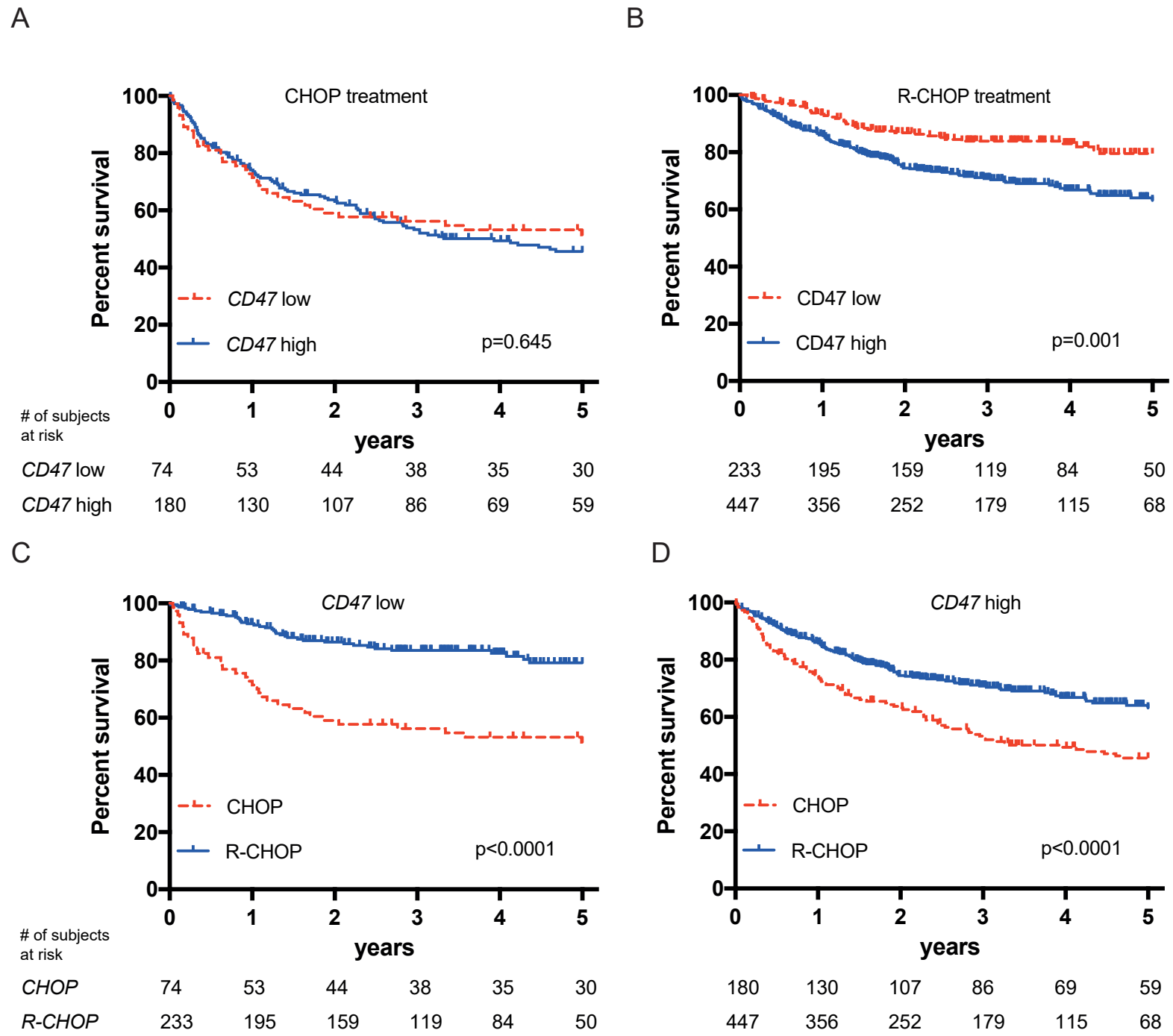

Figure 1

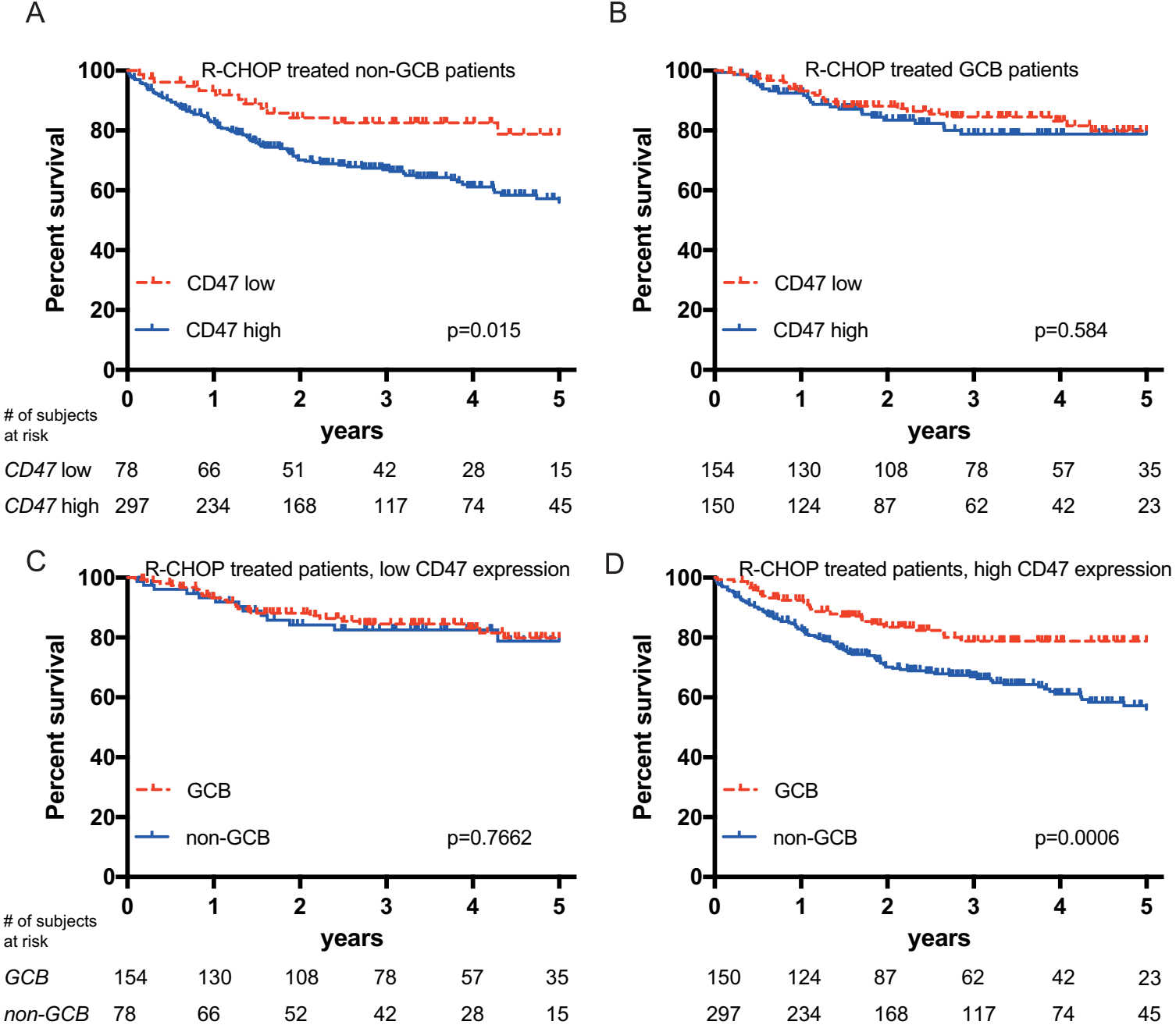

Figure 2

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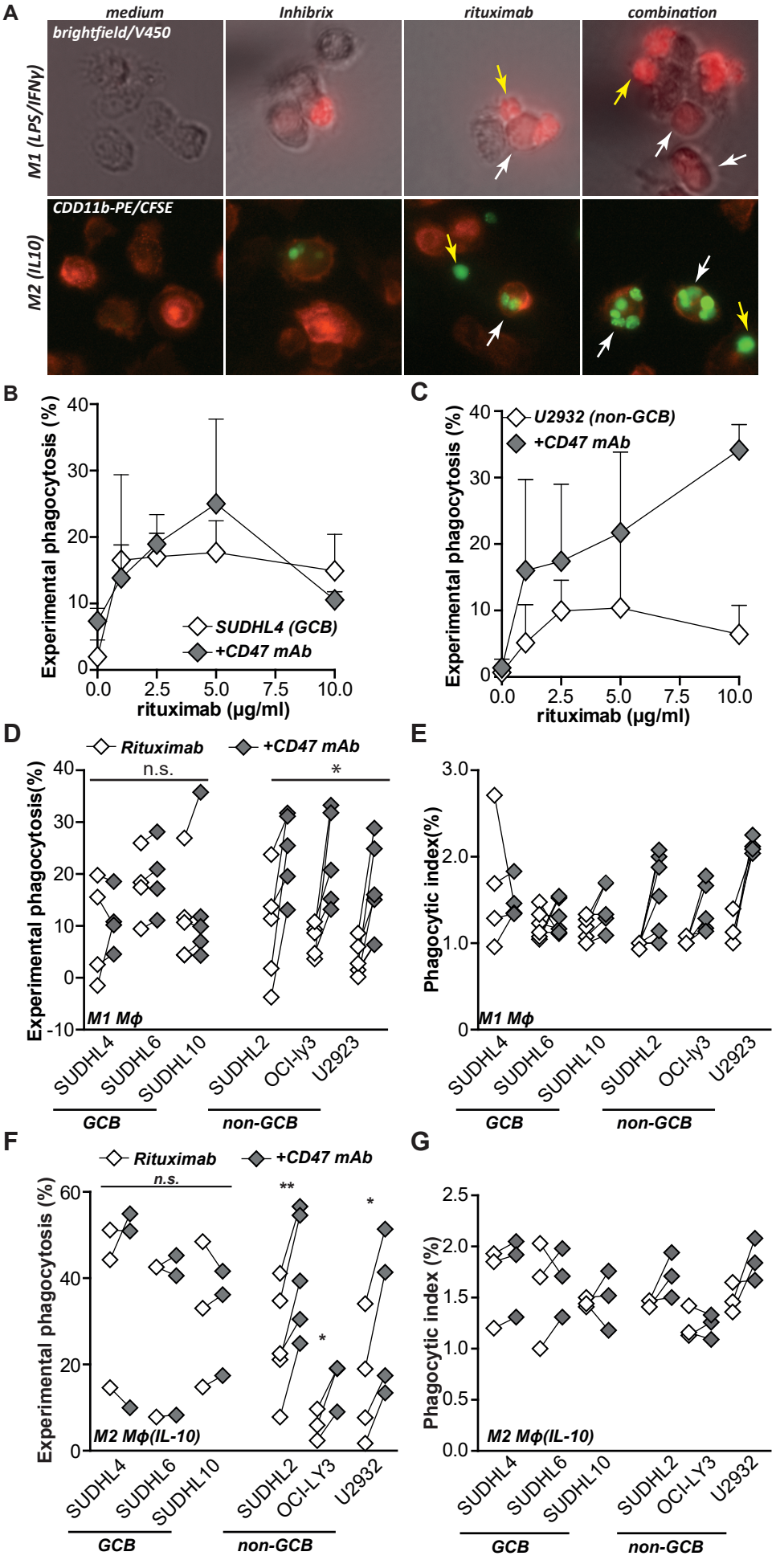

Figure 3

AACR American Association for Cancer Research

Cancer Immunology Research

CD47 expression defines efficacy of rituximab with CHOP in Non-Germinal Center B-cell (non-GCB) Diffuse Large B-Cell Lymphoma patients (DLBCL), but not in GCB DLBCL

Renee Bouwstra, Yuan He, Janneke Willemien de Boer, et al.

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