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Alterations in calreticulin and CD47 expression dynamics in myeloid malignancies: therapeutic and prognostic implications in myelodysplastic syndromes and myeloproliferative neoplasms

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Data-sharing statement: The data supporting the findings of this study, including de-identified patient-level data on CALR and CD47 expression in myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPN), as well as cell line experimental datasets, are available from the corresponding author upon reasonable request (crinaldi@lincoln.ac.uk). Data will be shared in accordance with institutional and ethical guidelines, and a data access agreement may be required.

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Letter to the Editors

Dear Editor,

Myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPN) are distinct yet biologically related clonal hematopoietic disorders that originate from acquired somatic mutations in hematopoietic stem and progenitor cells. These conditions are characterized by deregulated differentiation and proliferation, which are often associated with mutations affecting transcription factors, epigenetic modifiers, and signal transduction pathways, particularly those involving JAK/STAT signaling.[1,2] Clinically, MDS is marked by ineffective hematopoiesis leading to peripheral blood cytopenias and a high risk of transformation to acute myeloid leukemia (AML). In contrast, MPNs typically present with excessive myeloid proliferation, often resulting in hypercellular bone marrow, thrombotic complications, and, in advanced stages, myelofibrosis or leukemic transformation.

Despite the use of risk-adapted treatment strategies, including cytoreductive agents and hypomethylating agents (HMAs) such as azacytidine and decitabine for MDS, or JAK inhibitors such as ruxolitinib for MPN, the therapeutic landscape remains limited. These agents are not curative, and only about 40–50% of patients derive clinical benefit, with responses often being partial or transient. Allogeneic stem cell transplantation remains the only potentially curative option, yet it is applicable to a minority of patients due to age, comorbidities, or disease burden. Consequently, there is a critical need for novel therapeutic strategies that can either synergize with current treatments or offer alternatives for patients with refractory disease.

In recent years, attention has turned to the role of the innate immune system in recognizing and eliminating malignant hematopoietic cells. Among the immune checkpoint regulators implicated in immune escape are CD47 and calreticulin (CALR), two molecules that function as opposing modulators of macrophage-mediated phagocytosis.[3–5] CD47 acts as a “don’t eat me” signal by binding to signal regulatory protein alpha (SIRPα) on macrophages, thereby inhibiting phagocytosis. CALR, on the other hand, serves as a pro-phagocytic or “eat me” signal when translocated to the cell surface, where it engages with the LDL receptor-related protein-1 (LRP1) on phagocytes. The balance between CD47 and CALR surface expression is thus thought to critically determine whether a cell is targeted for clearance by the innate immune system.

Under physiological conditions, CALR resides within the endoplasmic reticulum and is involved in calcium homeostasis and protein folding. However, in the context of malignant transformation, CALR can translocate to the plasma membrane, where it serves as an immunogenic signal. Interestingly, in many malignancies, including AML and other leukemias, CD47 is overexpressed, effectively overriding the prophagocytic signals and allowing malignant cells to evade immune detection.[3,5] The dynamics of CALR and CD47 expression in myeloid neoplasms such as MDS and MPN, however, remain incompletely understood.[6,7]

To investigate this, we examined CALR and CD47 expression in MDS and MPN using a combination of *in vitro* models and patient-derived peripheral blood mononuclear cells (PBMCs). Cell line models included SKM-1 and MOLM-13 for MDS, and HEL-92 and GDM-1 for MPN. These were treated with disease-relevant therapies: azacytidine (5 μM, 48 hours) for MDS and ruxolitinib (5 μM, 48 hours) for MPN. Drug concentrations were selected based on titration studies, with re-dosing at 24-hour intervals to maintain consistent drug exposure. This study has been approved by a formally constituted review board.

In MDS cell lines, treatment with azacytidine led to a significant reduction in surface membrane CALR expression as detected by Western blotting, decreasing from baseline levels of 17–18% to 13–15% post-treatment ($p = 0.0001–0.004$). In contrast, CD47 expression on the cell membrane increased modestly by 3–4% following treatment ($p = 0.03$). A comparable pattern was seen in MPN models. Ruxolitinib reduced CALR membrane expression in HEL-92 cells from 29% to 13% and in GDM-1 cells from 16% to 11% ($p = 0.03$). Simultaneously, CD47 expression rose significantly from 11% to 17% in HEL-92 ($p = 0.008$), with similar trends in GDM-1. These findings indicate a consistent shift in the CD47/CALR balance in response to therapeutic agents, favoring immune evasion through reduced prophagocytic and increased antiphagocytic signaling.

To translate these findings to patient samples, we analyzed PBMCs from 28 individuals: 10 with MDS (IPSS-R low=3, int-1=2; int-2=3; high=2), 18 with MPN (subdivided into polycythemia vera [PV=8], essential thrombocythemia [ET=6], and primary myelofibrosis [PMF=4]), and 4 healthy controls. Treated MPN patients had received hydroxyurea, interferon-alpha, or ruxolitinib. Due to sample size constraints, treatment-specific subgroup analyses were not statistically powered. All patients were diagnosed using WHO 2016 criteria.8,9 Samples were processed via Ficoll-Paque separation.

Analysis of total protein expression using Western blotting with densitometry revealed interpatient variability in CD47 and CALR levels. Although absolute differences were not statistically significant across disease groups, the CALR:CD47 expression ratio was significantly different ($p = 0.0008$), particularly between healthy controls and untreated PV ($p = 0.0202$), and between untreated and treated PV samples ($p = 0.0004$). This suggests that therapy alters immune checkpoint expression in vivo, though the direction and magnitude of this change may depend on the MPN subtype (Figure 1).

Further investigation focused on membrane-specific expression. After Ficoll-Paque extraction of PBMCs from MDS samples, subcellular fractionation was performed using NucPer and MembranePlus kits to isolate membrane and cytoplasmic protein fractions. Western blotting together with densitometry was then used to analyze protein membrane expression vs cytosol, cytoplasm and nuclear expression. In MDS, CD47 membrane expression increased progressively with disease risk, reaching a peak in high-risk patients ($p = 0.0001$). Post hoc comparisons confirmed statistically significant differences between control vs. low-risk, control vs. high-risk, and low vs. high-risk groups ($p = 0.0011$ to <0.0001). CALR membrane expression did not vary significantly, but the membrane CALR:CD47ratio decreased with increasing disease severity ($p < 0.0001$), suggesting a

mechanism for immune escape through CD47 dominance in advanced disease (Figure 2). While CD47 was clearly higher in higher-risk categories, no consistent or statistically significant association was found with blast count or treatment duration, likely reflecting the small cohort size.

Interestingly, MPN subtypes showed more heterogeneity. While PV samples exhibited a consistent decline in CALR/CD47 ratio following treatment, patterns in ET and PMF were variable, highlighting intrinsic differences in immune modulation across MPN phenotypes. These differences may have implications for personalized immunotherapeutic strategies targeting CD47 or enhancing CALR-mediated phagocytosis.

To evaluate the functional relevance of CD47 modulation, we performed siRNA-mediated knockdown experiments in cell line models. Partial knockdown of CD47 (~40% reduction) was achieved using Lipofectamine LTX and siRNA. Complete knockdown led to loss of cell viability, indicating a survival role for CD47. In azacytidine-treated MDS cells, western blot analysis of CD47 membrane levels revealed they dropped significantly upon siRNA treatment ($p = 0.0058$), enhancing susceptibility to treatment. In contrast, in ruxolitinib-treated HEL-92 cells, CD47 knockdown did not significantly alter membrane CD47 expression or response, suggesting pathway-specific roles. While CALR localization was not dramatically altered by CD47 knockdown, its inability to translocate effectively to the membrane may limit the success of innate immune-mediated clearance (Figure 3).

These findings imply that the modulation of CD47 expression may enhance treatment efficacy in MDS, particularly in combination with HMAs. In MPN, particularly in JAK2-mutant cases treated with ruxolitinib, additional or alternative immune modulators may be necessary to synergize with CD47 inhibition. Our results thus support a disease- and subtype-specific approach to immune checkpoint blockade in myeloid malignancies.

The clinical relevance of CD47 blockade is being actively investigated. Anti-CD47 antibodies such as magrolimab have shown promising activity in AML and high-risk MDS. However, recent clinical trial data have prompted questions regarding optimal timing, patient selection, and potential mechanisms of resistance.10 Our data suggest that CD47 expression correlates with disease risk and treatment exposure, and could serve as a biomarker for stratifying patients likely to respond to anti-CD47 therapies. In addition, new anti-CALR approaches in MPN, must be carefully engineered to only target specific mutant CALR protein and avoid neutralizing a key prophagocytic signal .

Nonetheless, our study has limitations. The sample size was modest, and while findings were consistent across models, in vitro conditions cannot fully replicate the immunological complexity of the bone marrow microenvironment.11 Moreover, the intracellular regulation of CALR remains incompletely understood and may involve post-translational modifications or endoplasmic reticulum stress responses.

Importantly, emerging evidence suggests that the macrophage phenotype itself evolves during disease progression in MDS and MPN, potentially affecting response to CD47-

targeting agents.12–14 As such, future therapeutic strategies must consider not only the tumor cell's immune checkpoint profile but also the activation state, polarization, and function of the surrounding immune effector cells.

In conclusion, our study demonstrates that CD47 is upregulated and CALR downregulated at the cell surface in MDS and MPN, particularly after exposure to standard therapies. This shift promotes immune evasion by suppressing macrophage-mediated clearance. The balance of CD47 and CALR, particularly at the membrane level, may serve as a biomarker of disease progression and a predictor of response to immunotherapeutic agents. Targeting CD47 in combination with therapies that restore CALR presentation or enhance macrophage activation may provide a powerful strategy to overcome resistance and improve outcomes in patients with myeloid malignancies.

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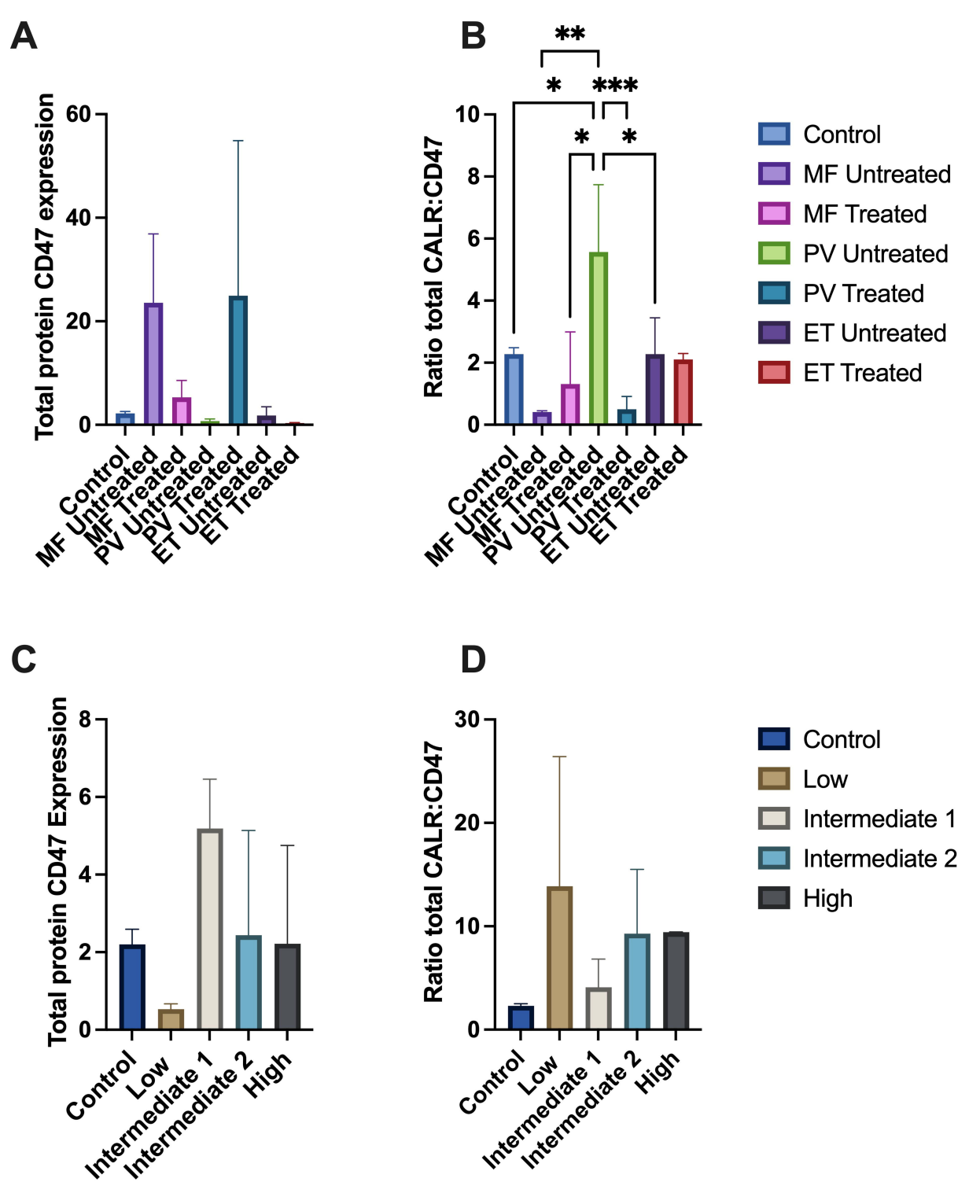
Figure 1. Total CD47 protein expression and ratio of total Calreticulin (CALR) to total CD47 in MDS and MPN patient samples. Total protein was extracted from patient PBMC samples using the Ficoll-Paque method, with subsequent western blotting with densitometry undertaken using a digital developer (Li-Cor Biosciences, UK) to look at total CD47 & CALR expression (Primary antibodies were obtained from Abcam, UK & secondary antibodies from Li-Cor Biosciences, UK). Protein expression was normalised to housekeeping protein GADPH. All experiments were done to n=3. MPN cohort : MF untreated n=2; MF treated n=2; PV untreated n=4; PV treated n=4; ET untreated n=4; ET treated n=4. MDS cohort: IPSS-R low=3, int-1=2; int-2=3; high=2. Graph A) No significant difference in total CD47 protein was found across MPN patient subgroups (p = 0.1347). Graph B) Significant difference in the ratio of CALR:CD47 total protein in MPN (p = 0.0008), post hoc testing revealed significant differences between healthy control and PV Untreated (p = 0.0202), MF Untreated and PV treated (p=0.0036), MF Treated and PV Untreated (p = 0.0134), PV Untreated and PV Treated (p = 0.0004), and PV Untreated and ET Treated (p = 0.0202). Graph C) no significant difference in total CD47 in MDS patient subgroups. Graph D) no significant difference in ratio of total CALR:CD47 in MDS patient subgroups. Notably there was a large amount of variability between patients even within patient subgroups as highlighted by the large error bars. Statistical significance is shown on the graphs. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 2. Membrane fractionation of CD47 in MDS patient samples. Total protein was extracted from patient PBMC samples. Patient samples were subject to fractionation into membrane, cytoplasm, cytosol and nuclear compartments using two kits as per manufacturer instructions. The Nucper kit (Thermofisher scientific, UK) was used to separate the sample into cytoplasm and nucleus samples and the Membrane plus kit (Thermofisher scientific, UK), separated the sample into membrane and the cytosol. Expression of CD47 and Calreticulin (CALR) was then examined for each cellular fraction via western blotting as details in Figure 1. The membrane expression percentages show the level of CD47 in the membrane vs the level of CD47 across all remaining fractions (the cytosol, cytoplasm and nucleus). . For our MDS cohort we had the following IPSS-R risk-stratified patients: Low risk: n=3, Int-1: n=2, Int-2: n=3, High risk: n=2 & healthy controls: n=4. All experiments were done to n=3. The amount of CD47 was normalised to the total protein extracted. Graph A) Membrane CD47 expression in MDS patient subgroups, significant difference in CD47 levels (p=0.0001), post hoc testing identified significant differences between control and high-risk patients (p=0.0005), low and high-risk patients (p = 0.0002), Int-1 and high-risk patients (p = 0.0001) and int-2 and high-risk patients (p=0.0011). Graph B) ratio of the membrane fractions of CALR to CD47, significant difference (p<0.0001), post hoc testing identified significant differences between control and low risk patients (p <0.0001), low and Int-1 patients (p=0.0001), low and Int-2 patients (p=0.0001) and low and high-risk patients (p=0.0002). Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****p < 0.0001.

Figure 3. Percentage membrane expression of CD47 and Calreticulin (CALR) in treated and untreated CD47 knockdown of HEL-92 and SKM-1 cell lines. CALR and CD47 knock downs were undertaken using siRNA and lipofectamine LTX (Life technologies, US). Lipofectamine was applied for 48 hours and the lipofectamine concentration used was shown to knock down CALR or CD47 levels but have no effect on the lipofectamine control or the scatter siRNA. The membrane expressions percentages show the level of CD47 (in panels A & B) and CALR (in panels C & D) in the membrane vs the level of CD47 across all remaining fractions (the cytosol, cytoplasm and nucleus). All experiments were done to n=3. Graph (A) Treatment of Hel-92 CD47 knock down with Azacitidine resulted in significantly greater percentage membrane expression of CD47 than untreated Hel-92 CD47 knockdown ($p = 0.0041$) or Ruxolitinib treated Hel-92 CD47 knockdown ($p = 0.0065$). Graph (B) Treatment of SKM-1 CD47 knockdown with Azacitidine resulted in significantly lower percentage membrane expression of CD47 than untreated SKM-1 CD47 knockdown ($p = 0.0058$) or Ruxolitinib treated SKM-1 CD47 knockdown ($p = 0.0030$). Graph (C) CALR membrane expression post treatment of Hel-92 CD47 knock down with Azacitidine and Ruxolitinib was not significantly different to control knockdown. Graph (D) CALR membrane expression in CD47 SKM-1 knockdown post treatment with azacitidine was significantly higher than either untreated knockdown ($p=0.0032$) or Ruxolitinib treatment ($p=0.0006$). Similarly, treatment with Ruxolitinib resulted in significantly lower CALR than untreated knockdown ($p=0.0070$). Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$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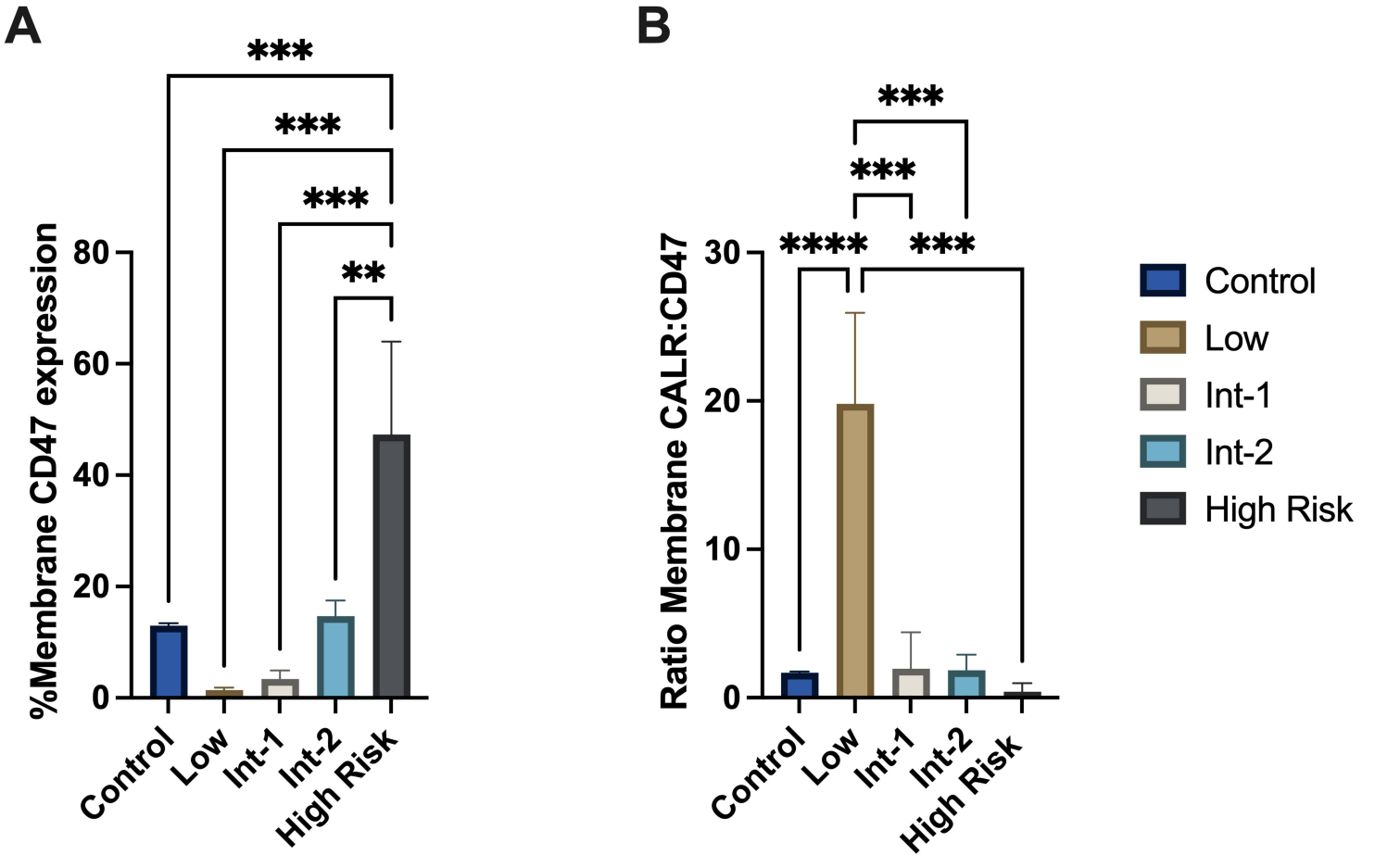
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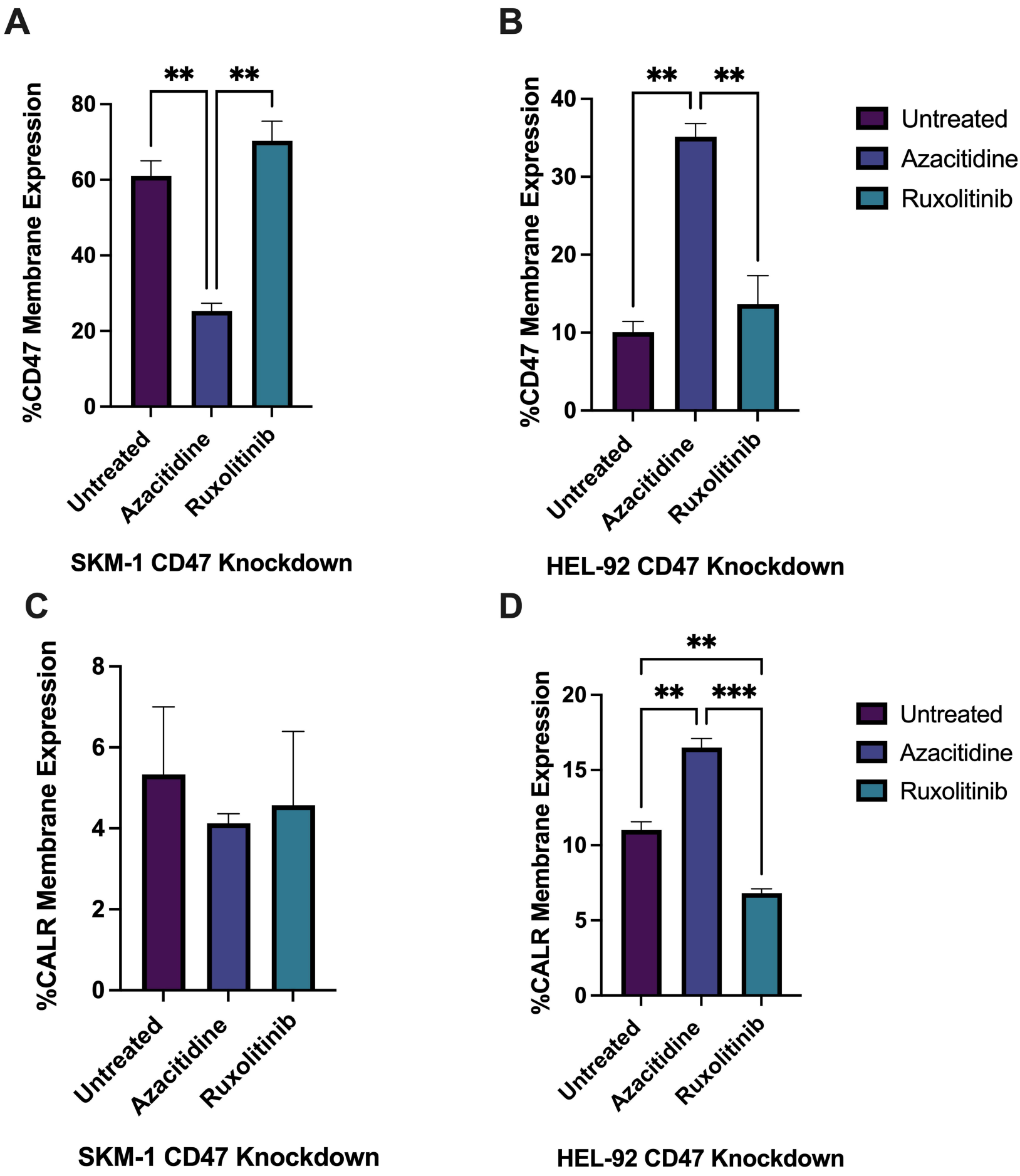
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