CD74 is expressed in a subset of pediatric acute myeloid leukemia patients and is a promising target for therapy: a report from the Children's Oncology Group

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Abstract

As curative therapies for pediatric acute myeloid leukemia (AML) remain elusive, identifying potential new treatment targets is vital. We assessed the cell surface expression of CD74, also known as the major histocompatibility complex-II invariant chain, by multidimensional flow cytometry in 973 patients enrolled in the Children's Oncology Group AAML1031 clinical trial (*clinicaltrials gov. Identifier: NCT01371981*). Thirty-eight percent of pediatric AML patients expressed CD74 at any level and a comparison to normal hematopoietic cells revealed a subset with increased expression relative to normal myeloid progenitor cells. Pediatric AML patients expressing high intensity CD74 typically had an immature immunophenotype and an increased frequency of lymphoid antigen expression. Increased CD74 expression was associated with older patients with lower white blood cells and peripheral blood blast counts, and was enriched for t(8;21), trisomy 8, and CEBPA mutations. Overall, high CD74 expression was associated with low-risk status, however 26% of patients were allocated to high-risk protocol status and 5-year event-free survival was 53%, indicating that a significant number of high expressing patients had poor outcomes. *In vitro* preclinical studies indicate that anti-CD74 therapy demonstrates efficacy against AML cells but has little impact on normal CD34$^+$ cells. Together, we demonstrate that CD74 is expressed on a subset of pediatric AML at increased levels compared to normal hematopoietic cells and is a promising target for therapy in expressing patients. Given that nearly half of patients expressing CD74 at high levels experience an adverse event within 5 years, and the availability of CD74 targeting drugs, this represents a promising line of therapy worthy of additional investigation.

Introduction

Even with the widespread application of intensive chemotherapy and hematopoietic stem cell transplantation, curative treatments for pediatric patients with acute myeloid leukemia (AML) remain elusive.[1] While outcomes in pediatric AML from first diagnosis have improved in recent years they still lag behind pediatric acute lymphoblastic leukemia (ALL) and outcomes of high-risk patients, and in particular patients who relapse, remain dismal, highlighting the need for new approaches for treatment.[1-8] Identifying biomarkers at the time of diagnosis is one such possible approach. These biomarkers could be associated with prognosis, such as the published RAM phenotype.[9] Alternatively, with the increased availability of targeted and immunotherapeutic treatments, identified candidate biomarkers could serve as a potential source of targets for treatment, as has been seen in B-cell malignancies.[10-15] While AML has proved more difficult to treat via these methods, gemtuzumab ozogamicin, an anti-CD33 antibody-drug conjugate is approved for use in AML and other targets, including CD123 and others, are under investigation.[16-20] However, both biomarker-based risk

assessments and targeted therapies require the identification of molecules that are sufficiently and stably expressed and relatively specific to leukemia cells.

CD74, also known as the invariant chain of major histocompatibility complex (MHC)-II, is a single pass type II membrane protein originally identified as an MHC class II chaperone, facilitating antigen loading and presentation.[21,22] More recently, CD74 has been identified as having roles in monocyte/macrophage activation, stem cell maintenance, B-cell differentiation, and T-cell function, all of which rely on the capability of CD74 to initiate cell signaling through multiple pathways.[23-31] Due to its diverse functions, previous studies have shown CD74 to be expressed in a variety of normal hematopoietic cell types (B cells, macrophages, and dendritic cells), as well as in AML, B-cell lymphomas, and multiple myeloma.[32-38]

CD74 has generated interest as an immunotherapeutic target, as it is expressed on the surface of multiple hematopoietic neoplasms and is rapidly internalized.[39,40] Previous studies have shown an association between expression of CD74 and clinical outcome in adult patients treated with bortezomib, though the exact nature of this association is unclear.[32,41] Additionally, anti-tumor activity has been noted in preclinical models of B- and T-cell lymphomas, multiple myeloma, and solid tumors.[34,37,42-44] To date, phase I trials in B-cell lymphomas have demonstrated limited response.[45] However, additional trials with newer therapeutics (STRO-001),[44,,46-48] in multiple myeloma and B-cell lymphomas, are underway and preliminary analysis has shown that the drug is well tolerated.[49,50] Recently published preclinical data has demonstrated the efficacy of STRO-001 against AML and B-ALL cells both *in vitro* and in patient derived xenograft (PDX) mouse models.[51]

Despite the association with both myeloid cells and hematologic malignancies, data regarding the quantitative expression of CD74 in pediatric AML is limited. In this study, we assessed CD74 cell surface expression by flow cytometry in 973 pediatric AML patients enrolled in the Children's Oncology Group AAML1031 phase III clinical trial and demonstrate that a subset of pediatric AML express increased levels of CD74 on the cell surface compared to normal myeloid progenitors. We further correlated cell surface CD74 expression levels with known risk factors and patient outcomes. Together, the data suggest that CD74 may be a useful biomarker and a possible therapeutic target in pediatric AML.

Methods

Patients and treatment

Pediatric patients with newly diagnosed *de novo* AML (excluding acute promyelocytic or Down syndrome-associated AML) enrolled (February 2011 to January 2016) on the Children's Oncology Group (COG) AAML1031 phase III clinical trial (*clinicaltrials gov. Identifier: NCT01371981*) were eligible for this study. Details of this study have been previously published.[2] AAML1031 tested the efficacy of the first-generation proteasome inhibitor bortezomib along with a four-cycle multi-agent chemotherapy backbone in a randomized fashion. Multi-dimensional flow cytometry (MDF) analyses were performed at central reference laboratory (Seattle, WA, USA). The National Cancer Institute's central institutional review board (IRB) as well as the IRB at each enrolling center approved the study; patients and families provided informed consent or assent as appropriate and was conducted in accordance with the Declaration of Helsinki.

CD74 assessment

Specimens were processed and analyzed by a difference from normal analysis method as previously described.[52,53] CD74 fluorescence was collected on diagnostic samples prospectively and the intensity of CD74 was retrospectively assessed. Expression of CD74 on normal cells was collected on either banked $CD34^+$ cord blood cells from healthy births or bone marrows from patients without hematopoietic malignancies.

STRO-001

STRO-001 (CD74-directed antibody drug conjugate) and the unconjugated antibody SP7219 were a gift from Sutro BioPharma. Drug and antibody were diluted in culture medium before addition to cells.

Colony-forming unit assays

Two hundred and fifty cells, either treated with STRO-001 or control for 48 hours, were cultured at 37°C with 5% CO_2 for 10-14 days. Colonies were counted and characterized. Colony types were normalized to total number of colonies present in the untreated specimen.

In vitro cytotoxicity assays

Primary cells were plated at a density of 20,000 cells per well on 96 well plates (Corning, 3603) with the indicated dilution of STRO-001 with or without blocking antibody. Cell viability was determined via luminescence 3 days after continuous exposure using CellTiter-Glo (Promega, Cat#G7570). Data were normalized to untreated controls.

Statistical analysis

AAML1031 patient outcome data were analyzed with a data cutoff of June 30, 2022. The Kaplan-Meier method was used to estimate overall survival (OS) and event-free survival (EFS). A P value <0.05 was considered statistically significant.

Results

Study population

A total of 1,294 pediatric patients with newly diagnosed *de novo* acute AML were enrolled on AAML1031. Patients consid-

ered not eligible or not consented for correlative biological studies were excluded from this analysis. Patients in arm C were also excluded as they were exclusively *FLT3*-internal tandem duplication (ITD) high allelic ratio and received sorafenib as part of their treatment,[4] making them a poor comparator to the remaining patients. A bone marrow aspirate at time of diagnosis submitted for flow cytometry analysis was required, disease had to be identified in the marrow, and cell surface CD74 expression data had to be collected. These criteria resulted in 973 patients eligible for this analysis, who were evenly distributed between arms A (478, 49%) and B (495, 51%). A diagram of the study design can be found in the *Online Supplementary Figure S1*.

CD74 is expressed on the cell surface of a subset of pediatric acute myeloid leukemias

Bone marrow specimens at time of AML diagnosis were assessed by flow cytometry for quantitative surface expression of CD74 on AML cells identified by a difference from normal assessment using FITC-conjugated antibody. CD74 showed a log normal distribution of expression across the cohort (Figure 1A). The intensity and range of antigen expression was comparable to that observed for other cell surface antigens expressed in pediatric AML when stained with FITC-conjugated antibodies (Figure 1B). Thirty-eight percent of cases demonstrated mean CD74 expression two standard deviations above the mean FITC autofluorescence of the patients eligible for this study and were considered positive for CD74 (Figure 1C). For the purposes of this study, patients were divided into quartiles based on CD74 expression, and as described for other AML surface markers, the highest CD74 expressing quartile (Q4) was compared to the lower expressing quartiles (Q1-3) to assess associations with clinical variables.[5,54,55] The median CD74 mean fluorescent intensities (MFI) were 6.23 (range, 2.24-7.84) for quartile 1 (Q1), 9.35 (range, 7.86-10.84) for quartile 2 (Q2), 13.26 (range, 10.85-16.55) for quartile 3 (Q3), and 24.09 (range, 16.57-275) for quartile 4 (Q4) (Figure 1C). Patients in each quartile were equally distributed between study arms (Q4: 129 [53%] arm A, 114 [47%] arm B; Q1-3: 349 [48%] arm A, 381 [52%] arm B).

Samples allocated to Q4 showed increased levels of expression on AML cells of immature markers (CD34 and CD117) as well as class II MHC (HLA-DR) and CD38 as assessed by mean MFI compared to cases in Q1-3 (Figure 1D). Patients in Q4 also showed an increased rate of aberrant expression of lymphoid antigens (CD56, CD7, and CD19), with positive expression defined as an MFI greater than two standard deviations above mean autofluorescence (Figure 1E). CD74 expression at time of relapse was only available for two patients in CD74 Q4, but in both patients CD74 was expressed at levels similar to diagnosis (Figure 1F), suggesting that CD74 may be a durable biomarker for leukemia in these patients and could possibly be utilized in MRD analysis.

CD74 is expressed on normal monocytes and myeloid progenitors at lower intensity than high expressing pediatric acute myeloid luekemias

In order to determine how the expression of CD74 on pediatric AML cells compares to what is observed on normal hematopoietic cells, we assessed CD74 (stained with PE antibody) expression on MRD-negative recovering pediatric bone marrows post chemotherapy. CD74 was consistently expressed on monocytes and $CD34^+$ myeloid progenitor cells (Figure 2A, B). While it was not consistently expressed on lymphocytes, analysis revealed the consistent presence of a subset expressing CD74, most likely a subset of B cells (Figure 2B). No expression was seen on mature granulocytes (Figure 2A, B). In order to confirm that exposure to chemotherapy was not impacting CD74 expression, we assessed $CD34^+$ cells isolated from cord blood from healthy births and found similar levels of expression (Figure 2A, B). Additionally, assessment of 14 normal bone marrows (B-lymphoma staging or to rule out myeloid neoplasm) revealed comparable CD74 expression. Expression was slightly decreased compared to regenerating marrow in all lineages except lymphocytes, though this did not reach significance (Figure 2A, B). When the expression on normal myeloblasts was compared to other canonical myeloid antigens (CD38, CD13, CD33, CD117, and CD123), CD74 had the lowest level of expression of the antigens assayed, expressed just above autofluorescence (Figure 2C).

Analysis of bone marrow in patients without evidence of hematopoietic malignancy revealed expression of CD74 lower than monocytes and only slightly above autofluorescence (*Online Supplementary Figure S2A*). In order to compare the level of expression on AML cells to normal cells within an AML patient, we analyzed several index cases randomly selected from Q4 (N=16). As noted above, AML patients were stained with FITC-conjugated CD74 antibody, which has a lower intensity than the PE antibody used for the recovering and normal/resting bone marrows. As seen in non-leukemic patients, low levels of CD74 expression was identified on a subset of lymphocytes and monocytes in these cases, but not on normal mature myeloid (granulocytic) cells (*Online Supplementary Figure S2B*). However, quantification of expression revealed that CD74 expression was significantly higher on the AML cells compared to all other normal cell lineages in the same sample for this subset of cases. This includes the monocytes, which are the highest expressors of CD74 in the normal bone marrows assessed ($P<0.002$ for all comparisons) (Figure 2D).

CD74 Expression level is associated with good prognostic markers but is expressed on a subset of high-risk patients

Patients enrolled in AAML1031 with the highest levels of CD74 expression (Q4), as assessed with FITC-conjugated antibody, had a higher median age, lower median white blood cell (WBC) count, and lower peripheral blood blast

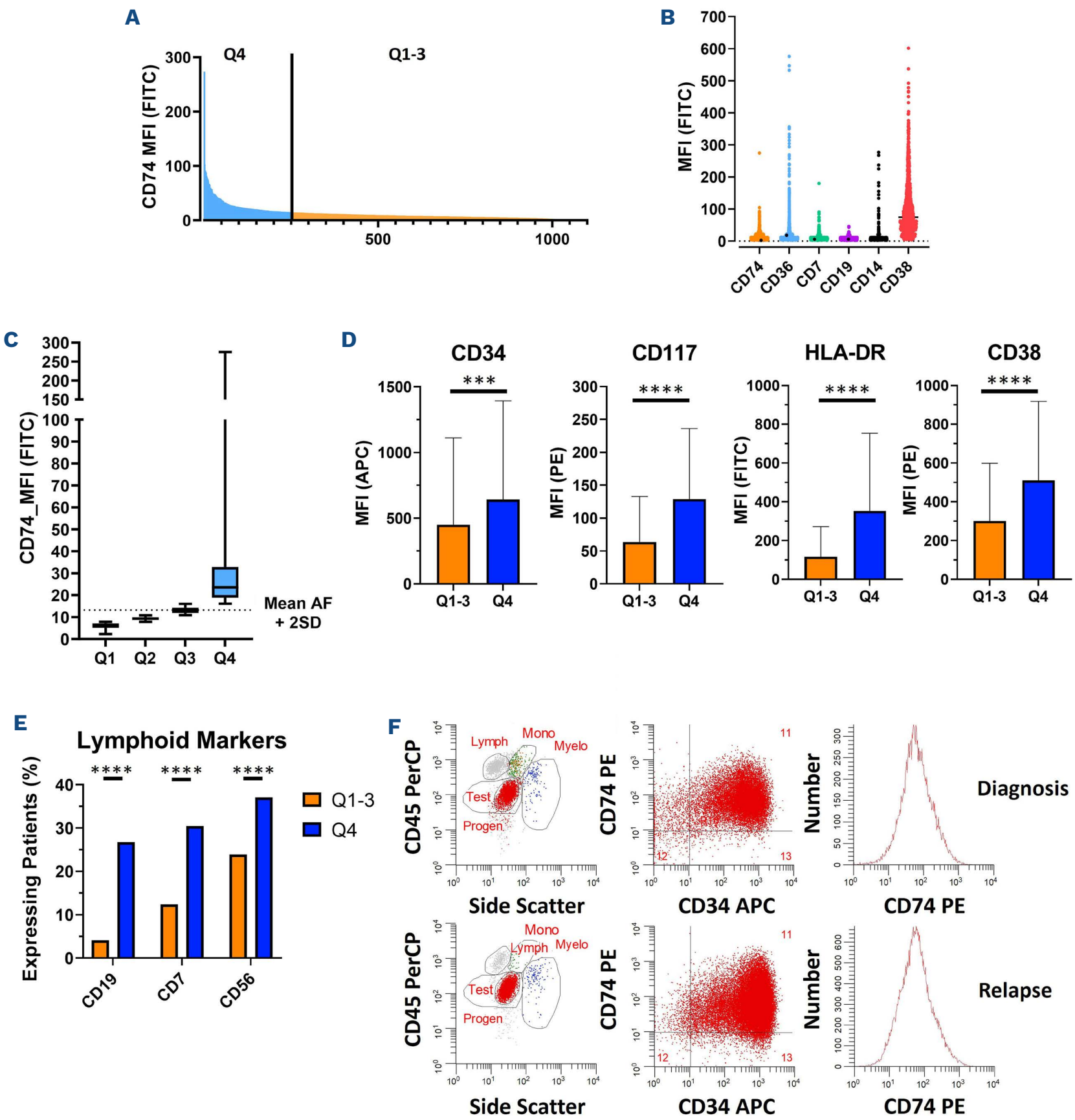


Figure 1. Flow cytometric assessment of CD74 on pediatric acute myeloid leukemia patients. (A) Across a cohort of 973 pediatric acute myeloid leukemia (AML) patients CD74 expression showed a log normal distribution. The top quartile (Q) of patients is shown in blue. (B) CD74 expression was similar to other antigens expressed on AML cells when assessed with the same fluorochrome. (C) Thirty-eight percent of eligible patients expressed CD74 more than 2 standard deviations above the mean autofluoresence of pediatric AML, with Q4 (blue) notably higher than the remaining patients. (D) Patients in CD74 Q4 had increased mean intensity of immature markers CD34 and CD117 as well as HLA-DR and CD38. (E) The number of patients in Q4 aberrantly expressing lymphoid markers was also increased compared to the rest of the cohort. (F) A representative specimen showing preserved expression of CD74 at relapse at levels similar to diagnosis. (unpaired *t* test [panel D] or Fisher's exact test [panel E)]; ***$P \leq 0.001$; ****$P \leq 0.0001$).

counts compared to all other patients (Q1-Q3) (Figure 3A-C). Patients in Q4 had a higher rate of achieving CR. However, there was no difference in rate of MRD positivity post induction chemotherapy, either for the entire cohort, or a sub analysis of the treatment arms (Figure 3D, E). In a comparison of genetic risk factors, CD74 Q4 was associated with a lower frequency of inv(16) but an enrichment of CEBP-α mutations, t(8;21), and trisomy 8 (Figure 3F).

We next assessed the protocol risk status, as outlined in the AAML1031 trial protocol.[2] Patients in CD74 Q4 were more

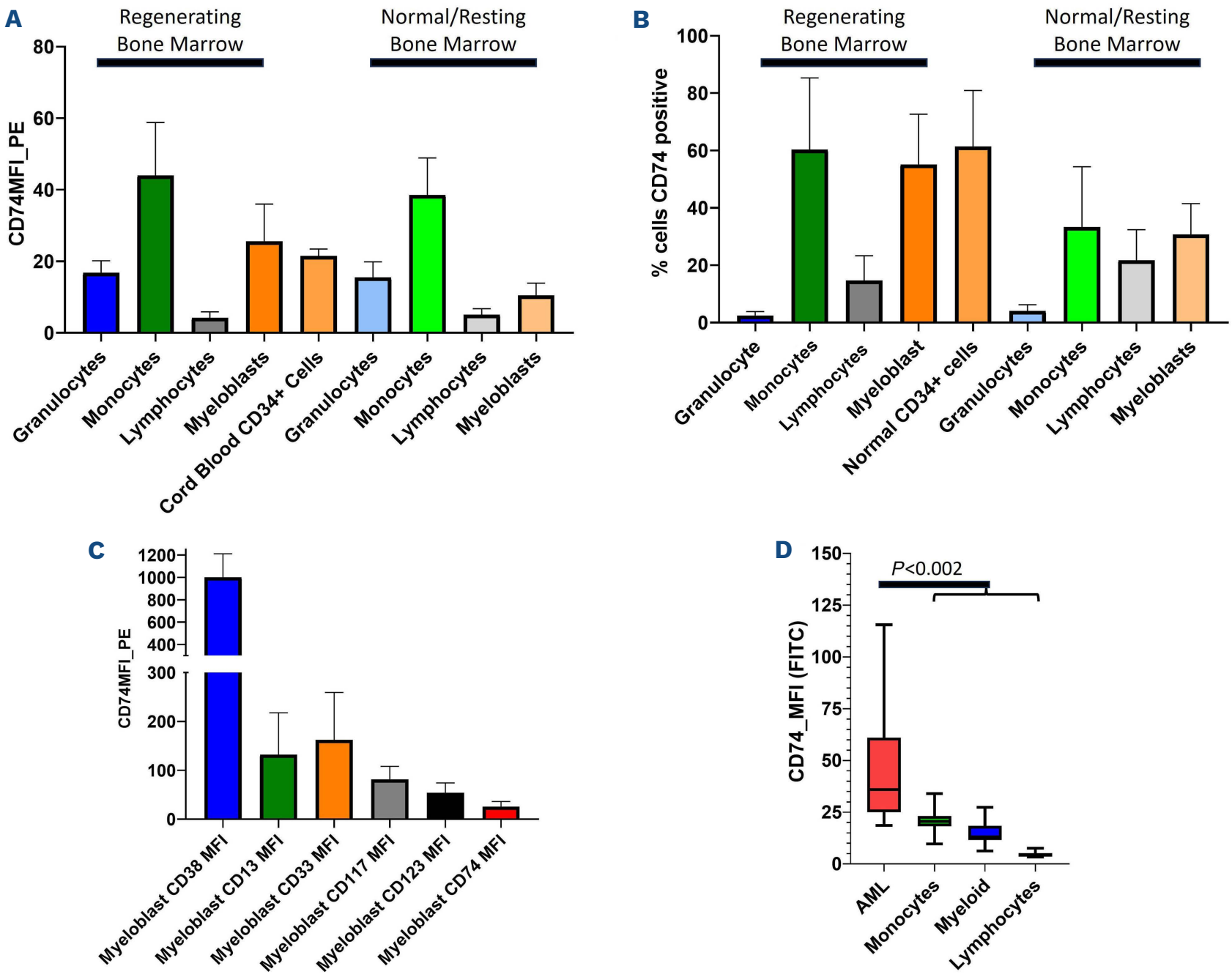


Figure 2. Flow cytometric assessment of CD74 on normal heamtopoietic cells. (A) Intensity of expression of CD74 on normal hematopoetic cells (blue, green, orange, grey) in normal regenerating bone marrow (N=20), cord blood CD34⁺ cells (N=3), or normal resting bone marrow specimens (N=14) (B) Percentage of cells expressing CD74 above patient-defined autofluoresence on normal hematopoetic cells (blue, green, orange, grey) in normal regenerating bone marrow (N=20), cord blood CD34⁺ cells (N=3), or normal resting bone marrow specimens (N=14). (C) Comparison of intensity of canonoical myeloid antigens (CD38, CD13, CD33, CD117, and CD123) stained with PE (phycoerythrin) antibody on normal myeloblasts (N=20 minimal residual disease [MRD]-negative pediatric bone marrows). (D) Quantification of CD74 expression on acute myeloid leukemia (AML) blasts compared to normal cells from the same patients (N=16 pediatric AML).

likely to be allocated to low-risk status than patients in CD74 Q-3 (Q4: low-risk: N=196, 82%, high-risk: 43, 18% *vs.* Q1-3: low-risk: 509, 72%, high-risk: 198, 28%; *P*=0.02) (Figure 3G). When risk factors for the more recent AAML1831 trial are retrospectively utilized,[56] 21 Q4 and 80 Q1-3 patients are moved to high-risk status, bringing the totals to 64 (26.3%) and 278 (38.1%) high-risk patients for CD74 Q4 and Q1-3 respectively (Figure 3G). Risk factors that differed in Q4 compared to Q1-3 were an increase in the prevalence of the favorable-risk markers CEBP-α and t(8;21), a decrease in favorable-risk factor inv(16), and a decrease in the frequency of high-risk factors CBFA2T3-GLIS2 fusions and RAM phenotype (Figure 3H). A full characterization of clinical differences based on CD74 quartile assignment can be found in the *Online Supplementary Table S2*.

High levels of CD74 expression is associated with lower risk and improved outcome

The clinical outcome for patients in CD74 Q4 was improved compared to Q1-3, by both overall and EFS (Figure 4A). High levels of CD74 expression were associated with favorable risk in a univariable COX analysis (hazard ratio [HR]= 0.76, 95% confidence interval [CI]: 0.59-0.99, *P*=0.04). However, in a multivariable analysis incorporating other risk factors, CD74 lost significance (HR=0.99), suggesting the association with other good risk factors drives the improved outcome in CD74 Q4 patients (*Online Supplementary Table S3*). As mentioned previously, CD74 expression is associated with low-risk protocol status, so to determine if CD74 expression had any impact on outcome in risk-matched patients, we compared OS and EFS of Q4 *versus* Q1-3 in low-risk

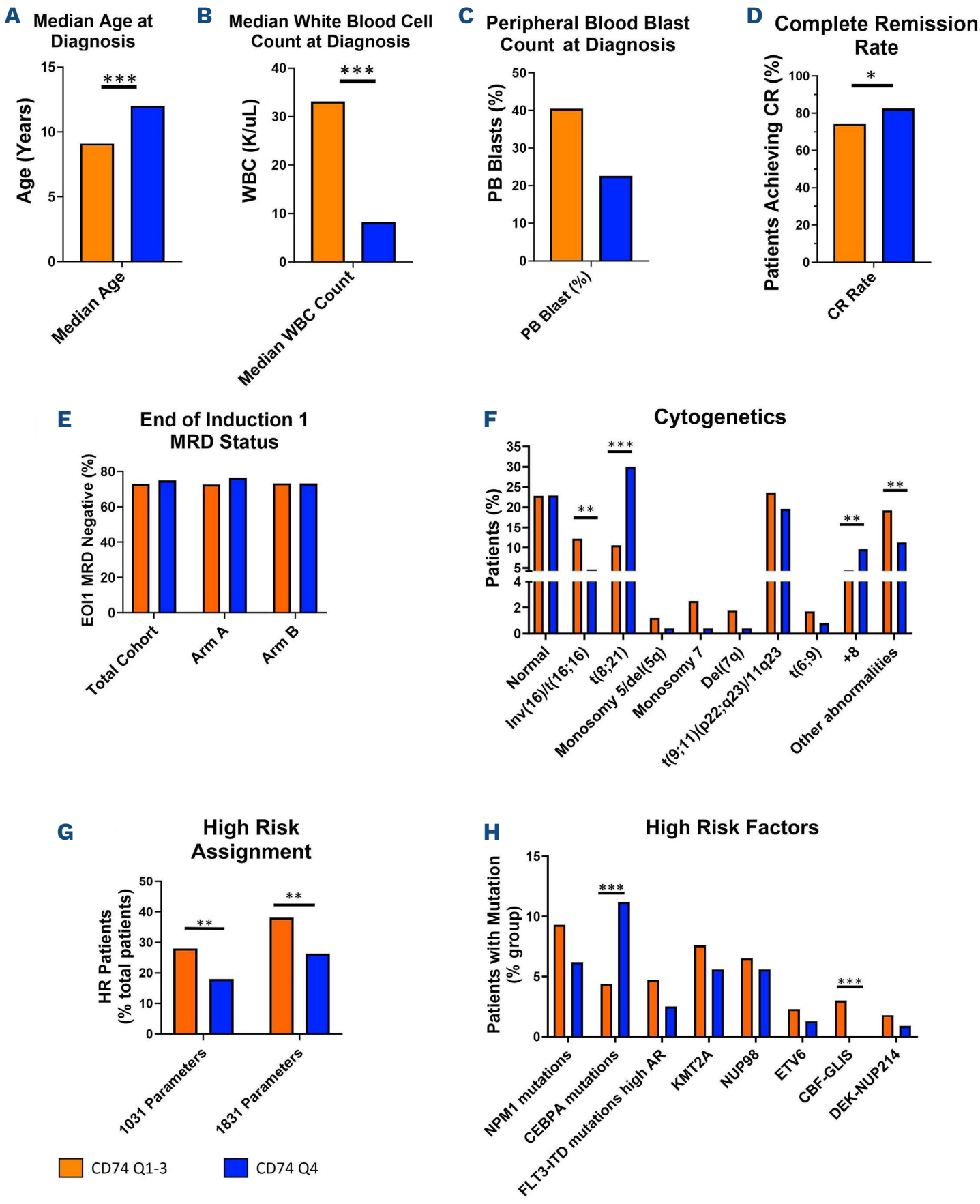


Figure 3. Clinical characteristics of CD74 Q4 *versus* CD74 Q1-3 patients. The median age of diagnosis of acute myeloid leukemia (AML) (A), median white blood cell count (B), and peripheral blood blast count at diagnosis (C) in patients in the highest quartile (Q) of CD74 expression (Q4, blue), *versus* other patients (Q1-3, orange). Comparison of the rate of achieving a complete remission (CR) (D) and minimal residual disease (MRD)-negative status (E) at end of induction cycle 1. Comparison of risk-associated cytogenetic abnormalities (F). The rate of assignment to high-risk status by both the definitions of the AAMl1031 study as well as the updated AAML1831 study (G) for Q4 and Q1-3, as well as a breakdown of the individual molecular risk factors utilized, in addition to cytogenetics and MRD, leading to the assignment (H) (χ^2 test; *P<0.05; **P≤0.01; ***P≤0.001).

and high-risk patients separately. Low-risk Q4 patients had an improved EFS but no difference in OS (Figure 4B), while in high-risk patients CD74 expression did not have a statistically significant impact on patient outcome (*Online Supplementary Figure S3A*). Given the previously published association with bortezomib efficacy, we assessed any impact of treatment arm on the outcome in CD74 expressing patients. A sub-analysis of each treatment arm showed no significant differences in outcome based on treatment with or without bortezomib among the CD74 expression quartiles (*Online Supplementary Figure S3B, C*). An analysis of just CD74 Q4 showed bortezomib treatment had no impact

on outcome in these patients demonstrating high levels of CD74 expression (Figure 4C).

CD74 treatment is cytotoxic to CD74-expressing acute myeloid leukemia cells with limited impact on normal cells

In our comparison of CD74 expression on normal hematopoietic cells to that of other myeloid antigens, we noted that CD74 was more dimly expressed on myeloid progenitors than other cell surface proteins utilized as targets for treatment (CD33 and CD123) (Figure 2C). Based on this, we hypothesized that utilizing CD74 as a therapeutic target may result in less hematopoietic toxicity than other targeted treatments. In order to determine the potential of CD74 as a target for therapy, we performed cytotoxicity studies on primary AML samples. Treatment with anti-CD74 antibody drug conjugate (ADC) STRO-001 effectively killed AML cells expressing CD74, with half maximal inhibitory concentration (IC_{50}) values ranging from 14.07 to 17.4 nM, but had no impact on AML that did not express CD74, with IC_{50} from 5,051 nM to not reached (Figure 5A). In contrast, even though they express CD74, $CD34^+$ cells isolated from cord blood from healthy births were only minimally impacted by treatment with STRO-001, with much higher IC_{50} than CD74-expressing AML cells and only slightly decreased compared to treatment in the presence of excess blocking antibody (Figure 5B). In order to further assess the functional impact of anti-CD74 treatment on normal myeloid progenitors, we performed colony-forming assays on $CD74^+/CD34^+$ cord blood cells. While the total number of colonies decreased after treatment

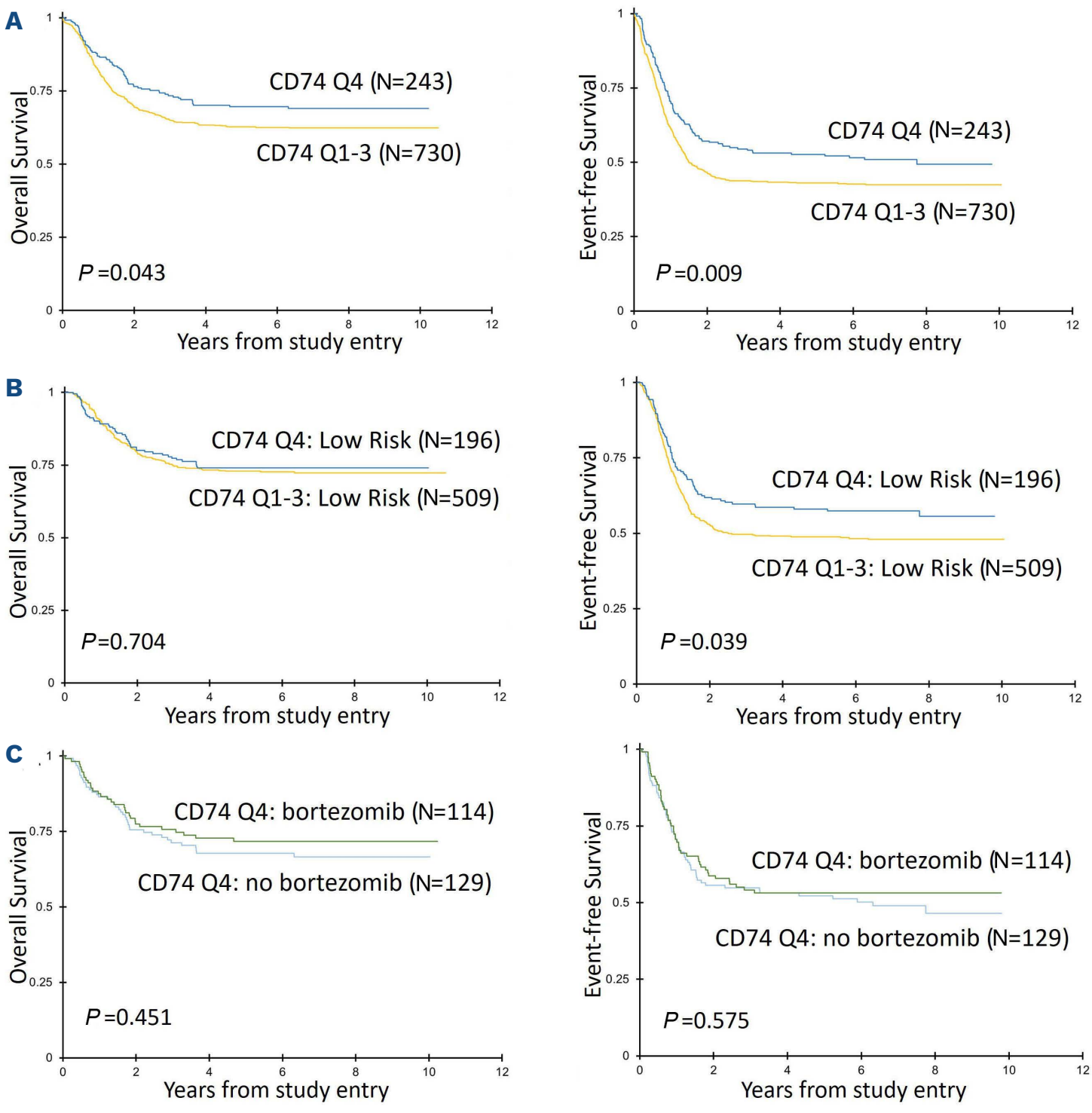


Figure 4. Outcome analysis of CD74 Q4 *versus* Q1-3. (A) The overall survival (OS) and event-free survival (EFS) of high CD74-expressing patients (quartile [Q] 4, blue) compared to all other patients (Q1-3, orange). (B) OS and EFS of low-risk patients assigned to either quartile 4 (Q4) or Q1-3. (C) OS and EFS of patients of patients in Q4 who were treated with bortezomib (arm B) or placebo (arm A) in addition to standard chemotherapy.

with STRO-001 in absence of blocking antibody in all three specimens, it only reached significance in donor 3 (Figure 5C). In all three cases, the number of granulocyte colonies significantly decreased while the effect on the other colony types was variable (*Online Supplementary Figure S4*).

Discussion

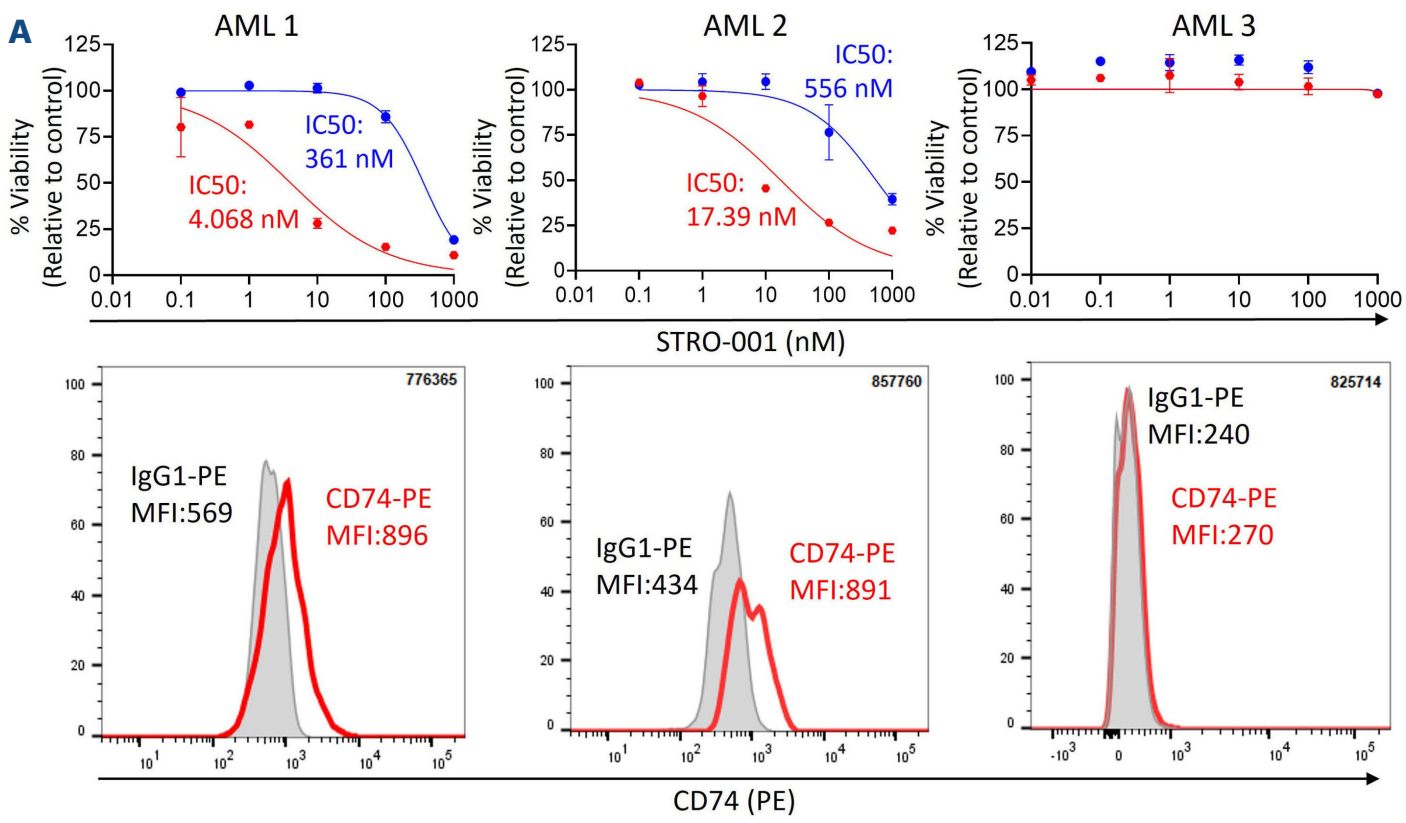
Herein, we report on the cell surface expression of CD74 on leukemia cells from pediatric patients with newly diagnosed AML enrolled on the Children's Oncology Group (COG) AAML1031 study. This study employed standardized processing methods, as well as a difference from normal algorithm that has been extensively validated in previous COG AML trials,[1,2,9,52] providing a strong underlying foundation for the characterization of CD74 expression in pediatric AML and its association with other clinical variables.

In total, 38% of patients expressed CD74 at the level of detection of this study, and CD74 expression was associated with an immature phenotype and aberrant expression of lymphoid antigens. Since our studies found that CD74 was expressed at low levels on normal myeloid progenitor cells, we focused on the clinical features of patients with the highest levels of expression (Q4), a method previously published for analyzing clinical comparisons of cell surface antigen expression in pediatric AML.[54,55,57] Comparison to normal bone marrow cells, both in the AML patients themselves and patients with normal bone marrow, revealed that in Q4 patients, CD74 was significantly increased, indicating that it could be a possible target for treatment. Additionally, this increased expression raises the possibility that CD74 could be used in a difference from normal analysis of AML at diagnosis and/or in post-therapy MRD monitoring.

In addition to older age at diagnosis and lower peripheral blood WBC and blast counts, patients in Q4 had higher rates of achieving CR, indicating that blast counts may decrease more rapidly in patients expressing high levels of CD74. However, there was no difference in MRD rates, meaning that a portion of patients in Q1-3 were MRD-negative but not in CR. Given published reports that these patients have a prognosis similar to MRD-negative patients in CR,[52,53] the clinical significance of this finding is unknown, but the association of CD74 high expression with blast clearance may be notable and worth investigation.

We found that CD74 high expressing patients were enriched for favorable prognostic genetic markers and had improved OS and EFS compared to all other patients. This was true using the AAML1031 risk stratification protocol as well as the updated AAML1831 definitions. While CD74 high expression is associated with good-prognostic genetic features and patients generally had improved outcomes compared to the remaining patients in the study, 5-year EFS remained just over 50%, meaning nearly half of patients still experienced adverse outcomes. Furthermore, patients with high levels of CD74 expression who were allocated to high-risk did no better than other high-risk patients. The significant number of patients with high levels of CD74 expression who experience poor outcomes suggests further studies regarding targeted therapy should be considered, particularly in a group such as CD74 high-expressors allocated to high-risk status or who relapse.

Previous work has demonstrated the potential of CD74 as a therapeutic target in hematologic malignancies, including ongoing phase I trials evaluating STRO-001 in mature B-cell malignancies.[50] Anti-CD74 treatment with STRO-001 has displayed target dependent *in vitro* cytotoxicity against CD74-expressing AML cell lines and primary samples as well



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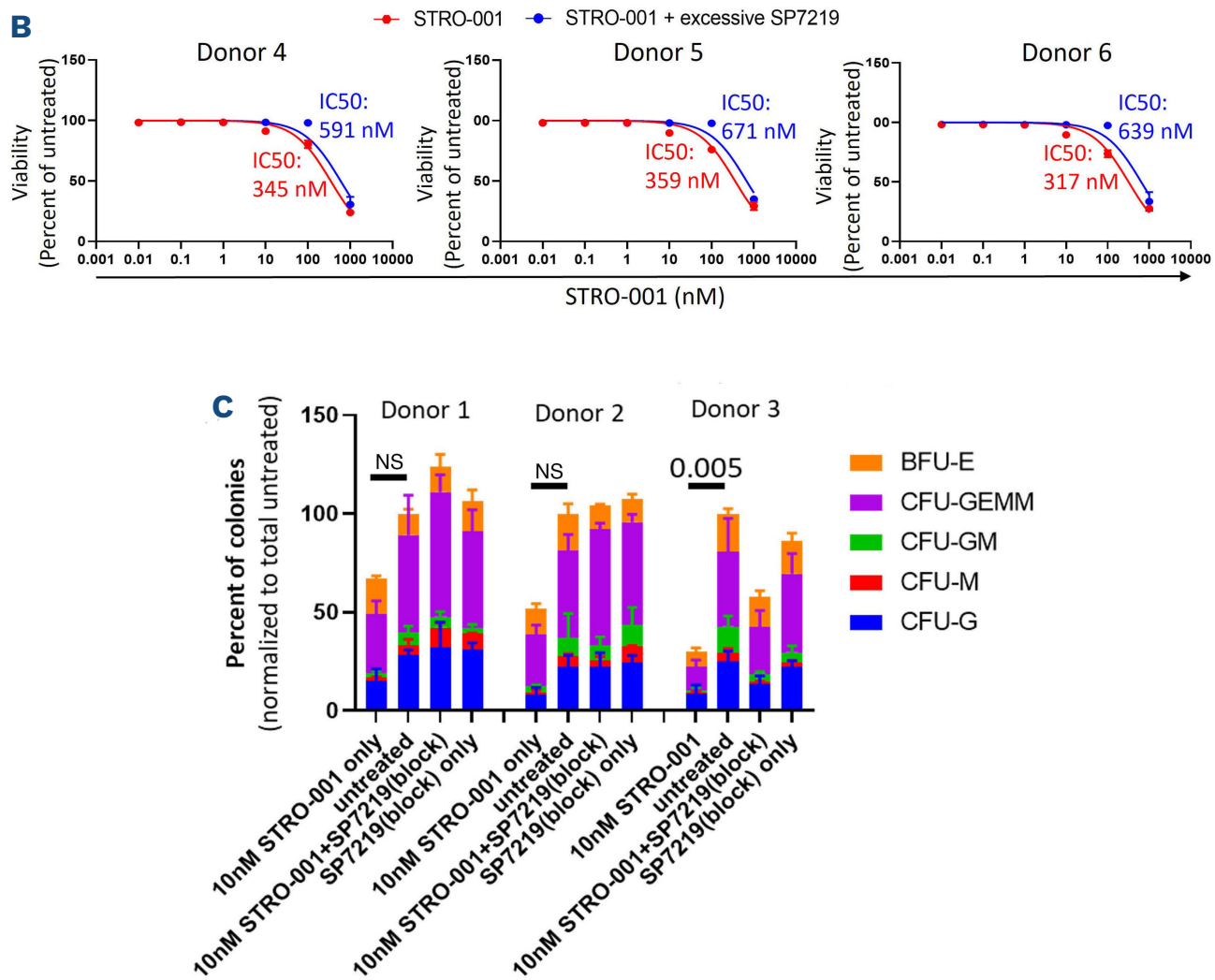

Figure 5. Treatment of acute myeloid leukemia and normal myeloid progenitor cells with anti-CD74 antibody drug conjugate STRO-001. (A) Cytotoxicity data from acute myeloid leukemia (AML)-expressing CD74 (AML1 and 2) or not expressing CD74 (AML3) treated with anti-CD74 ADC STRO-001 with (blue) or without (red) excessive CD74 blocking antibody (SP7219). Representative plots from 3 total experiments each. (B) Cytotoxicity data from CD34$^+$ cells isolated from cord blood from healthy births treated with STRO-001 with (blue) or without (red) excessive CD74 blocking antibody (SP7219). (C) Colony-forming unit assays of CD34$^+$ cells isolated from cord blood of healthy births either treated with anti-CD74 (STRO-001), untreated control, treated with blocking antibody and anti-CD74, or treated with blocking antibody only. Data is normalized to the total number of colonies in the untreated group. BFU-E: burst-forming unit erythroid; CFU-GEMM: colony-forming unit granulocyte, erythroid, macrophage, megakaryocyte progenitor cells; CFU-GM: colony-forming unit granulocyte macrophage; CFU-M: colony-forming unit macrophage; CFU-G: colony-forming unit macrophage.

as *in vivo* growth inhibition of CD74$^+$ cells (both cell lines and PDX model) in NSG mice.[51] In addition, we have confirmed the toxicity of STRO-001 to AML cells, but only when CD74 is expressed. Additionally, our *in vitro* experiments demonstrate that STRO-001 treatment has little impact on normal cells, with only granulocyte colonies consistently impacted in CFU assays and no significant difference from controls in cytotoxicity assays.

The combined preclinical data, previously published as well as presented here, indicate that anti-CD74 therapy is effectively cytotoxic, but only towards cells expressing the highest levels of CD74, potentially including high CD74-expressing pediatric AML. Additional studies are required, including assessing toxicity in AML patients (preliminary data from phase I dose escalation trials in mature B-cell malignancies has shown STRO-001 to be well tolerated)[50] and determining what level of expression is required for efficacy. Our data indicate that in at least some patients, it may be possible to administer treatment at a dose toxic to AML cells with limited impact on normal hematopoiesis, suggesting continued investigation of this potential targeted treatment is warranted. Furthermore, the idea that molecules expressed at low levels on normal cells can be targets for treatment, provided expression on AML cells is significantly higher, could open the door for other treatment targets previously not pursued due to expression on normal cells.

Most CD74 Q4 patients designated high-risk were allocated based on positive MRD post-induction cycle 1, as assessed by difference from normal flow cytometry. While major strides have been made in the identification of prognostically relevant genetic markers, both cytogenetic and molecular, there remains a significant fraction of patients for whom there is no prognostically informative genetic information. The prognostic relevance of MRD assessment by difference from normal flow cytometry at the end of first induction, combined with the ability to rapidly assess treatment response, highlights flow cytometry's continued clinical importance in AML, particularly in patients lacking prognostically relevant genetic markers at diagnosis.

In addition to the prognostic and monitoring roles of flow

cytometry, this study also highlights the importance of assessing cell surface expression of potential biomarkers when determining possible treatment targets. While RNA expression data is a useful tool for initial target identification,[51] targeted treatments largely rely on binding to cell surface molecules to be efficacious. As a result, a lack of sufficient cell surface expression often precludes a molecule from being an effective target, even if it is expressed at the RNA level. Together with previous publications, this demonstrates a potential pathway for the identification of therapeutic targets: RNA sequencing, followed by assessment of cell surface expression, and finally preclinical testing. CD74 serves as an example of this pathway, as after its initial identification based on RNA sequencing, our studies presented here show it to be expressed to a greater degree on AML cells than normal cells in a subset of patients and demonstrate preclinical efficacy.

Identifying patients with sufficient levels of expression is vital for effective use of targeted treatments, as has been shown previously for anti-CD33 therapy.[5,57,58] While dividing patients by quartiles is useful for associating immunophenotypic features with clinical variables, it may not represent the ideal cutoff for treatment eligibility. Further research should be carried out to determine the appropriate threshold for clinical use based on the difference in toxicity between high expressing CD74 AML cells and normal progenitor cells. In this study, 38% of patients expressed CD74 on at least a portion of the leukemic blasts, however dim or partial expression may not be sufficient to render a leukemia susceptible to targeted treatment, particularly in the case of a marker such as CD74, which is expressed on normal cells. Furthermore, a threshold such as the top 25% of patients ignores the biological reality that the range of antigen expression, both positive/negative as well as the degree of positivity, varies significantly based on the antigen of interest. For this reason, further investigation should be carried out to determine a robust data-driven definition for classifying antigen expression based on flow cytometry. Additionally, work should be done to determine the optimal level of antigen expression required for effective targeted treatment, for both CD74 and other current or future targets.

In summary, our data show that CD74 is expressed at some level on the cell surface of 38% of pediatric AML cases, and in a subset is expressed at levels much higher than normal hematopoietic cells in both the AML patients themselves as well as healthy controls. The highest quartile of CD74 expression is associated with low-risk genetic factors and improved EFS, but a meaningful number of high-risk patients do show significant levels of expression and nearly half of patients experience an adverse event within 5 years. Finally, our preclinical and clinical data demonstrates that CD74 may be a promising target for therapy and further research should be carried out to determine how and to whom it can be best implemented to help the patients who remain at high risk.

Disclosures

AJM, CAH, LP, Hsu FC, LLL, FD, CF, KG, JAC, DAW, MRL and LEB were all employees of Hematologics Inc. at the time the work was done. DAW and MRL have equity ownership in Hematologics Inc.

Contributions

AJM, CAH, LP, FCH, LL, CF, KG, TH and LEB performed research. AJM, TA, RG, AL, JAC, FD and LEB performed data analysis. KT, KRL, DAW and MRL provided scientific input. TA, RG, EAK, TC, JP, RA, SM and LEB oversaw the study. AJM and LEB wrote the manuscript.

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Data-sharing statement

Data were generated by authors and available on request from the corresponding author.

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