ARTICLE

CD38 knockout natural killer cells expressing an affinity optimized CD38 chimeric antigen receptor successfully target acute myeloid leukemia with reduced effector cell fratricide

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Ferrata Storti Foundation

Haematologica 2022
Volume 107(2):437-445

ABSTRACT

There is a strong biological rationale for the augmentation of allogeneic natural killer (NK) cell therapies with a chimeric antigen receptor (CAR) to enhance acute myeloid leukemia (AML) targeting. CD38 is an established immunotherapeutic target in multiple myeloma and under investigation as a target antigen in AML. CD38 expression on NK cells and its further induction during *ex vivo* NK cell expansion represent barriers to the development of a CD38 CAR-NK cell therapy. We set out to develop a CD38 CAR-NK cell therapy for AML, first by using an NK cell line which has low baseline CD38 expression and subsequently NK cells expanded from healthy donors. To overcome anticipated fratricide due to NK cell CD38 expression when using primary expanded NK cells, we applied CRISPR/Cas9 genome editing to disrupt the *CD38* gene during expansion, achieving a mean knockdown efficiency of 84%. The resulting CD38 knockdown expanded NK cells, after expression of an affinity optimized CD38 CAR, showed reduced NK-cell fratricide and an enhanced ability to target primary AML blasts. Furthermore, the cytotoxic potential of CD38 CAR-NK cells was augmented by pretreatment of the AML cells with all-*trans* retinoic acid which drove enhanced CD38 expression, offering a rational combination therapy. These findings support the further investigation of CD38 knockdown - CD38 CAR-NK cells as a viable immunotherapeutic approach to the treatment of AML.

Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, accounting for approximately 2% of all cancer deaths.[1] Curative treatment approaches remain chemotherapy-based, with allogeneic stem cell transplant consolidation for selected patients. The introduction of molecularly targeted therapies has provided important incremental improvements for specific AML subtypes.[2-4] Relapsed disease, mediated by the persistence of chemotherapy-resistant leukemic stem cells (LSC) is particularly difficult to treat, and accounts for much of the mortality burden associated with AML. For many older patients, treatment options that are both tolerable and efficacious do not yet exist. Anti-CD19 chimeric antigen receptor (CAR) T-cell therapies have provided a ground-breaking approach to cancer immunotherapy in B-cell acute lymphoblastic leukemia and B-cell non-Hodgkin lymphomas.[5,6] While there is considerable interest in applying the principle of CAR technology in other diseases, progress in AML has been limited to date by the absence of an ideal antigenic target, concerns about 'on-target off-tumor' toxicity including that to normal hematopoietic stem cells, and blast cell heterogeneity which exists both within and between patients.[7-9].

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Received: September 11, 2020.

Accepted: December 22, 2020.

Pre-published: December 30, 2020.

https://doi.org/10.3324/haematol.2020.271908

The multifunctional cell surface glycoprotein CD38, a breakthrough immunotherapeutic target in multiple myeloma, is also considered a potential target antigen in AML. In contrast to the uniformly high CD38 expression on malignant plasma cells, blast cell CD38 expression is heterogeneous although frequently exceeds that of normal cell populations.[10] The CD38 monoclonal antibody daratumumab has been investigated in AML and has shown promising pre-clinical activity.[10] CD38 CAR-T cells have been evaluated mainly for their activity in multiple myeloma and cytotoxicity against primary AML samples has also been confirmed.[11] However, there remains concern about a potent myelosuppressive effect with a constituently expressed high-affinity anti-CD38 CAR due to CD38 expression on both mature myeloid cells and their precursors.[11,12] To circumvent this problem, an affinity-optimized CD38 CAR has been developed to minimize the targeting of positive, but low-expressing normal cell populations.[13]

There is a strong biological rationale for natural killer (NK) cell-based approaches to adoptive cell transfer immunotherapy for AML. NK cells confer a component of the graft-*versus*-leukemia effect of allogeneic stem cell transplant and infusions of purified alloreactive NK cells have proven therapeutic potential.[14-16] CAR-NK cell therapies are emerging as a complementary approach to CAR-T cells, with potential advantages including allogeneic cell sources and innate antigen independent anti-leukemic activity. An early clinical report of a cord-blood derived CD19 CAR-NK cell therapy has shown promising safety and efficacy in B-cell malignancies.[17] We set out to develop and evaluate an affinity optimized CD38 CAR-NK cell therapy for AML. We first used the NK cell line KHYG-1, which has naturally low levels of CD38 expression. While allogeneic expanded NK (eNK) cell approaches are more suited to clinical translation, *ex vivo* NK cell expansion has been shown to lead to upregulation of CD38, which we also encountered using a feeder-free, interleukin-2-based expansion protocol.[18] To reduce the anticipated NK cell fratricide that would occur using eNK cells, we applied CRISPR/Cas9 to disrupt the *CD38* gene during NK cell expansion, creating fratricide-resistant NK cells prior to CD38 CAR expression. Both KHYG-1 and CD38 knockdown (KD) eNK approaches lead to efficient targeting of AML blasts upon CD38 CAR expression, with the degree of cytotoxicity correlating with CD38 expression. Finally, we confirm a rational combination approach utilizing all-*trans* retinoic acid (ATRA) to enhance CD38 expression on the AML cells. Collectively, our data support the potential of CD38 as a therapeutic target in AML and help to define a CD38 CAR-NK cell approach suited to clinical development.

Methods

Ethical statement

Healthy donor blood and AML patients' bone marrow samples were collected with written informed consent and approval from the institutional review boards at each institution (ref: CA2219). Cryopreserved samples were obtained from the biobank of Blood Cancer Network Ireland.

Cells and reagents

The cell lines THP-1, KG1a, U937 and KHYG-1 were obtained from the American Type Culture Collection and their identities confirmed by short tandem repeat profiling (Eurofins Genomics™). CD38 CAR and mock KHYG-1 cells were generated by retroviral transduction with genomic integration confirmed by the inclusion of DsRed fluorescent protein. The development of the second-generation CD28-CD3ζ, optimized-affinity CD38 CAR was reported previously.[13] Primary NK cells were isolated from healthy donor peripheral blood mononuclear cells after Ficoll-Paque density gradient centrifugation and negative immunomagnetic selection (NK Isolation Kit, Miltenyi Biotec™). NK cells were expanded in NK MACS medium (Miltenyi Biotec™) containing NK MACS supplement, 5% heat-inactivated human AB serum and 100 U/mL. interleukin-2 (PeproTech™). Cultures were pre-treated for 48 h with ATRA (Sigma-Aldrich™) or dimethyl sulfoxide, in the relevant experiments.

CRISPR/Cas9 gene editing

Five days after isolation, $5x10^5$ NK cells were electroporated with sgRNA-Cas9 complexes targeting multiple sites within the *CD38* gene (Gene Knockout Kit V2, Synthego™) or control electroporated (MaxCyte™ GT flow transfection system). *CD38*-edited and control electroporated cells were expanded at a target density of $1x10^6$ cells/mL. On day 13-15 of expansion, CD38 expression was assessed by flow cytometry. Knockdown efficiency was calculated as (% CD38-positive cells [mock electroporated] - % CD38-positive cells [CRISPR/Cas9 edited]).

CD38 chimeric antigen receptor mRNA electroporation

CD38 CAR mRNA was synthesized (Trilink Biotechnologies™) and CD38 CAR expression in primary CD38 KD and control eNK cells was achieved by electroporation (100 μg/mL mRNA, Maxcyte™ GT Flow Transfection System). CAR expression was confirmed by flow cytometry using anti-IgG H+L specific goat anti-human antibody (Jackson-Immuno research™) and biotinylated protein L stain (ACRO Biosystems™).

Cytotoxicity assays

Co-culture experiments involved 10,000 target cells (cell lines), or 20,000-50,000 bone marrow mononuclear cells from AML patients' samples. NK cell numbers were determined by the desired effector to target (E:T) cell ratio. After co-culture for 18-24 h, target cell lines or bone marrow mononuclear cells were identified by flow cytometry, using a cell-tracking dye: Tag-IT BV™ proliferation and cell tracking dye (Biolegend™) or VioletTrace™ (Thermo Fisher). Primary blast cell populations were identified as $CD45^{int}/SSC^{low}$ (CD45 APC), supported by additional markers chosen based on clinical immunophenotyping data. Cell death was determined using propidium iodide (PI) or LIVE/DEAD Fixable Near-IR (Life Technologies L10119) staining and reported as '% specific (blast) cytotoxicity' ([sample cytotoxicity – background cytotoxicity]/[100 – background cytotoxicity] x 100%) or '% blast cell cytotoxicity' as indicated.

Statistical analysis

GraphPad Prism 8 software (San Diego, CA, USA) was used for statistical analysis. Comparisons were conducted using multiple two-sided *t*-tests for cytotoxicity assays at each E:T ratio or one-way analysis of variance for cell expression data with statistical significance indicated by asterisks (*P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001). Flow cytometry data were acquired on a BD FACS Canto II and analyzed using Flow Jo V10 software and Infinicyt (Cytognos™).

Results

CD38 chimeric antigen receptor expression enhances KHYG-1 acute myeloid leukemia targeting

To assess the feasibility of targeting CD38 with a CAR-NK cell approach in AML, we first defined the CD38 expression profile of AML cell lines. We classified THP-1 and U937 as CD38-positive, and KG1a as CD38-negative for further experiments (Figure 1A). We also confirmed a low level of CD38 expression on the KHYG-1 cell line, previously shown to be a NK cell line with significant cytotoxic potential, expressing a high concentration of active perforin and signaling kinases.[19] The low CD38 expression of KHYG-1 is in contrast to that of NK-92 cells, another NK cell line which has been investigated clinically as an adoptive cell therapy, but is strongly CD38-positive.[18]

CD38 CAR-KHYG-1 cells were generated by retroviral transduction using an 'affinity-optimized', second-generation anti-CD38 CAR (CD3ζ-CD28).[13] CD38 CAR-KHYG-1 cells displayed similar characteristics and kinetics in cell culture as those of mock-transduced KHYG-1

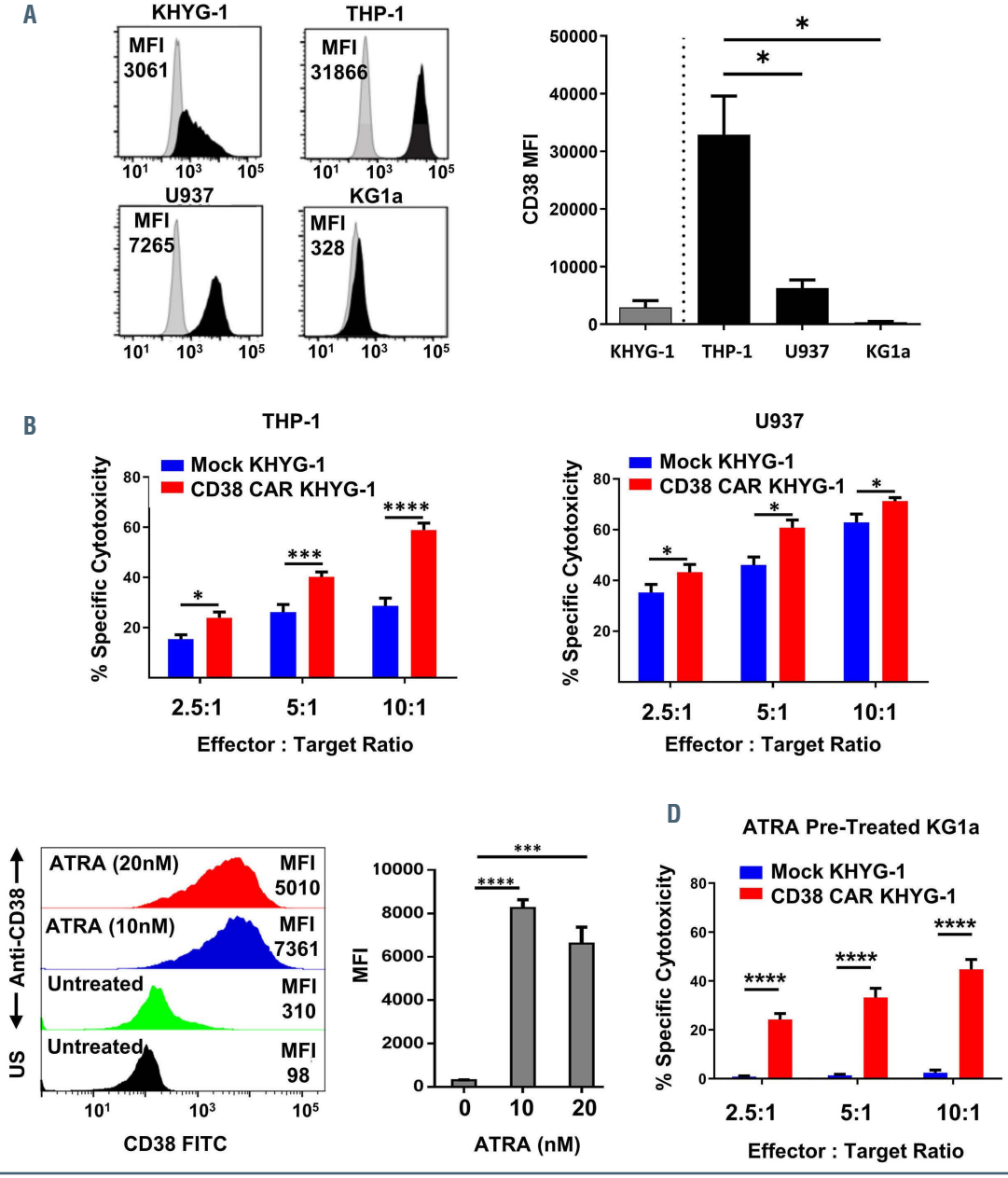

Figure 1. Affinity-optimized CD38 CAR-KHYG-1 cell cytotoxicity against acute myeloid leukemia cell lines. (A) Histograms depict CD38 expression of the KHYG-1 NK cell line and acute myeloid leukemia (AML) cell lines THP-1 (CD38-positive), U937 (CD38-positive) and KG1a (CD38-negative) with mean fluorescence intensity (MFI) indicated. Bars represent relative CD38 expression of KHYG-1 and AML cell lines (n=3 individual repetitions). Mean values for AML cell lines compared by an unpaired *t*-test. (B) Bar chart depicting the specific cytotoxicity of mock-transduced and CD38 CAR-KHYG-1 cells at varying effector to target (E:T) ratios against the CD38-positive cell lines THP-1 and U937. Comparisons of four independent experiments made by an unpaired *t*-test at each effector to target (E:T) ratio. (C) Representative histograms depicting CD38 upregulation and MFI values in KG1a cells after 48 h of treatment with all-*trans* retinoic acid (ATRA) at 10 nM and 20 nM concentrations, compared to dimethylsulfoxide (DMSO) control treated KG1a cells. The bar chart summarize data from four independent experiments with comparisons by one-way analysis of variance. (D) Bar chart depicting specific cytotoxicity of mock-transduced and CD38 CAR-KHYG-1 cells in co-culture with 48 h, 10 nM ATRA-pretreated KG1a cells at varying E:T ratios (summary of 4 experiments). Error bars indicate standard error of mean (SEM). Statistical significance is defined as *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

cells. To assess whether CD38 CAR targeting has an additional cytotoxic effect, we tested CD38 CAR-KHYG-1 cells and mock-transduced KHYG-1 cells against CD38-positive AML cell lines (Figure 1B). CD38 CAR-KHYG-1 cells demonstrated greater cytotoxic effects against CD38-positive cell lines relative to mock-transduced KHYG-1 cells at all E:T ratios tested, with relatively greater increases seen against the strongly CD38-positive THP-1 cells (specific cytotoxicity 58% *vs*. 28% for THP-1, 10:1 E:T ratio; $P<0.0001$).

ATRA has been shown to upregulate CD38 expression across all AML subtypes, mediated by a retinoic acid response element in the first intron of the *CD38* gene.[20] Pretreatment with ATRA at 10 nM for 48 h led to marked induction of CD38 expression on KG1a cells, which do not express detectable levels of CD38 in resting conditions (Figure 1C). CD38 CAR-KHYG-1 cells were cytotoxic to ATRA-pretreated KG1a cells, while mock-transduced KHYG-1 cells showed little cytotoxicity despite ATRA pretreatment (Figure 1D).

To better mimic the CD38 expression profile encountered in AML, we tested the efficacy of CD38 CAR-KHYG-1 cells against primary bone marrow mononuclear cells from AML patients (Figure 2A, B). CD38 CAR-KHYG-1 cells displayed greater specific cytotoxicity against AML blasts relative to mock-transduced KHYG-1 cells across a range of blast cell CD38 expression, with the degree of specific cytotoxicity correlating with blast cell CD38 expression (Figure 2C).

CRISPR/Cas9 gene editing of CD38 in primary NK cells reduces NK cell fratricide upon CD38 chimeric antigen receptor expression

While alloreactive NK cell approaches have shown some success in treating AML, we hypothesized that increased expression of CD38 during *ex vivo* NK cell expansion could be sufficient to trigger effector cell fratricide after expression of a CD38 CAR, despite affinity optimization. Indeed, we observed a consistent, mean 4-fold increase in CD38 expression during feeder-free expansion of NK cells in interlueukin-2-containing media. Increases in CD38 from baseline (mean fluorescence intensity [MFI] 11,903) were detectable by day 5 (MFI 40,948) and persisted to at least day 13 (MFI 38,600) (Figure 3A). Extrapolating from our previous work on THP-1 cells (MFI 31,866), we concluded that this degree of CD38 expression would lead to a fratricidal effect upon CD38 CAR expression thus limiting the cytotoxic capacity of *ex vivo*-expanded CD38 CAR-NK cells.

We, therefore, set out to use CRISPR/Cas9 gene editing technology to disrupt the *CD38* gene in primary NK cells. We used a multi-sgRNA format, introducing sgRNA-Cas9 complexes using a high-efficiency, electroporation-based approach on a platform scalable to Good Manufacturing Practice (GMP) grade development. CD38 KD and mock-electroporated cells were further expanded for use in functional assays. A consistent KD effect was achieved across all NK cell donors (mean 84%; range, 75-92%) (Figure 3B). CD38 KD was detectable 48 h after CRISPR/Cas9 gene editing, peaked by day 3-7 after electroporation and was stable across the duration of expansion suggesting minimal differences in the growth potential of CD38 KD and mock-electroporated NK cells in this expansion system (*Online Supplementary Figure S1*).

To confirm that CD38 KD eNK cells showed greater resistance to fratricide than wild-type eNK cells, we introduced mRNA coding for an affinity-optimized CD38 CAR. CAR expression was confirmed by complementary staining techniques – an anti-human IgG with light chain specificity, and biotinylated protein L, with control (background) and CAR staining depicted in Figure 3C. CD38 KD eNK cells displayed significantly less cell death than wild-type eNK cells, measured 18 h after CD38 CAR mRNA electroporation in the absence of target cells (18% *vs*. 37%, $P=0.002$) (Figure 3D), confirming a greater resistance to fratricide. Furthermore, the biphasic CD38 expression pattern (representing the small residual CD38-positive NK cell population after CRISPR/Cas9 gene editing) was lost in the CD38 KD population after CD38 CAR mRNA transfection, but not after non-specific (CD16) mRNA electroporation (Figure 3E). This emphasized the tendency of the CD38 CAR-NK cells to target CD38-positive eNK cells despite affinity-optimization of the CD38 CAR binding domain.

CD38 knockdown - CD38 chimeric antigen receptor-NK cells efficiently target primary acute myeloid leukemia blasts

To confirm that CD38 CAR expression in CD38 KD eNK cells enhances the activity of alloreactive NK cells against AML, CD38 KD eNK cells were electroporated with CD38 CAR mRNA or mock-electroporated prior to co-culture with bone marrow mononuclear cells from AML patients with a variety of molecular AML subtypes (*Online Supplementary Table S1*). CD38 KD - CD38 CAR-NK cells showed enhanced cytotoxicity relative to mock-electroporated CD38 KD cells, with the effect being most prominent at the highest E:T ratios tested (Figure 4A, B). Enhanced cytotoxicity was observed for all AML patients and cytotoxicity at the 5:1 E:T ratio correlated with blast cell CD38 expression ($R^2=0.81$) (Figure 4C).

We investigated the potential of ATRA pretreatment as a means of modulating CD38 expression and potentiating the effects of CD38 CAR targeting using CD38 KD - CD38 CAR-NK cells. ATRA pretreatment induced a mean 5-fold upregulation of surface CD38 expression in blast cells (Figure 4D). The increased CD38 expression was associated with greater sensitivity to CD38 KD - CD38 CAR-NK cells compared to dimethylsulfoxide-treated bone marrow mononuclear cell samples tested at the 2:1 and 5:1 E:T ratios (Figure 4E).

Discussion

We set out to augment the potential of NK cell adoptive transfer strategies in AML through expression of an affinity-optimized CD38 CAR. We demonstrated two potential approaches to CD38 CAR-NK cell therapy in this setting. We confirmed that CD38 KD eNK cells show reduced fratricide after CD38 CAR expression, allowing effective targeting of primary AML blasts. As an alternative approach we modified the NK cell line KHYG-1 to express a CD38 CAR, successfully targeting AML cell lines and primary samples. Both approaches could be enhanced by induction of CD38 expression using ATRA. We chose a NK cell line with naturally low CD38 expression to ensure viability after introducing a CD38 CAR. KHYG-1 cells have previously been shown to maintain

cytotoxicity after irradiation and could be applied clinically in a similar manner to the NK-92 cell line.[21] However, irradiation limits the potential for *in vivo* expansion and persistence - important variables in determining the clinical efficacy of cellular therapies. This requirement for irradiation may be avoided by using donor-derived, eNK cells, although this approach is further complicated by robust CD38 upregulation encountered during *ex vivo* expansion. Our CRISPR/Cas9 CD38 KD eNK cells reduce effector cell fratricide, representing an approach that could be explored clinically.

CD38 was a breakthrough immunotherapeutic target in multiple myeloma. While there is greater variability in CD38 expression in AML, CD38 is a potential target antigen in this disease. Daratumumab was shown to be active in an *in vivo* model of AML, while isatuximab has recently been examined in a large-scale, *in vitro* study.[10,22] The expression pattern of CD38 in AML, in which there is often overlap with normal cell populations including myeloid and monocytic populations, raises concerns about considerable 'on-target, off-tumor' toxicity when a potent effector cell is directed toward CD38. High-affinity CD38 CAR strategies may maximize the proportion of patients for whom a CD38-directed therapy is likely to have activity, at the expense of considerable myelosuppressive effects. It is important to consider that not all off-tumor effects are undesirable: in the case of CD38, elimination of CD38-positive immunoregulatory cell subsets may lead to a beneficial therapeutic effect.[23,24] The efficacy of lower-affinity CD38 CAR strategies is likely to be limited to cases with strong expression or pharmacological upregulation of CD38. Herein we investigated an approach to CD38 CAR targeting in AML which aims to strike the balance of efficacy, applicability, and off-tumor

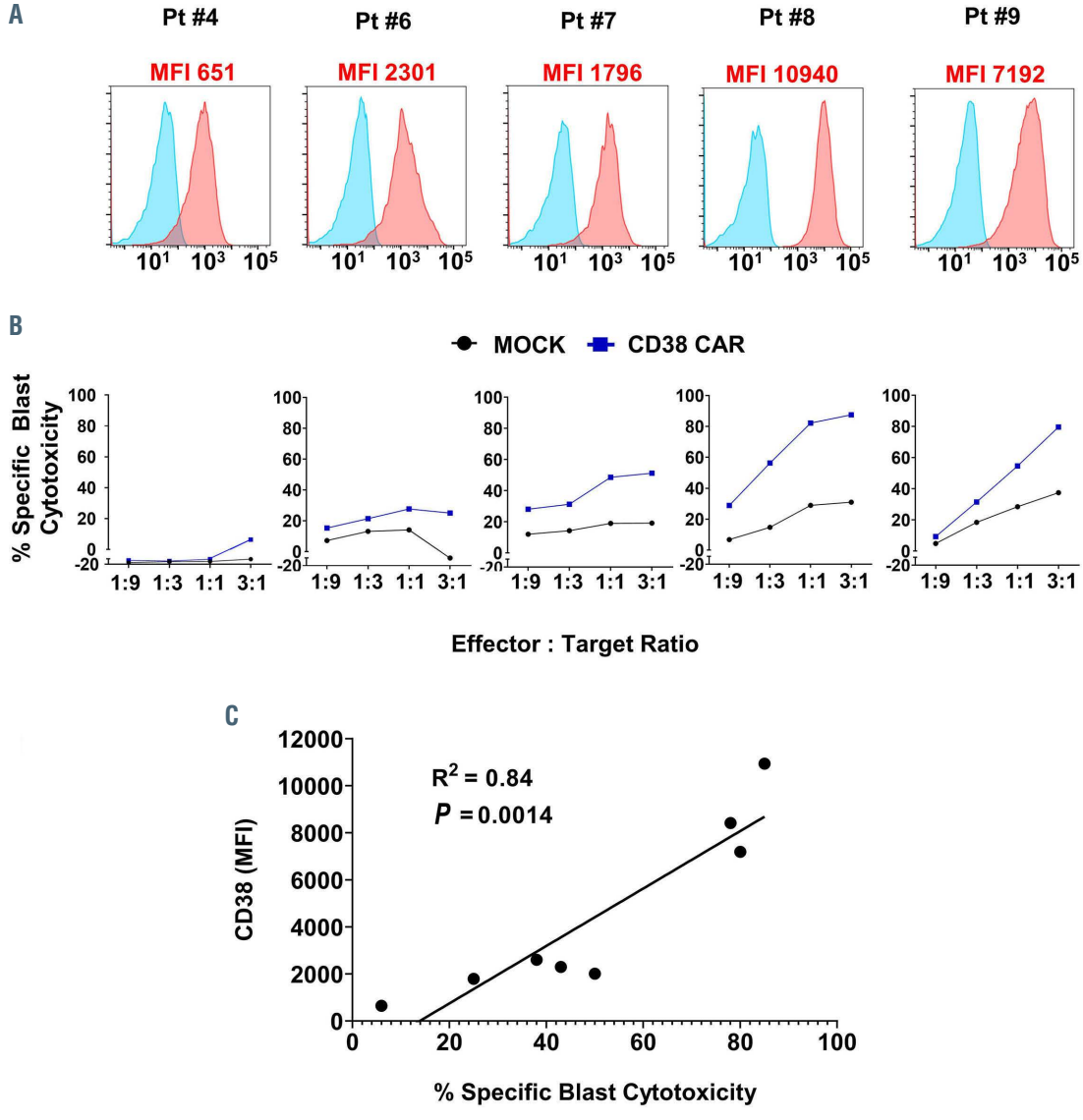

Figure 2. CD38 CAR-KHYG-1 activity against primary acute myeloid leukemia samples. (A) Histograms depict unstained controls (blue) and anti-CD38 stained blast cells (red), from a range of acute myeloid leukemia (AML) patients chosen to represent a spectrum of CD38 expression. Relative mean fluorescence intensity (MFI) figures for stained samples are reported. (B) Graphs represent specific blast cytotoxicity after co-culture assays with CD38 CAR transduced KHYG-1 (blue) and mock-transduced KHYG-1 (black) at specified effector to target (E:T) ratios for each corresponding patient's sample in Figure 2A. (C) The correlation plot and linear regression line depicts specific blast cell cytotoxicity at the E:T ratio of 3:1, *versus* CD38 expression (relative MFI) of primary AML samples from all co-culture experiments carried out in 2A, (n=8 experiments).

effects. The established anti-leukemic activity of alloreactive NK cells in AML provides a rationale for the development of CAR-NK cell approaches.[14,15] Alloreactive NK cells can be expected to retain their innate anti-leukemic activity, with enhancement against CD38-positive cells conferred by an anti-CD38 CAR. Toxicity against normal cell populations can be minimized through the use of an optimized-affinity CD38 CAR variant.[11]

While many target antigens are being considered in AML, CD38 is also unique in the availability of a licensed and well-tolerated oral agent capable of modulating target antigen expression, ATRA.[20] Furthermore, it has been shown that malignant blast cells are particularly sensitive to the CD38-inducing effect of ATRA, acting directly

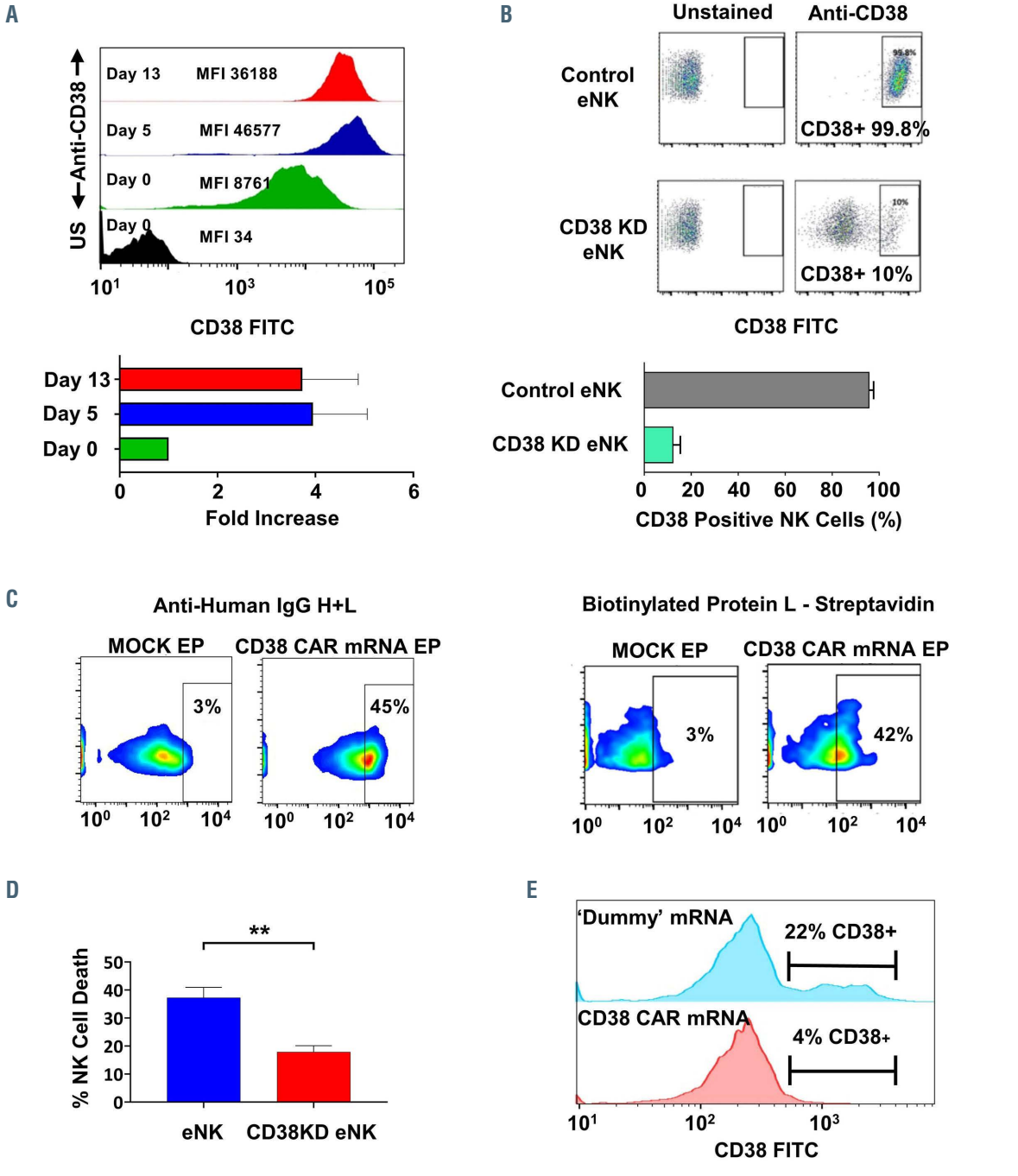

Figure 3. CRISPR/Cas9 gene editing of *CD38* in primary expanded natural killer cells to reduce natural killer cell fratricide upon CD38 CAR expression. (A) CD38 expression in freshly isolated (day 0), and expanded natural killer (eNK) cells at day 5 and day 13, as measured by flow cytometry and presented as a representative histogram and summary bar chart of three unique expansions. (B) Residual CD38 expression measured on day 8-10 after CRISPR/Cas9 gene editing of *CD38*. Dot plots from representative donor for mock-electroporated and CD38 knockdown (KD) conditions. Bar chart represents summary data for four donors. (C) Confirmatory CAR staining performed 18 h after CD38 CAR mRNA electroporation. Pseudo-colored plots depict results from one representative experiment. (D) NK cell death after 18 h of culture after CD38 CAR mRNA electroporation comparing CD38 KD and control eNK cells across three individual experiments and NK cell donors. Comparison of mean cell death by an unpaired *t*-test. (E) Histogram depicting a representative residual CD38 expression profile of viable CD38 KD eNK cells demonstrating loss of residual CD38-positive eNK cell population after CD38 CAR electroporation but not after 'dummy' mRNA (CD16) electroporation. EP: electroporated; H + L: heavy and light chain specific. Error bars indicate standard error of mean (SEM). Statistical significance is defined as $*P \leq 0.05$, $**P \leq 0.01$.

through a retinoic acid response element within the *CD38* gene.[25] The vitamin D receptor agonist inecalcitol represents another investigational approach to CD38 modulation which could enhance the efficacy of CD38-directed therapies in AML through a similar principle.[26] The timing of a clinically applied combination therapy using ATRA and a CD38 CAR-NK cell would require careful planning. Evidence suggests that NK cell exposure to ATRA may have a net inhibitory effect on NK cell function, suggesting that the preferred approach may be ATRA prior to adoptive cell transfer.[27]

CD38 targeted therapies are complicated by NK cell CD38 expression, observed clinically with the NK cell-depleting effects of daratumumab seen during the treat-

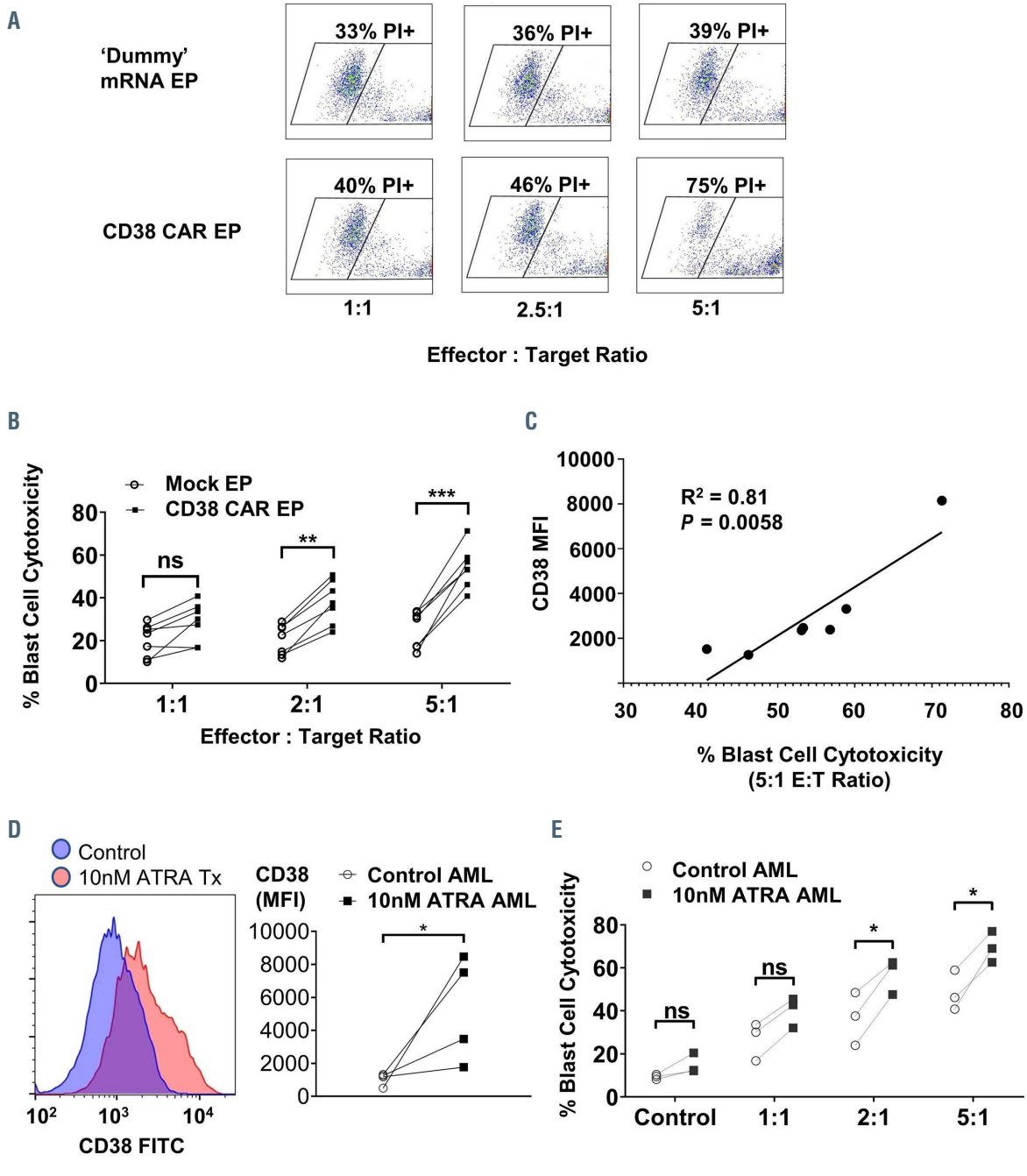

Figure 4. CD38 KD - CD38 CAR-NK cells show enhanced cytotoxicity against primary acute myeloid leukemia samples. (A) CD38 knockdown (KD) expanded natural killer (NK) cells were electroporated with CD38 CAR mRNA or 'dummy' mRNA (CD16) prior to co-culture with bone marrow mononuclear cells from acute myeloid leukemia (AML) patients. Blast cell cytotoxicity was measured by percentage of propidium iodide (PI)-positive cells (representative dot-plots are shown). (B) Summary data of co-culture assays as described in (A), for seven AML patients compared using unpaired t-tests for each effector to target (E:T) cell ratio. (C) The CD38 expression level of the blast population was correlated with the cytotoxic effect observed at an E:T ratio of 5:1 for experiments conducted in (B), and a linear regression model fitted using GraphPad Prism. (D) Bone marrow mononuclear cells from n=4 donors were treated with 10 nM all-*trans* retinoic acid (ATRA) or dimethyl sulfoxide (DMSO) for 48 h prior to anti-CD38 staining. A representative histogram is displayed and summary data of four pooled donors were compared using an unpaired t-test. (E) ATRA or DMSO pretreated cells were co-cultured with CD38 KD-CD38 CAR-NK cells. Summary data from three experiments. Analysis by unpaired *t*-test for each E:T ratio. Statistical significance is defined as $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

ment of multiple myeloma.[28] Indeed, overcoming the fratricidal effect of daratumumab through combination with *ex vivo* eNK cells is actively under investigation in multiple myeloma. In keeping with prior reports, we observed CD38 upregulation during NK cell expansion, which was sufficient to lead to a fratricidal effect despite the use of an optimized-affinity CD38 CAR design.[18] While it has been considered difficult to apply genetic engineering approaches to primary NK cells, we achieved a consistent, and high-efficiency disruption of the *CD38* gene using a multi-sgRNA approach coupled with a flow transfection system. Our findings are comparable to recent descriptions of CRISPR/Cas9 editing in primary NK cells but using a different sgRNA design and expansion approach.[27,29,30] The resulting CD38 KD eNK cells continued to expand and displayed reduced fratricide after CD38 CAR expression. With the availability of CRISPR/Cas9 and the relative ease of application to primary NK cells using clinically adaptable platforms now demonstrated by multiple groups, there are vast possibilities for this technology across NK cell therapeutics.

One potential limitation to CD38 targeting in AML is the limited capacity to target LSC populations, questioning the 'curative' potential of the therapies. LSC in AML are well-established, and while our understanding has evolved to include the existence of some CD38-positive LSC populations, it is likely that many LSC do reside within the traditional CD34-positive, CD38-negative compartment.[31] Considering this feature of AML LSC, a CAR-NK cell targeting CD38 could be expected to have greater LSC targeting potential than a CAR-T cell, because of the presence of the innate activating pathways of NK cells above and beyond the CD38-specific CAR. Indeed the potential for long-term disease control, and thus LSC targeting capabilities can be inferred from data establishing the importance of NK cell KIR-ligand mismatch in the efficacy of allogeneic stem cell transplantation.[16] Furthermore, a tandem CAR approach including a LSC-specific antigen and/or a variant of TRAIL (tumor necrosis factor related apoptosis inducing ligand) could be incorporated to augment LSC targeting.[32] Tailored approaches using CAR modified NK cells targeting combinations based on the specific identified LSC immunophenotype in each case may ultimately be required given the absence of an identified universal LSC marker. This approach is becoming feasible with current and emerging technologies.

Antibody- and protein-based approaches have been considered previously in attempts to overcome fratricide in a CD38-directed CAR-T cell platform.[33] CRISPR/Cas9-generated, CD38 KD eNK cells have recently and successfully been applied to reducing the NK cell fratricidal effects of daratumumab with a focus on multiple myeloma.[27] Interestingly, while a magnetic separation step was utilized to enhance the purity of the KD population in this innovative study, our data suggest that expression of a CD38 CAR combined with a highly efficient CRISPR/Cas9 KD will likely lead to a self-selecting KD population without additional processing. While not the focus of our experiments, Kararoudi *et al.*[27] also explored the cellular bioenergetic benefit of deletion of CD38 in eNK cells. CD38 converts nicotinamide adenine dinucleotide (NAD^+) to cyclic adenosine diphosphate-ribose through an enzymatic function. Additional NAD^+ availability due to loss of CD38 supplies an important co-factor favoring oxidative phosphorylation within NK cells. FT538, a NK cell product derived from induced pluripotent stem cells being developed by FATE Therapeutics, incorporates a CD38 deletion to overcome fratricide when combined with daratumumab. The group also demonstrated greater resistance to oxidative stress conferred by deletion of CD38, a characteristic likely to be favorable within the tumor microenvironment.[34] These enhancements to NK cell biology suggest a broad range of applications for CD38 KD eNK cells beyond CD38 targeting and fratricide concerns. Simple and consistent approaches to their generation will likely be of clinical utility.

In conclusion, we present two viable approaches to CD38 CAR-NK cell therapies applied to AML. Both our CD38 CAR-KHYG-1 cells and CD38 KD eNK cell platforms overcome effector cell fratricide relating to NK cell CD38 expression. Furthermore, we report an efficient approach to CRISPR/Cas9 genome editing adapted to primary eNK cells and suitable for GMP expansion.

Disclosures

MG has received educational funding from Janssen Pharmaceuticals and Takeda. AS and SS have received research funding from ONK Therapeutics Limited. LKM is an employee of ONK Therapeutics Limited. SK and RS are employees of Maxcyte Inc. SZ has received research funding from Takeda, Celgene, and Janssen and is a member of the board of directors or an advisory committee for Takeda, Celgene, and Janssen. NWCJvdD has received research funding from Janssen Pharmaceuticals, Amgen, Celgene, Novartis, and BMS and has participated in advisory boards for Janssen Pharmaceuticals, Amgen, Celgene, BMS, Takeda, Roche, Novartis, Bayer, and Servier. TM has received research funding from Gilead, Celgene, Novartis, ONK Therapeutics Limited, Genmab, Janssen and has been a member of an advisory board for Janssen. ES has collaborated in research projects with Janssen, Roche, Celgene, and Takeda. MOD has received research funding from ONK Therapeutics Limited, BMS, Celgene, and Glycomimetics; is a member of the board of directors or an advisory committee for Janssen, Abbvie, and ONK Therapeutics Limited; and owns equity in ONK Therapeutics Limited. EN has no potential conflicts of interest to disclose.

Contributions

SS and MOD conceived the research. EN, MG, AS and LKM performed functional assays. SK and RS contributed to electroporation optimization. ES contributed to acquisition of patients' samples and the primary AML assay design. TM, SZ and NVD developed CD38 CAR-KHYG1 cells and associated functional assays. MG, SS and AS wrote the manuscript and prepared the figures. All authors contributed to editing and reviewing the final manuscript prior to submission.

Acknowledgments

Reagents for electroporation were contributed by Maxcyte inc.

Funding

This research was supported by Irish Clinical Academic Training (ICAT) Programme fellowship funding to MG. ICAT is supported by the Wellcome Trust and the Health Research Board (grant number 203930/B/16/Z), the Health Service Executive National Doctors Training and Planning and the Health and Social Care, Research and Development Division, Northern Ireland. Work performed in collaboration with Blood Cancer Network Ireland was funded by Science Foundation Ireland and the Irish Cancer Society (Blood Cancer Network Ireland, 14/ICS/B3042).

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