Human Effectors of Acute and Chronic GVHD Overexpress CD83 and Predict Mortality

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ABSTRACT

Purpose: Acute and chronic GVHD remain major causes of transplant-related morbidity and mortality (TRM) after allogeneic hematopoietic cell transplantation (alloHCT). We have shown CD83 chimeric antigen receptor (CAR) T cells prevent GVHD and kill myeloid leukemia cell lines. In this pilot study, we investigate CD83 expression on GVHD effector cells, correlate these discoveries with clinical outcomes, and evaluate critical therapeutic implications for transplant recipients.

Experimental Design: CD83 expression was evaluated among circulating $CD4^+$ T cells, B-cell subsets, T follicular helper (Tfh) cells, and monocytes from patients with/without acute or chronic GVHD ($n = 48$ for each group), respectively. CD83 expression was correlated with survival, TRM, and relapse after alloHCT. Differential effects of GVHD therapies on CD83 expression was determined.

Results: CD83 overexpression on $CD4^+$ T cells correlates with reduced survival and increased TRM. Increased $CD83^+$ B cells and Tfh cells, but not monocytes, are associated with poor posttransplant survival. CD83 CAR T eliminate autoreactive $CD83^+$ B cells isolated from patients with chronic GVHD, without B-cell aplasia as observed with CD19 CAR T. We demonstrate robust CD83 antigen density on human acute myeloid leukemia (AML), and confirm potent antileukemic activity of CD83 CAR T *in vivo*, without observed myeloablation.

Conclusions: CD83 is a promising diagnostic marker of GVHD and warrants further investigation as a therapeutic target of both GVHD and AML relapse after alloHCT.

Introduction

Despite recent advances in GVHD prophylaxis, like posttransplant cyclophosphamide (PTCy) or abatacept, acute and chronic GVHD remain major causes of morbidity and mortality after allogeneic hematopoietic cell transplantation (alloHCT; refs. 1, 2). Nonselective targeting of donor T cells with anti-thymocyte globulin reduces the incidence of grade II–IV acute GVHD, but increases the risk of disease relapse, and ultimately limits survival after alloHCT compared with T cell–replete GVHD prophylaxis (3). A strategy that can simultaneously reduce GVHD and relapse would be advantageous to the field. We have demonstrated that CD83 is differentially expressed on alloreactive $CD4^+$ T cells implicated in acute GVHD, with negligible expression on regulatory T cells (Treg) or $CD8^+$ T cells (4). Our human CD83 chimeric antigen receptor (CAR) T prevent and treat xenogeneic GVHD in mice (4). Importantly, CD83 CAR T also spare antiviral responses by $CD4^+$ and $CD8^+$ T cells against cytomegalovirus or influenza A (4). Acute myeloid leukemia (AML) is a leading indication for alloHCT. We demonstrated that AML blasts express CD83 and are readily killed by CD83 CAR T (4). Thus, CD83 CAR T have the potential to reduce the two main causes of death among alloHCT recipients, GVHD and AML relapse. This discovery is a major departure from contemporary GVHD prophylaxis, which offers little to no specific protection against AML.

In this pilot study ($n = 48$ with/without acute GVHD and $n = 48$ with/without chronic GVHD) we investigated CD83 expression on pathogenic cells critically involved in the biology of GVHD; $CD4^+$ T cells in acute GVHD and B cells as well as T follicular helper (Tfh) cells in chronic GVHD (4, 5). Further, we surmised a better understanding of CD83 expression on GVHD effectors could inform clinical trial design and future applications of CD83 CAR T. We also evaluated the impact of FDA-approved therapies for acute and chronic GVHD on effector cell CD83 expression. Importantly, we provide proof-of-concept evidence that increased CD83 expression on $CD4^+$ T cells or B cells and Tfh cells discriminates patients with acute or chronic GVHD, respectively, at time of diagnosis. We also show that increased GVHD effector cell CD83 expression is associated with decreased survival after alloHCT.

While others and we have shown CD83 is relevant to acute GVHD, this pilot study is the first to demonstrate important correlations between GVHD effector cell CD83 expression and diagnosis or survival in both acute and chronic GVHD (4, 6). In combination with our CD83 CAR T, this discovery provides early evidence that CD83 could be a cell-based therapeutic target and a potential marker of

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Translational Relevance

Modern GVHD prophylaxis, like posttransplant cyclophosphamide and abatacept-based strategies, have achieved the lowest rates of grade III–IV, life-threatening acute GVHD since the introduction of allogeneic hematopoietic cell transplantation (alloHCT). However, transplant outcomes and recipient quality of life are still marred by disease relapse and chronic GVHD. We have shown that anti-CD83 chimeric antigen receptor (CAR) T can eliminate GVHD and acute myeloid leukemia (AML), the most common indication for alloHCT in adults. We now demonstrate CD83 is specifically overexpressed on critical effector cells of GVHD, including alloreactive $CD4^+$ T cells as well as B cells and T follicular helper cells during acute and chronic GVHD, respectively. High CD83 expression on these cells also correlate with reduced survival. Further, we show CD83 CAR T offers a therapy to selectively target autoreactive B cells without inducing B-cell aplasia, reducing the risk of hypogammaglobulinemia and opportunistic infections. While prospective validation of these findings is underway, this discovery provides proof-of-concept evidence that CD83 selectively identifies critical immune effectors of acute and chronic GVHD, informs transplant recipient survival, and provides a universal cell therapy target to concurrently eliminate GVHD and AML relapse.

GVHD. Of the contemporary GVHD biomarkers, like suppression of tumorigenicity 2 (ST2) and regenerating islet-derived 3α (REG3α), only ST2 has demonstrated potential as a therapeutic target to reduce acute GVHD severity in mice (7, 8). Herein, we show that CD83 stands out as a promising cell-based correlate for the full spectrum of GVHD and to inform mortality risk after alloHCT; in addition, it has clinical relevance as a therapeutic target to concurrently reduce GVHD and AML relapse (4).

Materials and Methods

Patient samples

Written informed consent was obtained from eligible alloHCT recipients at the University of Minnesota to collect blood samples via an Institutional Review Board (IRB)-approved biospecimen collection study, in accordance with the Declaration of Helsinki. Peripheral whole blood (∼60 mL) was acquired by venipuncture or a central intravenous catheter in green top, heparin-containing tubes and transported to the Translational Therapy Laboratory, Masonic Cancer Center, at room temperature. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation, frozen in a control-rate freezer, and stored at −180°C in 40% Iscove's modified Dulbecco's medium, 30% heat-inactivated FBS, and 30% DMSO using a temperature-monitored liquid nitrogen freezer. Samples were collected at time of acute (9) or chronic (10) GVHD diagnosis before treatment was initiated. Control alloHCT samples from patients without GVHD were matched as closely as possible to the cases to maintain similar sample acquisition times. No randomization was performed. Patient characteristics and demographics are summarized in **Table 1**. Inclusion was not restricted by age, sex, or transplant conditioning. Acute GVHD clinical grading was performed according to the 1995 consensus guidelines (11). Chronic GVHD was diagnosed according to the NIH consensus criteria (12). Prior to cell staining for flow cytometry, vials of cells were thawed in a water bath at 37°C for approximately 3 to 5 minutes, and then washed in RPMI supplemented with 10% heat-inactivated pooled human serum. The cells were stained by 5 to 8 minutes.

Fluorochrome-conjugated antibodies, flow cytometry, and measurement of CD83

To evaluate CD83 expression on $CD4^+$ T cells from acute GVHD patients and controls, PBMCs were surface stained with live/dead Aqua, CD4, and CD83. CD83 expression was determined by gating on live $CD4^+$ T cells in the lymphocyte gate. For chronic GVHD patient samples and controls, PBMCs were stained for live/dead Aqua, CD4, CD19, CD45RA, CXCR5, and CD83 or live/dead Aqua, CD1c, CD14, CD15, CD16, CD45, and CD83. B cells were identified as $CD19^+$, $CD4^-$, and Tfh cells were characterized as $CD4^+$, $CXCR5^+$, $CD45RA^{neg}$ (13–15). B-cell subsets were characterized as transitional ($CD19^+$, IgD^+, $CD38^+$, $CD27^-$), age-associated (ABC; $CD19^+$, $CD21^-$, $CD11c^+$), IgD^+ memory ($CD19^+$, IgD^+, $CD38^{lo}$, $CD27^+$), pregerminal ($CD19^+$, IgD^+, CD38+, $CD27^+$), postgerminal memory ($CD19^+$, IgD^{lo}, $CD38^{lo}$, $CD27^+$), and plasmablast ($CD19^+$, IgD^{lo}, $CD38^{hi}$, $CD27^+$) B cells (16, 17). Monocytes were characterized as $CD14^{high}$, $CD15^{int}$, $CD16^{low}$. Fluorescence minus one (FMO) staining controls were used for each fluorochrome. Fc block was used to reduce nonspecific binding of antibodies. The frequency of $CD83^+$ cells and the geometric mean florescence intensity (gMFI) were recorded (4). Supplementary Table S1 details the fluorochrome-conjugated antibodies used.

Allogeneic mixed leukocyte reactions

Purified donor T cells were allo-stimulated with cytokine-matured, allogeneic monocyte-derived dendritic cells at a DC:T cell ratio of 1:30, as previously described (4, 18). Clinically relevant concentrations of ruxolitinib (JAK1 and JAK2, 300 nmol/L or 1 μmol/L), itacitinib (JAK1, 1 μmol/L), fedratinib (JAK2, 1 μmol/L), a combination of itacitinib and fedratinib, or DMSO control 0.25% was added once on day 0 (19–21). After a 5-day culture, $CD4^+$ T-cell proliferation was evaluated by Ki-67, and CD83 expression was assessed by flow cytometry (4, 18). The percent proliferation was normalized to untreated T cells analyzed under the same assay conditions.

CAR T-cell production

The SFG retroviral backbone was modified to include an anti-human CD19 or anti-human CD83 scFv with CD8α signal peptide and transmembrane plus a hinge domain followed by the co-stimulatory domain 41BB and CD3ζ (ref. 4; Genewiz). Plasmids were calcium phosphate transfected into H29 cells, and retroviral supernatants were used to transduce RD114 producer cells. Retroviral supernatant of producer cells was harvested, 0.45-μm filtered, and used to transduce T cells as described (4). Leukocytes obtained from healthy donor apheresis products (AllCells) were isolated by density gradient centrifugation. T cells were magnetically isolated (Stem Cells Inc.) and stimulated with human CD3/CD28 activation beads (Thermo Fisher Scientific) in supplemented RPMI1640 medium (10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin) with recombinant human IL2 (100 IU/mL). Activated T cells were transduced with the respective γ-retrovirus on RetroNectin-coated (TaKaRa Bio Inc.) plates, or with virus-free media for untransduced T cells. For viral transduction, the same volume of viral particles was used to transduce both CAR constructs. CD83 CAR T cells were debeaded after 5 days. On day 9 after T-cell isolation, transduction efficiency was assessed by flow cytometry using biotinylated-Protein L

Table 1. Patient characteristics. Patient characteristics and clinical variables are detailed.

Characteristic	Acute GVHD *N* = 24	No acute GVHD *N* = 24	*P*	Chronic GVHD *N* = 24	No chronic GVHD *N* = 24	*P*
Age (years)	54 (29–73)	55 (20–70)	0.699	41 (6–72)	50 (20–67)	0.432
Sample collection, days posttransplant	D+34 (25–61)	D+28 (25–30)	0.156	D+209 (91–1,206)	D+212 (167–372)	0.149
Sample collection, days post-GVHD diagnosis	3 (1–7)	*NA*	*NA*	12 (1–21)	*NA*	*NA*
Disease			0.312			0.447
AML	7	13		7	10	
Myelodysplasia	7	5		3	6	
Myeloproliferative neoplasm	2	0		0	0	
Acute lymphoblastic leukemia	6	3		6	2	
Chronic myeloid leukemia	0	1		3	1	
Chronic lymphocytic leukemia	0	0		0	1	
Non–Hodgkin lymphoma	1	2		2	1	
Hodgkin lymphoma	0	0		0	1	
Multiple myeloma	0	0		1	0	
Other	1	0		2	2	
GVHD prophylaxis			0.139			0.066
CSA/MMF	18	11		15	10	
TAC/MMF	3	4		1	6	
SIR/MMF	1	7		0	3	
CSA/MTX	1	1		5	2	
TAC/MTX	0	1		0	1	
SIR/TAC/Ruxo	1	0		0	0	
SIR/TAC/MTX	0	0		0	1	
PTCy/TAC/MMF	0	0		1	0	
Acute GVHD clinical grade			*NA*			*NA*
Grade II	4	*NA*		*NA*	*NA*	
Grade III	11	*NA*		*NA*	*NA*	
Grade IV	9	*NA*		*NA*	*NA*	
Conditioning Intensity			0.221			0.221
Reduced intensity	14	18		14	18	
Myeloablative	10	6		10	6	
Stem cell source			0.221			0.133
Bone marrow	2	1		7	3	
Peripheral blood	13	8		12	10	
Cord blood	9	15		5	11	
Donor relationship			0.745			0.773
Related	6	7		12	11	
Unrelated	18	17		12	13	

Abbreviations: CSA, cyclosporine; MMF, mycophenolate mofetil; MTX, methotrexate; NA, not applicable; Ruxo, ruxolitinib; SIR, sirolimus; TAC, tacrolimus.

and Streptavidin for CD19 CAR detection and recombinant human CD83-Fc chimeric protein (R&D Systems) and an anti-human IgG1 secondary antibody (ThermoFisher) for CD83 CAR detection. Untransduced, anti-hCD19, and anti-hCD83 CAR T cells were harvested, enumerated, and normalized to CAR transduction efficiency for use in the described culture experiments.

Autoreactive B-cell experiments

B cells (100,000) from patients with chronic GVHD were purified by magnetic bead purification (Miltenyi Biotec) and then stimulated with 5,000 OP9-DL1 cells and 0.625 μg/mL of donkey anti-human IgM antibody for 72 hours in 100 μL of complete RPMI media supplemented with 10% heat-inactivated, pooled human serum (GeminiBio) using flat-bottom 96-well plates (5). Where indicated, ibrutinib (BTK inhibitor, 1 μmol/L), belumosudil (ROCK2 inhibitor, 1 μmol/L), ruxolitinib (1 μmol/L), or DMSO vehicle was added once on day 0 (19, 22, 23). B-cell proliferation and CD83 expression were assessed by flow cytometry. In separate experiments, 10,000 untransduced T cells, CD19 CAR T, or CD83 CAR T were added to 100,000 stimulated B cells for 72 hours. The anti-CD19 and anti-CD83 CAR T used were previously described (4). No small molecule inhibitors or DMSO were added to the CAR T coculture experiments.

Cell lines

MV411 cells (RRID:CVCL_0064) were obtained from ATCC, with appropriate authentication provided in the certificate of analysis. Cryopreserved cells were stored in liquid nitrogen freezers at the Betts or Davila Labs, confirmed to be *Mycoplasma*-free. MV411 cell line passages were kept < 10.

Statistical analysis

Statistical differences in patient characteristics were determined by Fisher exact test for proportions or *t* test for numeric variables. All *in vitro* experiments were replicated with at least 3 independent experiments. ROC curves evaluated the sensitivity and specificity of

CD83 expression on $CD4^+$ T cells for acute GVHD and B cells and Tfh cells for chronic GVHD. The area under the ROC curve (AUC) was estimated non-parametrically and compared with 0.5 with a hypothesis test (24). For samples collected by day +100, a CD83 expression intensity on $CD4^+$ T cells of 740.5 gMFI was selected to evaluate overall survival among alloHCT recipients with high or low CD83 expression. For samples collected after day +100, a CD83 expression intensity on B cells or Tfh cells of 396 and 469, respectively, was used to determine the risk of mortality after alloHCT. Cut points were chosen to maximize Youden's index to give the highest AUCs for acute or chronic GVHD. For comparisons of independent data sets, the unpaired Mann–Whitney test was used. ANOVA with a Sidak or Dunnett multiple comparison test was used for group comparisons. Geometric mean fluorescence intensity was log-transformed prior to analysis. The Log-rank test was used to evaluate differences in survival based on CD83 expression or GVHD. The cumulative incidences of transplant-related mortality (TRM) and relapse were estimated treating each event as a competing risk. The cumulative incidences of TRM and relapse were compared between groups using Grey test (25). The statistical analyses were conducted by use of Prism software (GraphPad Software, San Diego, CA) or R version 4.1.2 "Bird Hippie" with the cmprsk, survminer, and survival packages. Statistical significance was defined by $P < 0.05$.

Data availability

For de-identified, original data, please contact Brian C. Betts, MD at bett0121@umn.edu. If needed, the Office of Technology Commercialization at University of Minnesota and/or the Office of Innovation and Industry Alliances (OIIA) at Moffitt Cancer Center will assist with negotiating material transfer agreements.

Results

Patient characteristics

AlloHCT recipients were consented to a biorepository protocol approved by the IRB at the University of Minnesota from 2009 to 2021. Descriptive data were collected and are summarized as patient characteristics in **Table 1**. Patient characteristics, clinical variables, and time of sample acquisition were statistically similar among the GVHD cases and controls (**Table 1**).

$CD83^+$, $CD4^+$ T cells are increased among alloHCT recipients with grade II–IV acute GVHD

Our current work determined that the frequency of circulating $CD83^+$, $CD4^+$ T cells are significantly increased among patients diagnosed with grade II–IV acute GVHD (**Fig. 1A**), compared with posttransplant controls without acute GVHD. Blood samples were acquired prior to the initiation of systemic glucocorticoid therapy for acute GVHD. ROC curves were generated to test the ability of $\%CD83^+$, $CD4^+$ T cells to discriminate between those with or without grade II–IV acute GVHD by day +100. The area under the curve (AUC) for $\%CD83^+$, $CD4^+$ T cells was 0.823, with a significant P value of 0.0001 (**Fig. 1B**). In addition, the gMFI of CD83 expression on $CD4^+$ T cells was significantly increased among those with grade II–IV acute GVHD after alloHCT (**Fig. 1C**). ROC curves generated from these data yielded an AUC of 0.789, with a significant P value of 0.0006 (**Fig. 1D**). Thus, either the frequency of $CD83^+$, $CD4^+$ T cells or the intensity of CD83 expression on $CD4^+$ T cells effectively discriminate patients with grade II–IV acute GVHD compared with healthy posttransplant controls.

Earlier acute GVHD diagnosis is associated with increased $CD4^+$ T cell CD83 expression

We then characterized the burden of $CD4^+$ T cell CD83 expression as a temporal function of acute GVHD diagnosis. The percentage of $CD83^+$, $CD4^+$ T cells and the gMFI of CD83 on $CD4^+$ T cells were both highest among patients diagnosed with acute GVHD at earlier time points (Supplementary Fig. S1A and S1B). These translational data provide evidence that early treatment with CD83 CAR T may be beneficial in ameliorating acute GVHD, as CD83 antigen is abundant prior to day +40.

High $CD4^+$ T cell CD83 expression before day +100 is associated with reduced survival and increased TRM after alloHCT

CD83 antigen content of the GVHD effector cell is functionally relevant to CAR T targeting and cytotoxicity (26). Therefore, a gMFI cutoff of 740.5 for $CD4^+$ T cell CD83 expression achieved a test sensitivity of 91.67%, specificity of 54.17%, and a positive likelihood ratio of 2 for discriminating acute GVHD (**Fig. 1E**). With a pool of 40 alloHCT patients evaluable for survival (20 with or without grade II–IV acute GVHD), we investigated the overall survival of patients based on a $CD4^+$ T cell CD83 expression cutoff of 740.5 prior to day +100. Those with high $CD4^+$ T cell CD83 expression (e.g., > 740.5) demonstrated a significant reduction in overall survival, compared with those with low CD83 expression (**Fig. 1E**). Importantly, only $CD4^+$ T cell CD83 expression intensity, but not the presence or absence of grade II–IV acute GVHD, was considered in this comparison. However, those diagnosed with grade II–IV acute GVHD by day +100 also exhibited a significant decrease in overall survival compared with healthy alloHCT controls (Supplementary Fig. S2A). Increased $CD4^+$ T cell CD83 expression also correlated with greater TRM (**Fig. 1F**). None of the evaluated patients experienced relapse in this pilot cohort (**Fig. 1G**). The clinical outcomes of overall survival and TRM analyzed by the frequency of circulating $CD83^+$, $CD4^+$ T cells (low < 30%, high > 30%) followed similar trends but did not reach statistical significance (Supplementary Fig. S2B and S2C).

Impact of JAK inhibition on $CD4^+$ T cell CD83 expression

The JAK1/2 inhibitor, ruxolitinib, is FDA approved for the treatment of steroid-refractory acute GVHD (27). We investigated whether ruxolitinib reduced CD83 expression on allo-activated $CD4^+$ T effectors of GVHD, as it could inform the use of CD83 CAR T as an experimental therapeutic after ruxolitinib failure. Consistent with work from others and our group, ruxolitinib significantly reduced the proliferative capacity of allo-antigen stimulated human T cells at clinically relevant concentrations (**Fig. 2A**; refs. 28, 29). To investigate whether JAK1, JAK2, or concurrent JAK1 and JAK2 inhibition could impact T cell CD83 expression, we treated allo-stimulated T cells with DMSO control, ruxolitinib (JAK1/2 inhibitor), itacitinib (JAK1 inhibitor; refs. 20, 30), fedratinib (JAK2 inhibitor; ref. 18), or a combination of itacitinib plus fedratinib. Overall, inhibition of JAK1, JAK2, or JAK1/2 similarly suppressed CD83 expression on allo-stimulated $CD4^+$ T cells (**Fig. 2B**).

CD83 expression on B cells and Tfh cells is increased upon chronic GVHD onset

Autoreactive B cells and Tfh cells are implicated in chronic GVHD pathogenesis (5). CD83 is expressed on mantle cell lymphoma and Reed-Sternberg cells in Hodgkin lymphoma, but CD83 expression on autoreactive B cells was previously unknown (31, 32). While CD83 expression on Tfh cells was unreported prior to our current work, Tfh

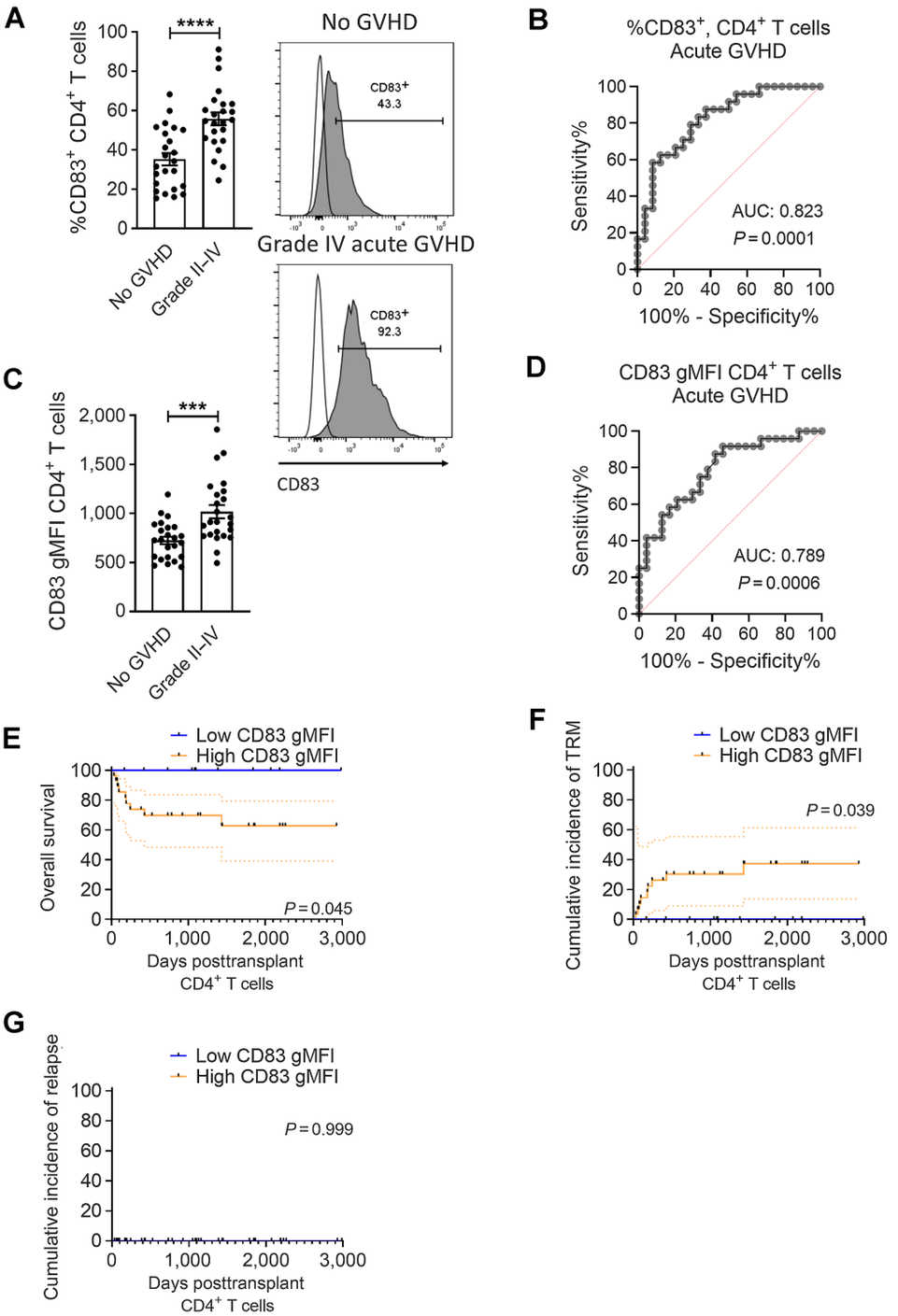

Figure 1.

CD83 expression on CD4$^+$ T cells is increased upon acute GVHD onset and is associated with poor clinical outcomes. CD83 expression was evaluated among patients post alloHCT with grade II–IV ($n = 24$) acute GVHD or without ($n = 24$) at similar time points before day +100. **A–D,** Bar graphs and ROC curves depicting %CD83 expression and geometric mean fluorescence intensity are shown. Representative histograms (included in **A**) show CD4$^+$ T cell CD83 expression from patients without or with acute GVHD. Unfilled histogram, CD83 FMO; filled histogram, CD4$^+$ T cell. **E,** Graph shows the overall survival of alloHCT recipients ($n = 40$) based on CD4$^+$ T cell CD83 expression. CD83 expression was determined at time of acute GVHD diagnosis ($n = 20$) or sampled among controls without acute GVHD ($n = 20$) at similar time points posttransplant. A gMFI cutoff of 740.5 was used to determine low versus high CD83 expression. Graphs show the cumulative incidence of (**F**) TRM and (**G**) relapse based on CD4$^+$ T cell CD83 expression among the evaluated alloHCT recipients. Dotted lines represent the 95% confidence intervals. ***, $P = 0.001$–0.0001; ****, $P < 0.0001$.

cells orchestrate critical aspects of autoreactive B-cell activation in chronic GVHD (15, 33). In addition, monocytes and related interferon-inducible genes are associated with end organ damage in chronic GVHD (34). Therefore, we investigated CD83 expression among circulating B cells, Tfh cells, and monocytes at time of chronic GVHD diagnosis, prior to the initiation of systemic glucocorticoid therapy. The frequency of CD83$^+$ B cells and Tfh cells, but not monocytes, were significantly increased among patients with chronic GVHD, compared with alloHCT controls without GVHD (**Fig. 3A**). ROC curves were generated to test the ability of %CD83$^+$ B cells or Tfh cells to discriminate between those with or without chronic GVHD after day +100. The AUC for %CD83$^+$ B cells was 0.979, with a significant P value of 0.0001 (**Fig. 3B**). Similarly, the AUC for %CD83$^+$ Tfh cells was 0.843, with a significant P value of 0.0001 (**Fig. 3C**). In addition, the gMFI of CD83 expression on B cells or Tfh cells were significantly increased among patients with chronic GVHD (**Fig. 3D**). The AUC for

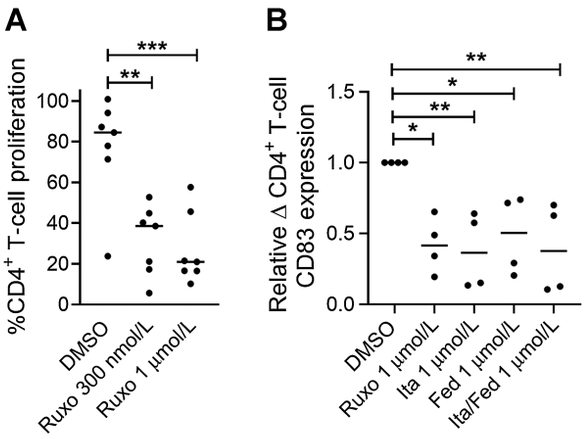

Figure 2.

Impact of JAK inhibition on CD4$^+$ T cell CD83 expression. Human T cells were cultured with allogeneic, monocyte-derived dendritic cells (DC:T cell ratio 1:30) in standard 5-day mixed leukocyte reactions. **A,** Graph shows %proliferation of CD4$^+$ T cells after the 5-day culture, with DMSO or ruxolitinib added once on day 0. **B,** Graph shows change in CD83 expression on CD4$^+$ T cells after treatment with ruxolitinib, itacitinib (JAK1 inhibitor), fedratinib (JAK2 inhibitor), or a combination of itacitinib and fedratinib, relative to DMSO control, after a 5-day coculture. $n = 7$ (for **A**) and 4 (for **B**) independent experiments. *, $P < 0.05$; **, $P = 0.01$–0.001; and ***, $P = 0.001$–0.0001.

CD83 expression on B cells or Tfh cells was 0.984 and 0.812, respectively (**Fig. 3E** and **F**), demonstrating the ability to discriminate those with chronic GVHD from healthy alloHCT controls. Samples from a group of the chronic GVHD patients ($n = 9$) were further phenotyped to characterize CD83 expression among B-cell subsets. CD83 expression favored pregerminal B cells (CD19$^+$, IgD$^+$, CD38+, CD27$^+$), with significantly greater antigen intensity compared with transitional (CD19$^+$, IgD$^+$, CD38$^+$, CD27$^-$), age-associated (ABC; CD19$^+$, CD21$^-$, CD11c$^+$), IgD$^+$ memory (CD19$^+$, IgD$^+$, CD38^{lo}, CD27$^+$), postgerminal memory (CD19$^+$, IgD^{lo}, CD38^{lo}, CD27$^+$), or plasmablast (CD19$^+$, IgD^{lo}, CD38^{hi}, CD27$^+$) B cells (refs. 16, 17; **Fig. 3G** and **H**).

Increased B cell or Tfh CD83 expression after day +90 is associated with reduced survival after alloHCT

A gMFI cutoff of 396 for B cell CD83 expression achieved a test sensitivity of 95.83%, specificity of 83.33%, and a positive likelihood ratio of 5.75 in discriminating those with chronic GVHD. We observed a significant reduction in overall survival among alloHCT patients with high versus low B cell CD83 expression (**Fig. 4A**). Alternatively, a gMFI of 469 for Tfh CD83 expression produced a sensitivity of 75%, specificity of 62.5%, and a positive likelihood ratio of 2 in separating patients with chronic GVHD versus healthy alloHCT controls. High Tfh CD83 expression also identified those at risk for significantly decreased overall survival compared with those with low Tfh CD83 expression (**Fig. 4B**). As described with grade II–IV acute GVHD, those with chronic GVHD after day +100 had a significant reduction in overall survival compared with healthy alloHCT recipients (Supplementary Fig. S3A). A trend toward greater TRM was observed among patients with increased B cell or Tfh CD83 expression after day +90, but this did not reach statistical significance (**Fig. 4C** and **D**). Increased effector cell CD83 expression did not discriminate relapse risk among the evaluated patients (**Fig. 4E** and **F**). Increased circulating CD83$^+$ B cells (> 30%) was associated with significantly worse overall survival and TRM (Supplementary Fig. S3B and S3C). Greater frequencies of CD83$^+$ Tfh cells (> 30%) demonstrated significantly worse survival, but not TRM (Supplementary Fig. S3D and S3E).

Impact of BTK, JAK1/2, or ROCK2 inhibition on autoreactive B cell CD83 expression from patients with chronic GVHD

Ibrutinib (BTK inhibitor), ruxolitinib, and belumosudil (ROCK2 inhibitor) are FDA-approved therapies for steroid-refractory chronic GVHD (35, 36). We investigated how ibrutinib, ruxolitinib, or belumosudil affected CD83 expression on autoreactive B cells from patients with chronic GVHD. Magnetic bead-purified B cells from newly diagnosed patients with chronic GVHD, prior to systemic glucocorticoid therapy, were collected and then stimulated with anti-human IgM antibody and OP9-DL1 cells to provide NOTCH signaling (5). The activated B cells were treated with clinically relevant concentrations of ibrutinib, ruxolitinib, belumosudil, or DMSO control for 72 hours (19, 22, 23). Ibrutinib significantly reduced the proliferation of the autoreactive B cells, but ruxolitinib did not (**Fig. 5A**). Belumosudil showed a trend toward reduced B-cell proliferation but did not meet statistical significance (**Fig. 5A**). In addition, BTK or ROCK2 inhibition significantly decreased CD83 expression on B cells from patients with chronic GVHD (**Fig. 5B**). Ruxolitinib had no impact on CD83 expression among autoreactive B cells (**Fig. 5B**).

Anti-CD83 CAR T eliminate autoreactive CD83$^+$ B cells from patients with chronic GVHD, but do not induce broad B-cell aplasia

We have previously shown that CD83 CAR T prevent and treat acute GVHD in mice (4). With our discovery that autoreactive B cells significantly express CD83, we investigated whether CD83 CAR T could selectively reduce autoreactive B cells while avoiding complete B-cell aplasia. This is relevant as CD19 CAR Tregs were recently shown to reduce GVHD and kill lymphoma in mice, but at the cost of circulating B cells (37). B cells from patients with chronic GVHD were purified, stimulated with anti-human IgM antibody and OP9-DL1 cells, and cultured with CD19 CAR T, CD83 CAR T, or untransduced T-cell controls for 72 hours. Anti-CD19 and -CD83 CAR T significantly reduced CD83$^+$ autoreactive B cells, compared with untransduced T cells (**Fig. 5C**). However, only CD19 CAR T induced complete B-cell elimination, compared with CD83 CAR T or untransduced T cells (**Fig. 5D**).

Selective targeting of AML with CD83 CAR T

In terms of CD83 CAR T safety, we confirmed our past findings (4) with an independent tissue microarray demonstrating that CD83 cell surface expression is absent to low in healthy organs (Supplementary Fig. S4). Prior *in vitro* studies also showed CD83 CAR T preserved human hematopoiesis, despite its ability to kill myeloid leukemia (4). Importantly, CD83 is largely absent on human HSCs and myeloid precursors in the marrow (Supplementary Fig. S5A). In mice reconstituted with human HSCs (~37% human CD45$^+$ chimerism on day 0), we compared recipients injected with CD83 CAR T to mice bearing other engineered effector cells that target AML, CD33, or CD123 CAR T (Supplementary Fig. S5B–S5F). Mice treated with CD83 CAR T exhibit significantly greater numbers of human HSCs and T cells compared with those injected with CD33 or CD123 T cells (Supplementary Fig. S5D–S5F). Further, CD83 CAR T also preserves B cell numbers *in vivo* (Supplementary Fig. S5G). Adding to our prior report, we show CD83 antigen density is robust among leukemia stem cells (38), like CD123, from bone marrow samples acquired from

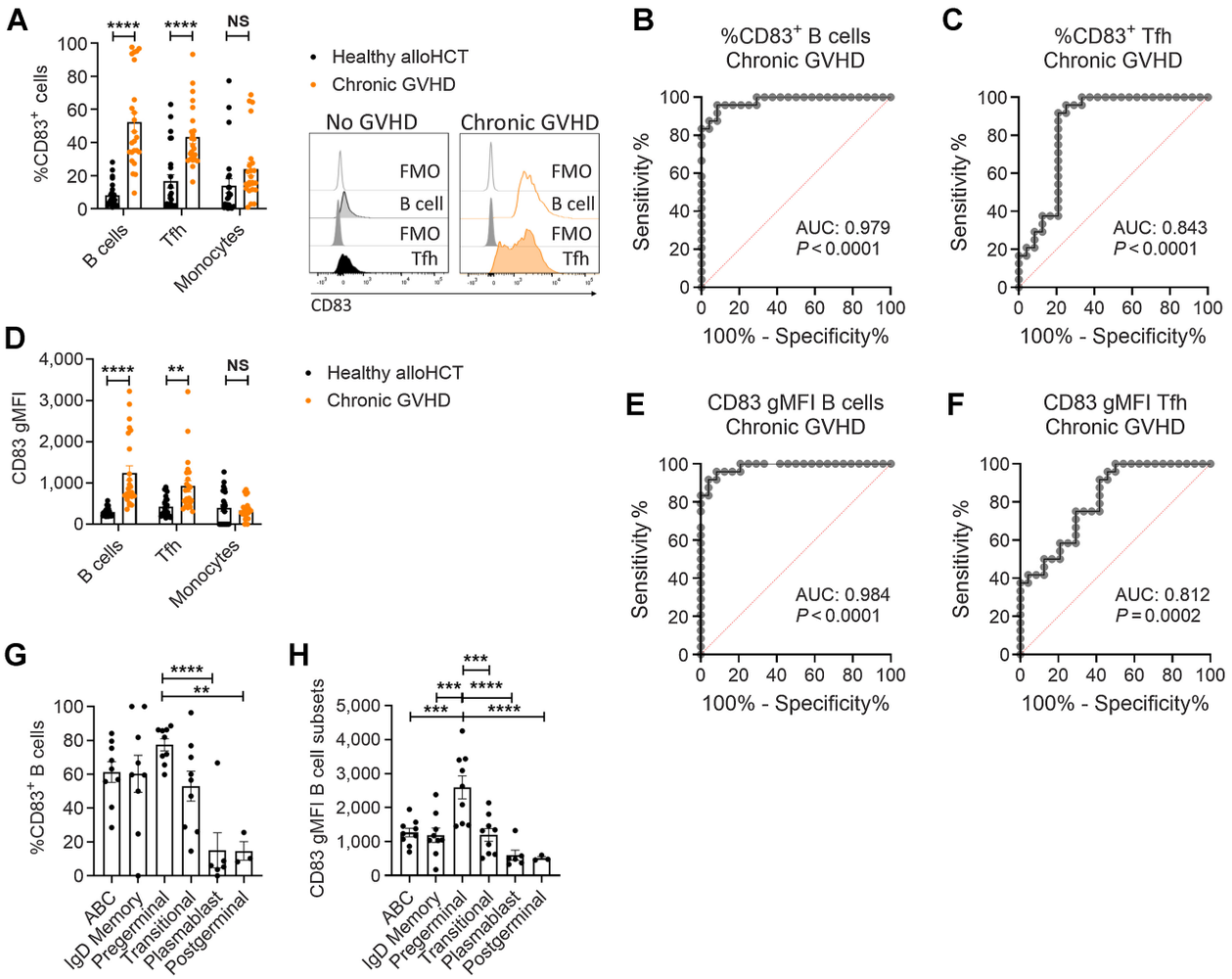

Figure 3.

CD83 expression on B cells and Tfh cells is increased upon chronic GVHD onset. CD83 expression was evaluated among B cells, Tfh cells, and monocytes from patients post alloHCT with ($n = 24$) or without ($n = 24$) chronic GVHD at similar time points posttransplant. **A–F,** Bar graphs and ROC curves depicting %CD83 expression and gMFI are shown. Representative histograms (included in **A**) show B cell and Tfh CD83 expression from patients without or with chronic GVHD. B cells were identified as $CD19^+$, $CD4^-$. Tfh cells were identified as $CD4^+$, $CD45RA^{neg}$, $CXCR5^+$ T cells. Monocytes were characterized as $CD14^{high}$, $CD15^{int}$, $CD16^{low}$. Graphs show the (**G**) $\%CD83^+$ and (**H**) CD83 gMFI among age-associated (ABC; $CD19^+$, $CD21^-$, $CD11c^+$), IgD^+ memory ($CD19^+$, IgD^+, $CD38^{lo}$, $CD27^+$), pregerminal ($CD19^+$, IgD^+, $CD38^+$, $CD27^+$), transitional ($CD19^+$, IgD^+, $CD38^+$, $CD27^-$), plasmablast ($CD19^+$, IgD^{lo}, $CD38^{hi}$, $CD27^+$), and postgerminal memory ($CD19^+$, IgD^{lo}, $CD38^{lo}$, $CD27^+$) B-cell subsets from a group of patients with chronic GVHD ($n = 9$). *, $P < 0.05$; **, $P = 0.01$–0.001; ****, $P < 0.0001$; NS, not significant.

patients with AML (Supplementary Fig. S6A; $n = 12$). Moreover, we confirm CD83 CAR T potently kills AML *in vivo*, as demonstrated against luciferase-transduced MV411 as well NSG-SGM3 mice bearing patient-derived AML blasts (Supplementary Fig. S6B–S6E). Together, this line of work supports the expected safety and efficacy of CD83 CAR T in treating GVHD and AML.

Discussion

Here we provide proof-of-concept evidence that CD83 expression on effectors of acute or chronic GVHD is increased upon diagnosis and associated with increased mortality among alloHCT recipients. Importantly, we have previously shown that CD83 CAR T can prevent or treat xenogeneic GVHD mediated by human T cells in mice (4). Our current work now supports future translational investigation of CD83 CAR T as a therapy for chronic GVHD too, as CD83 is highly expressed on autoreactive B cells and circulating Tfh cells, both implicated in chronic GVHD pathogenesis (5). We surmise the enhanced CD83 antigen intensity on pregerminal B cells is biologically relevant, as such circulating B cells are increased in chronic GVHD (17, 39). However, germinal center B cells, which were not evaluated for CD83 expression, are also implicated in the production of pathogenic autoantibody (40). Further, Tfh cells are necessary for pulmonary manifestations of chronic GVHD, while exhibiting enhanced homing capacity during active disease (15, 33). We submit that CD83 could bridge the continuum of GVHD care, providing clinical impact as a diagnostic correlate and a selective target of engineered cell therapy to mitigate GVHD (4).

While increased CD83 expression on $CD4^+$ T cells was observed beyond day +60 among patients diagnosed with acute GVHD, the

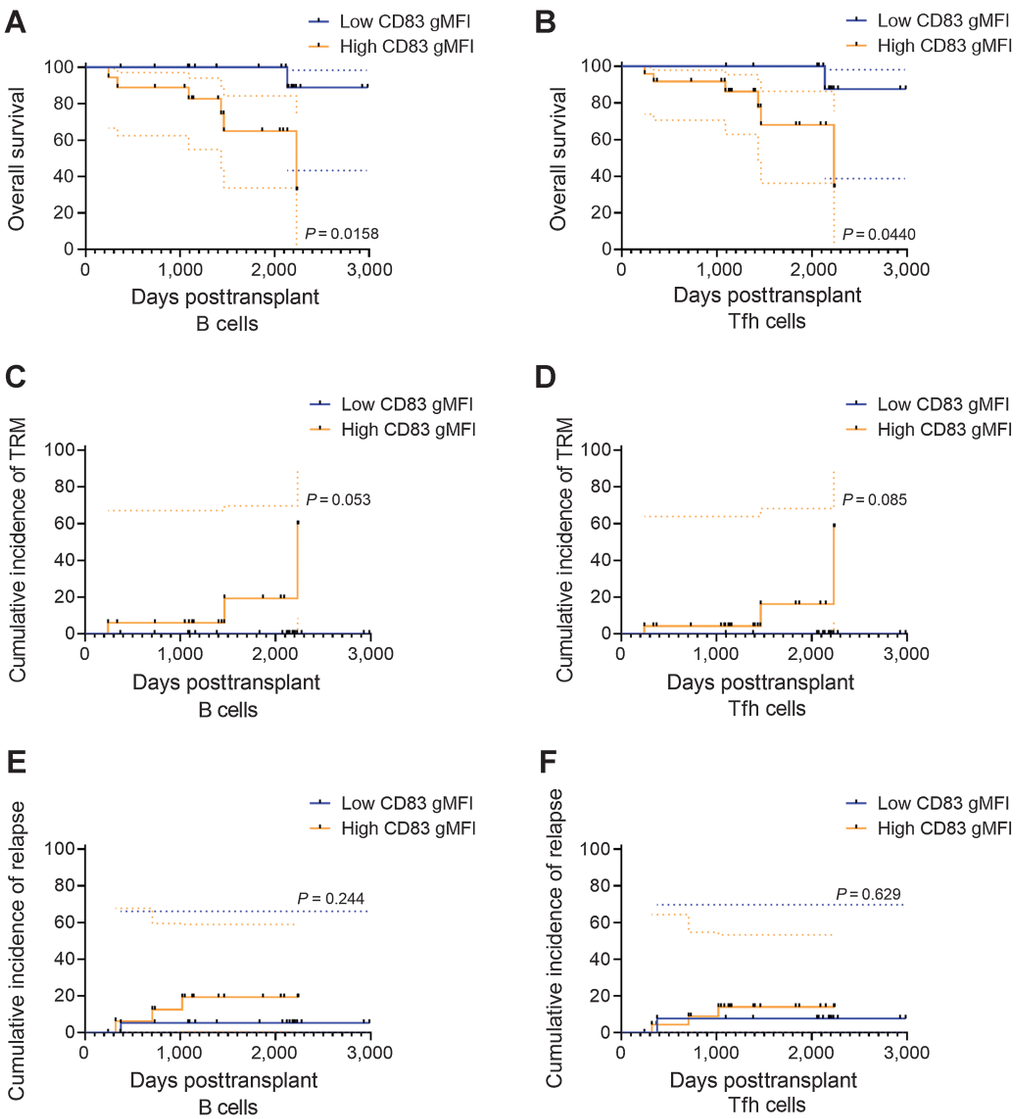

Figure 4.

Increased B cell or Tfh CD83 expression after day +90 is associated with reduced survival after alloHCT. Graph shows the overall survival (**A, B**), cumulative incidence of TRM (**C, D**), and cumulative incidence of relapse (**E, F**) of alloHCT recipients ($n = 37$) based on B cell or Tfh cell CD83 expression. CD83 expression was determined at time of chronic GVHD diagnosis ($n = 17$) or sampled among patients without chronic GVHD ($n = 20$) at similar time points posttransplant. A gMFI cutoff of 396 and 469 was used to determine low versus high CD83 expression for B cells and Tfh cells, respectively. Dotted lines represent the 95% confidence intervals.

highest CD83 intensities were measured before day +40. This suggests that serial monitoring of circulating $CD83^+$, $CD4^+$ T cells following engraftment could rapidly and comprehensively identify patients with, or possibly at risk of, acute GVHD. Our pilot data support that increased $CD83^+$ Tfh cells and B cells at time of diagnosis are discriminators of chronic GVHD. Active investigations will determine if early CD83 expression on B cells or Tfh cells can identify patients at risk of chronic GVHD prior to symptom onset. Clinical development of CD83 CAR T is underway. We surmise that initial clinical investigations of CD83 CAR T as GVHD therapy will focus on treatment first, then prophylaxis. Therefore, longitudinal tracking of $CD83^+$ effector cells after alloHCT may identify patients at greatest risk of acute or chronic GVHD, those with the highest potential for benefit from CD83 CAR T, and avoid over treating patients at lower risk of acquiring GVHD.

With the recent FDA indications awarded to ruxolitinib for the treatment of acute and chronic GVHD and belumosudil or ibrutinib for chronic GVHD, we investigated the impact of these small molecule inhibitors on CD83 expression among GVHD effector cells (22, 27, 36). Interestingly, we found that in addition to the JAK1/2 inhibitor, ruxolitinib, selective inhibitors of JAK1 or JAK2 all reduced CD83 expression on $CD4^+$ T cells by approximately 50%. The regulation of CD83 expression on human T cells is unknown. It is possible that IL2, IL6, IL12, and/or IL23 may partially influence T cell CD83 expression as JAK1 and/or JAK2 regulate the receptor signal transduction of these cytokines, respectively (41). Interestingly, ruxolitinib had no effect on

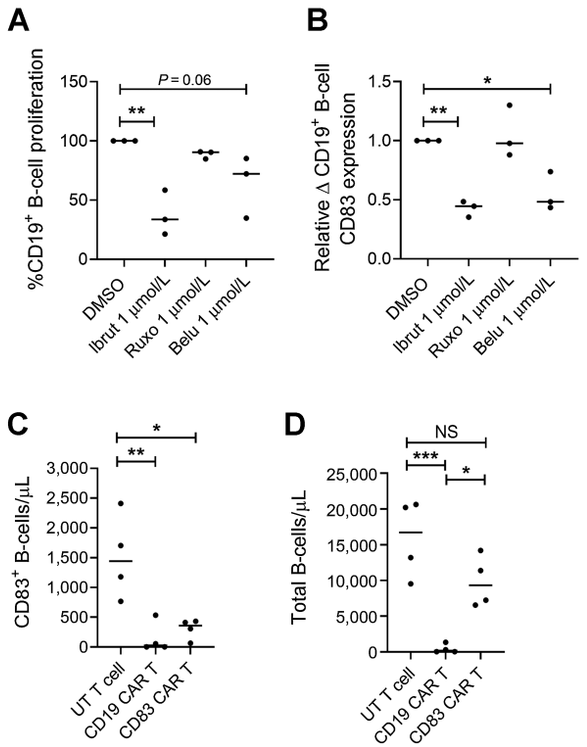

Figure 5.

Targeting autoreactive CD83$^+$ B cells from patients with chronic GVHD without inducing B-cell aplasia. B cells from untreated patients with chronic GVHD were stimulated with anti-human IgM antibody and OP9-DL1 cells, and exposed to ibrutinib (BTK inhibitor), ruxolitinib, belumosudil (ROCK2 inhibitor), or DMSO control for 72 hours. Graphs show (**A**) the %proliferation of B cells and (**B**) the relative change in CD83 expression on autoreactive B cells relative to DMSO control. $n = 3$ independent experiments. B cells from untreated patients with chronic GVHD were stimulated with antihuman IgM antibody and OP9-DL1 cells, and cocultured with anti-CD19 CAR T, anti-CD83 CAR T, or untransduced (UT) T cells for 72 hours. Graphs show the absolute number of (**C**) CD83$^+$ B cells and (**D**) the total number of B cells recovered after the 72-hour coculture. $n = 4$ independent experiments. *, $P < 0.05$; **, $P = 0.01$–0.001; ***, $P = 0.001$–0.0001; NS, not significant.

B cell CD83 expression. Conversely, ibrutinib and belumosudil significantly decreased autoreactive B cell CD83 expression. BTK is a critical regulator of B-cell receptor (BCR) function (36). Therefore, intact BCR activity may be required for CD83 upregulation on B cells. While ROCK2 and JAK1/2 critically regulate STAT phosphorylation, belumosudil uniquely suppresses STAT3 while increasing STAT5 activity (29, 42). The influence of belumosudil on this balance of STAT phosphorylation may induce a suppressive effect on CD83 expression among autoreactive B cells. The impact of ruxolitinib, belumosudil, or ibrutinib on T cell or B cell CD83 expression, respectively, may inform the timing and use of CD83 CAR T in acute and chronic GVHD treatment and will likely guide future clinical trial design and eligibility criteria.

Current therapies for chronic GVHD treatment broadly impair circulating B cells (43). Ibrutinib degrades B-cell activity by suppressing BCR function (43). Anti-CD20 antibody therapy eliminates B cells via several modes of cytotoxicity (43, 44). Further, CD19 CAR Tregs have potent activity as cytolytic immune effector cells that can kill B-cell lymphoma and concurrently reduce GVHD in mice (37). In addition, in other autoreactive syndromes, like multiple sclerosis and systemic lupus erythematosus, CD19 CAR T are actively being investigated as novel experimental therapy (45, 46). CD19 CAR conventional or Tregs distinctly induce B-cell aplasia as CD19 fails to distinguish healthy from autoreactive B cells (37, 47). Long-lasting B-cell aplasia can lead to hypogammaglobulinemia, severe opportunistic infections, and the need for intravenous immunoglobulin replacement (37, 47). CD83 expression is significantly lower on healthy B cells, compared with autoreactive B cells. Distinct from CD19 CAR T, we show that CD83 CAR T selectively eliminates autoreactive CD83$^+$ B cells but avoids B-cell aplasia. This important characteristic of CD83 CAR T may allow for selective and highly effective *in vivo* depletion of pathogenic B cells in patients with chronic GVHD, yet circumvent the long-term clinical complications of profound B cell loss. In addition, while other CAR T for AML carry a risk for marrow aplasia, like CD123 CAR T (48), CD83 CAR T treatment showed no evidence of human myeloablation *in vivo*. We further demonstrate robust CD83 antigen density among myeloid leukemia stem cells and confirm the potent cytolytic activity of CD83 CAR T against AML cell lines and patient-derived blasts *in vivo*.

Few chronic GVHD therapies target pathogenic Tfh cells. Belumosudil exhibits direct suppressive activity against Tfh cells (49) by increasing the intracellular ratio of pSTAT5 to pSTAT3. Reports from patients with systemic sclerosis demonstrate that ruxolitinib can also decrease circulating Tfh cells (50). CD83 CAR T could provide a therapeutic strategy to eliminate pathogenic Tfh cells and B cells in chronic GVHD with a high degree of precision.

CD83 could bridge a critical gap in GVHD diagnostic strategies by providing a direct therapeutic target for GVHD prophylaxis or treatment with CD83 CAR T. Prospective validation of CD83 as a potential biomarker of acute and chronic GVHD is in process (NCT03557749). One limitation of our dataset is that a single patient received PTCy-based GVHD prophylaxis, which may be more widely used pending the results of BMTCTN 1703 (NCT03959241). Therefore, our ongoing validation of CD83 in GVHD ensures the inclusion of PTCy-treated patients. Translational efforts are underway to develop, test, and if successful, deploy, CD83 CAR T as a novel strategy to eradicate acute and chronic GVHD, as well as AML relapse, with low risk of B-cell aplasia or myelosuppression.

Authors' Disclosures

S.G. Holtan reports other support from Incyte and VITRAC Therapeutics outside the submitted work; in addition, S.G. Holtan serves as a clinical trial adjudicator for CSL Behring. A.A. Eaton reports grants from NCI during the conduct of the study, as well as grants from NIH outside the submitted work. J. Maakaron reports other support from Gilead, ADC Therapeutics, CRISPR, and Precision BioSciences outside the submitted work. D.J. Weisdorf reports grants from Fate Therapeutics and Incyte outside the submitted work. M. Felices reports personal fees from GT Biopharma outside the submitted work. J.S. Miller consults for and holds stock in Fate Therapeutics, GT BioPharma, and Vycellix, as well as reports research funds from Fate Therapeutics and GT Biopharma; these interests have been reviewed and managed by the University of Minnesota in accordance with its conflict of interest policy. J.S. Miller serves on the Scientific Advisory Board of ONK Therapeutics, Wugan, and Sanofi; none of these relationships are related to the content of this manuscript. M.L. Davila reports grants from CRISPR during the conduct of the study. M.L. Davila also reports personal fees and other support from Bellicum and Adicet; personal fees from Capstan and CARGO; grants from Kite/Gilead and Novartis; and other support from Adaptive Biotechnologies outside the submitted work. In addition, M.L. Davila has a patent for CAR design pending to Atara and a patent for CD83 CAR pending to CRISPR. B.C. Betts reports personal fees and other support from CRISPR Therapeutics during the conduct of the study, as well as other support from VITRAC Therapeutics, Incyte, and CTI BioPharma outside the submitted work; in addition, B.C. Betts has a patent for WO2019165156 licensed to CRISPR Therapeutics. No disclosures were reported by the other authors.

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Note

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