Novel Targets in Acute Myeloid Leukaemia

By

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Statement of originality

This is to certify that to the best of my knowledge the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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Supervision

The research work presented in this thesis was supervised by Associate Professors Georgina Clark and Stephen Larsen. The thesis is sufficiently well presented to be examined and does not exceed the prescribed word limit.

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Publications of Thesis

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	6	**Abadir E**, Silveira S, Gasiorowski RE, Ramesh M, Romano A, Mekkawy A, Lo T, Kabani K, Sutherland S, Pietersz G, Ho PJ, Bryant C, Larsen S, Clark GJ. ***Targeting CD300f to enhance hematopoietic stem cell transplantation in acute myeloid leukaemia***. Blood Advances. 2020;4(7):1206–1216.	Published
	7	**Abadir E**, Bryant C, Larsen, S, Clark GJ. ***Targeting the niche: depleting haemopoietic stem cells with targeted therapy***. Bone Marrow Transplantation. 2019 Jul;54(7):961-968	Published
Appendix	A	Li Z, Ju X, Silveira PA, **Abadir E**, Hsu WH, Hart DN, Clark GJ. ***CD83: activation marker for antigen presenting cells and its therapeutic potential***. Frontiers in Immunology. 2019;10:1312.	Published
	B	Li Z, Ju X, Lee K, Clarke C, Hsu JL, **Abadir E**, Bryant CE, Pears S, Sunderland N, Heffernan S, Hennessy A. Lo T, Pietersz G, Kupresanin F, Fromm P, Silveira P, Tsonis C, Cooper W, Cunningham I, Brown C, Clark GJ and Hart DN. ***CD83 is a new potential biomarker and therapeutic target for Hodgkin lymphoma.*** Haematologica. 2018 Apr 1;103(4):655-65.	Published
	C	Li Z, **Abadir E**, Lee K, Clarke C, Bryant C, Cooper W, Pietersz G, Silveira PS, Hart DN, Ju X, Clark GJ. ***Targeting CD83 in Mantle cell lymphoma with anti-human CD83 antibody.***	Under Review (Clinical and Translational Immunology)

Authorship Attribution Statement

Chapter 3

Lo TH*, **Abadir E ***, Gasiorowski RE, Kabani K, Ramesh M, Orellana D, Fromm PD, Kupresanin F, Newman E, Cunningham I, Hart DN, Silveira PA, Clark GJ. *Examination of CD302 as a potential therapeutic target for acute myeloid leukemia*. PLOS ONE. 2019 May 10;14(5):e0216368. * contributed equally

EA, THL, GJC, PAS and REG designed the study and planned experiments. **EA**, THL, REG, MR, KK, FK and DO performed experiments. **EA** and THL analysed the data. **EA** and THL wrote the manuscript. REG, PAS, PDF, EN, IC, DNH and GJC revised the manuscript.

Chapter 4

Abadir E, Gasiorowski RE, Silveira PA, Larsen S, Clark GJ. *Is Hematopoietic Stem Cell Transplantation Required to Unleash the Full Potential of Immunotherapy in Acute Myeloid Leukemia? Journal of Clinical Medicine*. 2020 Feb;9(2):554.

This was an invited review for the Journal of Clinical Medicine's special on Acute Myeloid Leukaemia. **EA** developed the original concept, performed the literature review and prepared the manuscript. PAS revised the manuscript and prepared the figures. REG, SL and GJC revised the manuscript.

Chapter 5

Abadir E *, Gasiorowski RE *, Lai K, Kupresanin F, Romano A, Silveira PA, Lo TH, Fromm PD, Kennerson ML, Iland HJ, Ho PJ, Hogarth M, Bradstock K, Hart DN, Clark GJ. *CD300f epitopes are specific targets for acute myeloid leukemia with monocytic differentiation*. Molecular oncology. 2019 Oct;13(10):2107-20 * contributed equally

EA, REG, LK, FK, PAS, THL, PDF, and GJC performed experiments; **EA**, REG, HJI, PJH, and KB provided samples and intellectual input; **EA**, REG, PAS, and GJC analysed results and made figures; PJH and DNJH contributed intellectually to the project design; **EA**, REG, and GJC contributed to project design, analysis of results, and writing of manuscript. * contributed equally

Chapter 6

Abadir E, Silveira S, Gasiorowski RE, Ramesh M, Romano A, Mekkawy A, Lo TH, Kabani K, Sutherland S, Pietersz G, Ho PJ, Bryant C, Larsen S, Clark GJ. *Targeting CD300f to enhance hematopoietic stem cell transplantation in acute myeloid leukaemia*. Blood Advances. 2020;4(7):1206–1216.

EA and GJC designed the study and planned the experiments. **EA**, PS, REG, MR, AR, AM, THL, THL KK, SS and GP performed experiments. **EA**, PS, REG and GJC analysed the data. **EA** and GJC wrote the manuscript. PS, REG, SS, CB, PJH and SL assisted with revision of the manuscript.

Chapter 7

Abadir E, Bryant C, Larsen, S, Clark GJ. *Targeting the niche: depleting haemopoietic stem cells with targeted therapy. Bone Marrow Transplantation*. 2019 Jul;54(7):961-968

EA developed the original concept, performed the literature review, prepared the manuscript and figures. CB, SL and GJC revised the manuscript and figures.

Appendix A

Li Z, Ju X, Silveira PA, **Abadir E**, Hsu WH, Hart DN, Clark GJ. *CD83: activation marker for antigen presenting cells and its therapeutic potential*. Frontiers in Immunology. 2019;10:1312.

Paper writing by ZL, WHH, and PAS. Editing by XJ, **EA,** and GJC. Figure drawing by PAS and WHH. DNH provided the concept and supervised our CD83 work.

Appendix B

Li Z, Ju X, Lee K, Clarke C, Hsu JL, **Abadir E**, Bryant CE, Pears S, Sunderland N, Heffernan S, Hennessy A, Lo TH, Pietersz G, Kupresanin F, Fromm P, Silveira P, Tsonis C, Cooper W, Cunningham I, Brown C, Clark GJ and Hart DN. *CD83 is a new potential biomarker and therapeutic target for Hodgkin lymphoma*. Haematologica. 2018 Apr 1;103(4):655-65.

ZL, XJ, KL, CL, JLH, SP, SH, AH, THL, GP, FK, PDF and PS performed experiments; ZL, XJ, **EA** and GJC analysed results and made the figures. **EA** and WC interpreted clinical results. XJ, CT and DNH. designed the experiments; XJ and DNH wrote the paper.

Appendix C

Li Z, **Abadir E**, Lee K, Clarke C, Bryant C, Cooper W, Pietersz G, Silveira PA Hart DN, Ju X, Clark GJ. *Targeting CD83 in Mantle cell lymphoma with anti-human CD83 antibody*. Under Review in Clinical & Translational Immunology

ZL, XJ designed, performed experiments, analysed data and wrote the manuscript. DNH and GJC designed experiments, analysed data and edited the manuscript. **EA**, CB and WC recruited patients. KL and CC performed the immunohistochemistry staining and interpretation. GP designed 3C12C-MMAE and Herceptin-MMAE. PAS guided mouse experiments and edited the manuscript.

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Edward Abadir, Date 11/06/2020

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Associate Professor Georgina Clark, Date 11/06/2020

Presentations Related to Thesis

Invited Presentations

Mouse Xenograft Models in Onocoimmunology. Sydney Catalyst Immunology and Cancer Education Dinner, 2019

An Antibody Drug Conjugate Against Human CD300f Facilities Targeted Hemopoietic Stem Cell Conditioning in Acute Myeloid Leukaemia, DC Down Under Symposium, 2019

Oral Presentations

Abadir E, Silveira P, Gasiorowski R, Ramesh M, Romano A, Kabani K, Pietersz G, Larsen S, Clark G. *An Anti-CD300f Antibody Drug Conjugate Depletes Hematopoietic Stem Cells and Primary Acute Myeloid Leukemia (AML): Facilitating a Targeted Conditioning Regimen for Allogeneic Stem Cell Transplantation in AML*. The Combined Annual Meetings of the American Society for Transplantation and Cellular Therapy and the Center for International Blood & Marrow Transplant Research, 2020

Abadir E, Gasiorowski R, Silveira P, Ramesh M, Lo T, Romano A, Kupresanin F, Pietersz G, Sutherland S, Larsen S, Clark G**.** *An antibody drug conjugate against CD300f is promising for minimal toxicity conditioning in hemopoietic stem cell transplantation for acute myeloid leukaemia*. Blood Annual Scientific Meeting*,* 2019

Abadir E, Lee K, Li Z, Clarke C, Cooper W, Gasiororwski E, Ju X, Brown C, Bryant C, Hsu J, Fazekas de St Groth B, Hogarth M, Cunningham I, Clark G, Hart D. *CD83 is a promising biomarker and therapeutic target in Hodgkin Lymphoma.* University of Sydney Postgraduate and Early Career Researcher Cancer Research Symposium, 2017

Poster Presentations

Abadir E, Lee K, Li Z, Clarke C, Cooper W, Gasiororwski E, Ju X, Brown C, Bryant C, Hsu J, Fazekas de St Groth B, Hogarth M, Cunningham I, Clark G, Hart D. *CD83 is a leading therapeutic target in Hodgkin and mantle cell lymphoma*. Pathology Update, 2018

Abadir E, Lee K, Li Z, Clarke C, Cooper W, Gasiororwski E, Ju X, Brown C, Bryant C, Hsu J, Fazekas de St Groth B, Hogarth M, Cunningham I, Clark G, Hart D. *CD83 is a promising biomarker and therapeutic target in Hodgkin Lymphoma.* The Australasian Society of Immunology Annual Scientific Conference, 2017

Abstract

Background: Antibody based immunotherapies have revolutionised the treatment of haematological malignancies. Despite recent advances in Acute Myeloid Leukaemia (AML) most patients still have poor outcomes. Current surface targets in AML are not ideal and ongoing work is required to examine new antigens for meaningful clinical outcomes.

Hypothesis: CD302 and CD300f have inherent properties that make them promising potential targets in AML. Preclinical work will establish these antigens as suitable targets in AML for further study.

Methods: We looked at the distribution of CD302 on AML and Haematopoietic Stem and Progenitor Cells (HSPC) using flow cytometry from local patient cohorts and compared this data with AML gene expression profiling from public databases. The expression of CD302 from healthy organs was examined using PCR. The rate of internalisation of anti-CD302 antibodies was assessed using flow cytometry and fluorescent microscopy. The ability of unmodified antibodies to perform Antibody Dependant Cellular Cytotoxicity (ADCC) was examined. The impact of CD302 on AML cell migration was assessed using a transwell system. The influence of an anti-CD302 antibody upon AML cell line engraftment was tested *in vivo*.

CD300f on AML and HSPC was analysed using flow cytometry from local patient cohorts and we compared the data to gene expression profiling from public databases. The distribution of CD300f isoforms across AML and HSPC was assessed by PCR and confirmed with TCGA RNA sequencing data. Alterations in antibody binding epitopes using multiple CD300f antibodies was assessed in AML cell lines as well as primary AML and HSPC.

The cytotoxicity of an anti-CD300f based ADC was examined *in vitro* using cell lines. An anti-CD300f ADC was examined *in vitro* and *in vivo* against cell lines as well as primary AML and healthy HSPC. The rate of cytotoxicity and synergy of the ADC with Fludarabine was assessed *in vitro* with cell lines.

Results: In a cohort of 33 AML patients, 88% were found to express CD302 on the surface of blasts and 80% on the surface of CD34+ CD38- population. Expression of CD302 was found on the surface of HSPC. A monoclonal antibody (mAb) targeting human CD302 was effective in mediating ADCC and was internalised, making it amenable to toxin conjugation. Targeting CD302 with this antibody limited *in vivo* engraftment of the leukaemic cell line HL-60 in NOD/SCID mice. While CD302 was expressed in a hepatic cell line, HepG2, this molecule was not detected on the surface of HepG2, nor could HepG2 be killed using a CD302 antibody-drug conjugate.

CD300f antibodies bind to AML from 85% of patient samples. Transcriptomic analysis found that CD300f transcripts are expressed by healthy HSPC. Several CD300f protein isoforms exist as a result of alternative splicing. The extracellular region of CD300f can be present with or without the exon 4-encoded sequence. This results in CD300f isoforms that are differentially bound by CD300f-specific antibodies. Analysis of publicly available transcriptomic data indicated that CD34+ HSPC expressed fewer CD300f transcripts that expressed exon four compared to AML with monocytic differentiation. An anti-CD300f antibody, DCR-2, to CD300f exposes a structural epitope recognized by a second CD300f mAb, UP-D2. Analysis of a small cohort of AML cells revealed that this UP-D2 conformational binding site could be induced in cells from AML patients with monocytic differentiation but not those from other AML or HSPC.

CD300f is expressed evenly across HSPC subtypes. CD300f has equivalent transcription and protein expression as CD33 on AML. We have developed an anti-CD300f antibody which efficiently internalises into target cells and conjugated it with a PBD warhead that selectively depletes AML cell lines and AML Colony Forming Units (CFU) *in vitro*. CFU derived from healthy HSPC are depleted by the ADC. The ADC synergises with Fludarabine, which is often used in allogeneic Haematopoietic Stem Cell Transplant (allo-HSCT) conditioning. The ADC prolongs the survival of mice engrafted with human cell lines and depletes primary human AML engrafted with a single injection. In a humanised mouse model, a single injection of the ADC depletes CD34+ HSPC and CD34+ CD38- CD90+ HSC.

Conclusions: CD302 is a potential target in AML. The hepatic expression may limit potential therapeutics but this could be mitigated as hepatocytes appear to express CD302 predominantly intracellularly, further work is required in this field. CD302 is expressed on HSPC and any future therapeutics would likely need to be part of a conditioning strategy. CD300f is a more promising target given the lack of non-haematopoietic expression. Certain isoforms or epitopes may be targeted to generate selective binding to AML with monocytic differentiation and avoid HSPC. An anti-CD300f ADC has promise as a targeted conditioning agent that may deplete residual AML and facilitate allo-HSCT.

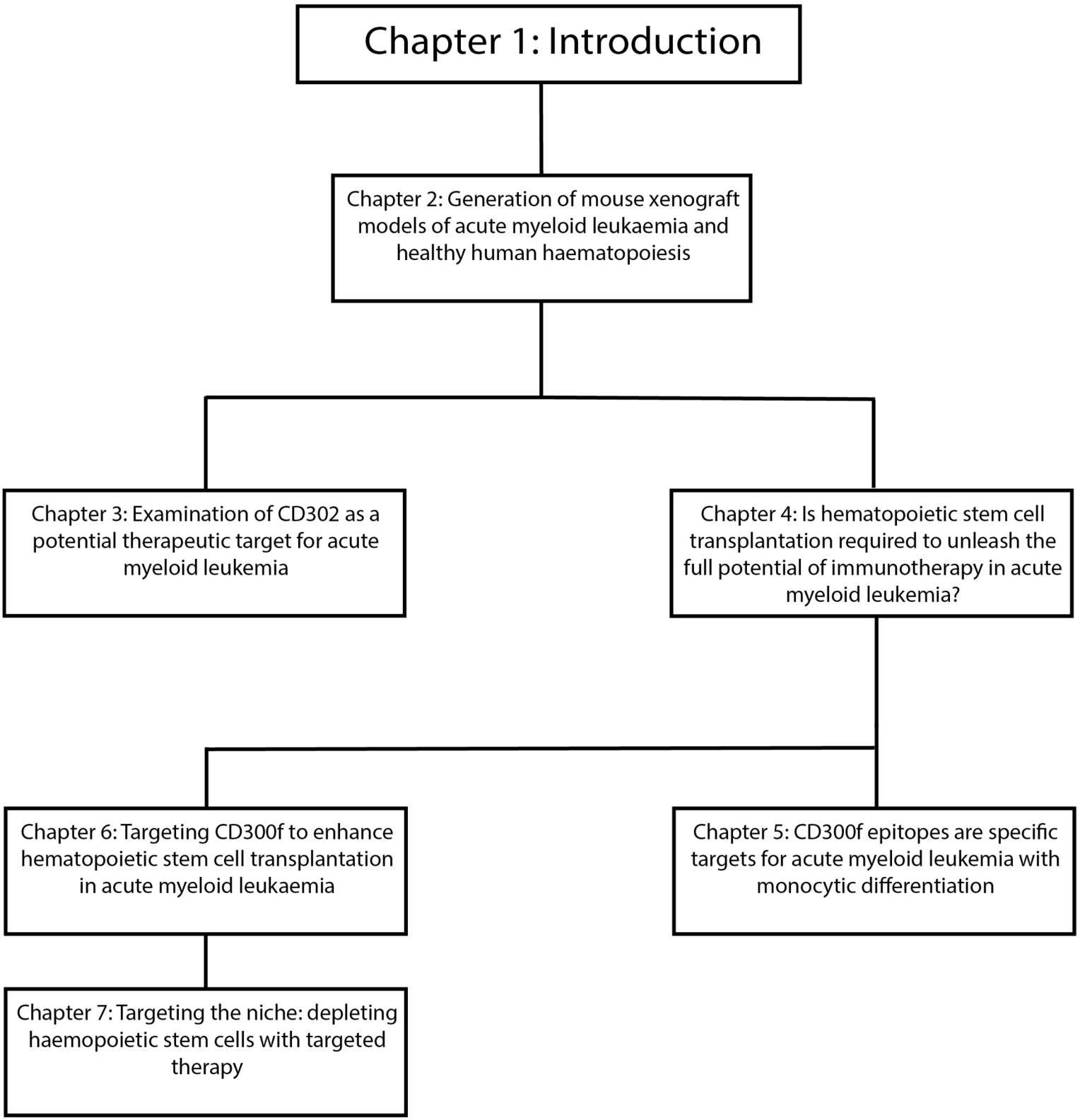
List of Abbreviations

ADC	Antibody drug conjugate
ADCC	Antibody dependent cell mediated cytotoxicity
ALL	Acute lymphoblastic leukaemia
allo-HSCT	Allogeneic haematopoietic stem cell transplant
AML	Acute myeloid leukaemia
BiTE	Bispecific T cell engager
BSA	Bovine serum albumin
CLR	C type lectin receptor
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CFU	Colony forming unit
CR	Complete remission
CRS	Cytokine release syndrome
CRi	Complete remission with incomplete count recovery
CRp	Complete remission with incomplete platelet recovery
DART	Dual affinity re-targeting
DCR	Dendritic Cell Research Group
GO	Gemtuzumab Ozogamicin
FLT3-ITD	Fms-like tyrosine kinase 3 internal tandem duplication
HL	Hodgkin lymphoma
HSC	Haematopoietic stem cells
HSPC	Haematopoietic stem and progenitor cells
LeY	Lewis Y antigen
LSC	Leukaemic stem cells
mAb	Monoclonal antibody
MAC	Myeloablative conditioning
MDS	Myelodysplastic syndrome
MDR	Multidrug resistant
MMAE	Monomethyl auristatin E

MRD	Minimal residual disease
NHL	Non-Hodgkin lymphoma
NHP	Non-human primate
NMA	Non Myeloablative conditioning
NPM1	Nucleophosmin 1
NOD/SCID	Non obese diabetic/Severe combined immunodeficiency
NSG	*NOD.Cg-Prkdc<scid> Il2rg<tm1Wjl>/SzJ* or NSG
NKG2D	Natural killer group 2D
ORR	Overall response rate
OS	Overall survival
PBD	Pyrrolobenzodiazepine dimer
PBS	Phosphate buffered saline
PDX	Patient derived xenografts
RIC	Reduced intensity conditioning
scFv	Single-chain variable fragment
SOS	Sinusoidal obstructive syndrome
TIM-3	T-cell immunoglobulin- and mucin-domain-containing molecule
VT	Vadastuximab Talirine or SGN-CD33A

Overview and Chapter Summaries

I joined the Dendritic Cell Research Group (DCR) in 2017, at the time led by Prof Derek Hart, to continue the group's study of novel of antigens as potential targets in Acute Myeloid Leukaemia (AML). The DCR had been exploring the antigens CD302 and CD300f in AML. The basic biology of CD302 was being developed by Drs Lo and Silveira within the group. Dr Gasiorowski had developed the anti-CD300f mAb, DCR-2, and characterised CD300f and CD302 expression in a local cohort of AML prior to departing the lab. Much of the following work happened concurrently. Throughout the project I continually developed and refined our mouse models, improving the repertoire of cell line models and developing primary models (Chapter 2). The first published material in this thesis (Chapter 3) examined the potential of CD302 as a target in AML, building off the work that Dr Lo had started. The expression of CD302 on Haematopoietic Stem and Progenitor Cells (HSPC) became apparent and we had to develop strategies to deal with this going forward. Chapter 4 reviews methods to either avoid depleting HSPC or to incorporate targeting them as part of allogeneic Haematopoietic Stem Cell Transplant (allo-HSCT) conditioning. During our examination of CD302 we decided future work may be better placed in CD300f as it has similar AML and HSPC expression profiles without the presence on non-haematopoietic cells. We developed this further to see if the isoforms of this molecule could be used to selectively target AML over HSPC. Chapter 5 studies CD300f isoforms and examines the differential expression which may facilitate selective depletion of AML with monocytic differentiation. As an alternative strategy we wanted to see if depleting CD300f+ cells eliminates AML but also facilitates conditioning for allo-HSCT. We explored this with an anti-CD300f Antibody Drug Conjugate (ADC) as a method to treat AML and deplete HSPC (Chapter 6). I concurrently summarised the literature on the new methodologies being employed as a part of targeted conditioning (Chapter 7).


Chapter 1: Introduction
Chapter 2: Generation of mouse xenograft models of acute myeloid leukaemia and healthy human haematopoiesis
Chapter 3: Examination of CD302 as a potential therapeutic target for acute myeloid leukemia
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Chapter 5: CD300f epitopes are specific targets for acute myeloid leukemia with monocytic differentiation
Chapter 7: Targeting the niche: depleting haemopoietic stem cells with targeted therapy

Chapter 2 outlines the methods and outcomes of the generation of new mouse models required to further the work. At the time of my arrival in the lab there was a working HL-60 systemic Non Obese Diabetic/Severe combined immunodeficiency (NOD/SCID) mouse model but we felt that this would be inadequate for our future experimental plans. I successfully developed subcutaneous and systemic AML cell line models. I concurrently developed primary AML models which could be moved forward into therapeutic testing. A robust healthy humanised mouse model was developed that would allow for the effect of therapeutics on normal human haematopoiesis to be further investigated.

Chapter 3 examines CD302 as a target in AML. CD302 is the simplest type I transmembrane C-type lectin receptor described. In human haematopoiesis CD302 is restricted to myeloid derived populations including monocytes, macrophages, dendritic cells, granulocytes and HSPC. Most cases of AML express CD302. Antibodies to CD302 were able to induce ADCC. AML cells coated with an antibody to CD302 displayed limited engraftment in mouse models. CD302 is expressed on the surface of haematopoietic cells and gets rapidly internalised upon antibody binding where it can deliver a toxin to AML cells. CD302 has high expression in hepatocytes, but appears to have a preferentially intracellular distribution, therefore limiting the toxicity of an ADC *in vitro*.

Chapter 4 reviews approaches for AML immunotherapy, either sparing HSPC or being used with allo-HSCT. The choice of AML target has proven to be complicated. No clear target has separated itself as ideal. As AML and HSPC share many surface antigens this would allow for potent anti-AML therapies as conditioning agents in addition to AML depletion. This strategy would require an allo-HSCT which would limit the ability of many patients to undergo this approach. Current preclinical and clinical studies are discussed in this chapter.

Chapter 5 explores a method of targeting AML with monocytic differentiation and sparing HSPC by targeting specific isoforms and conformations of CD300f. CD300f is a member of the CD300 immunoregulatory family and a transmembrane glycoprotein with both inhibitory motifs and PI3K phosphorylation sites in its cytoplasmic. Proteomic and transcriptomic analyses have shown that it is upregulated in AML samples. Its expression on HSPC had limited investigations prior to this work. One antibody suggested that it was not expressed on CD34+ CD38- primitive HSPC. Our work demonstrates CD300f expression across all major myeloid HSPC as well as most AML samples. CD300f has seven isoforms with two major extracellular configurations. There is differential binding of different antibodies to the isoforms. AML with monocytic differentiation has increased expression of the exon 4 extracellular region of CD300f compared to HSPC. The anti-CD300f mAb DCR-2 can alter the CD300f molecule for the preferential binding of another anti-CD300f mAb, UP-D2. UP-D2 binds to AML with monocytic differentiation significantly more than to HSPC in the presence of DCR-2. The difference in binding may allow for an immunological therapeutic window to target AML without depleting HSPC.

Chapter 6 takes a different approach to targeting CD300f. An antibody drug conjugate which binds to all extracellular CD300f isoforms was examined as a conditioning agent as well as anti-AML therapy. This work has shown that an anti-CD300f ADC can kill AML cells *in vitro* and *in vivo*. In addition, the ADC depletes HSPC both *in vitro* and *in vivo*. This ADC has a rapid onset of action and a synergistic effect with the commonly used conditioning agent Fludarabine. This approach has the potential to reduce the toxicity of conditioning by removing alkylating agents or radiation as well as the potential of increasing the efficacy with minimal toxicity.

Chapter 7 reviews work on immunologically targeted conditioning agents. This work is a natural extension from the concept of using anti AML therapies as part of HSCT conditioning. Embracing the

killing of HSPC may lead to improved developments in the field of transplantation outside of AML. HSCT is an inherently risky procedure, in part induced by the tissue damage of nonspecific conditioning agents. Replacing the agents with target therapies would allow for transplants in patients who would not normally be able to undergo the procedure as well as reducing morbidity in those that would normally proceed. This approach may make gene editing therapies in those with inherited disorders of haematopoiesis more appealing, especially in younger patients who would benefit most.

Appendices: I was originally invited to work with the DCR Laboratory by Prof Derek Hart while completing my training in haematology. This initial contribution was to be the clinical and pathology liaison in the CD83 project in Hodgkin lymphoma (HL). When I later joined the group, I switched my focus to AML. However I kept working on the CD83 projects in lymphoma while concurrently performing research in AML. The works in the appendix are my contribution to the understanding of CD83 in lymphoma. As I was not the primary author and they diverge from the main theme of the thesis I have placed them in the appendices.

Appendix A reviews the therapeutic potential of CD83 in human disease. CD83 plays a role in autoimmunity, HSCT and malignancy. These fields are being explored with either soluble CD83 or anti-CD83 antibodies. The review summarises the current understanding of CD83 and the potential clinical implications.

Appendix B examines the possibility of targeting CD83 in HL as a novel therapeutic. CD83 was expressed on most HL cells, in addition an excess of the soluble CD83 was found in nearly all HL cases and correlated with remission. A human anti-CD83 antibody was able to kill HL cell lines by ADCC or when bound to the toxin Monomethyl Auristatin E (MMAE) as an ADC. T cell division is impaired by sCD83 and the anti-CD83 antibody is able to overcome this effect. This work validates CD83 as a novel potential target in HL.

Appendix C examines CD83 as a target in MCL. MCL produces CD83 which is detectable in 50% of clinical cases. CD83 is expressed on the surface of MCL cell lines with upregulated canonical NF-κB pathways. An anti-CD83 ADC using MMAE was able to kill MCL cells *in vitro* and *in vivo*. The treatment of MCL with traditional chemotherapies including Cyclophosphamide and Doxorubicin was able to induce the NF-κB dependant expression of CD83 and synergise with the ADC. CD83 has been shown to be a promising target in MCL.

Chapter 1: Introduction

Immunotherapies have become the fourth pillar of oncology, joining chemotherapy, radiotherapy and surgery. Immune based strategies are now routinely incorporated into standard of care treatment of solid organ tumours and lymphoid malignancies.[1-3] The concept on immunotherapy for oncology began at the end of the 19th century where it was observed that infections could lead to the remission of malignancies.[4] Vaccination proved to be revolutionary in the early 1900s and the idea of a vaccine for cancer was developed by 1959.[5] Interferon use in chronic myeloid leukaemia was characterised by the 1980s.[6] These advances provided evidence that immunotherapy may have a role in malignancy, but targeted immunotherapy was brought upon by the understanding and use of antibodies.

Standard therapy for Acute Myeloid Leukaemia (AML) based on Cytarabine and anthracycline combinations have changed little over the previous three decades and only leads to a 20-30% long term disease free survival.[7] The use of allo-HSCT to improve disease free survival demonstrates the potential of immunotherapy in this disease.[8] Recent novel therapeutics including Chimeric Antigen Receptor (CAR) T cells and Bispecific T cell Engagers (BiTE) targeting CD19 have produced impressive results in ALL.[9, 10] As opposed to CD19 and CD20 for B cell malignancies no clear antigen presents itself as an ideal target in AML. This chapter will look at the development of AML immunotherapy to date, with a focus on agents that have made it to clinical trials or have provided scientific advancements in the field.

Antibody Based Therapy Target Selection

The ideal antigen on malignancies should have the following properties.[11, 12]

- Involved in the oncogenic process
- Expressed only on malignant cells
- Highly expressed on all malignant cells
- Expressed on cancer stem cells
- Expressed in high levels on all cases of the cancer
- Expressed on the cell surface with little circulating soluble antigen
- Evidence of therapeutic function in clinical trials

Meeting the requirements for all of these ideal characteristics is highly unlikely. Most successful cancer antigens in B cell malignancies are not specific for malignant cells, but are expressed across all B cells. As B cell aplasia is usually tolerable this does not significantly limit development of immunotherapy. Despite few targets meeting all ideal requirements these criteria are a useful list to guide cancer antigen discovery. The choice of immunotherapy construct impacts upon the choice of antigen as well. ADC require internalisation upon antigen binding, therefore certain antigens that would otherwise be ideal may not be suitable for ADC if they do not internalise.[13] Targets in AML are particularly difficult given their presence on HSPC, the inherent surface molecule heterogeneity of AML within and between cases as well as an unclear definition of leukaemic stem cells.[12]

Evolution of Treatment and Immunotherapy in Acute Myeloid Leukaemia

The first case of leukaemia was described by Velapeau in 1827, but the condition was only later characterised and named by Virchow and Bennett in 1845.[14] Virchow's initial postulation that leukaemia derived from blood cells was correct. Treatment regimens developed for leukaemia by the 1930s and some included arsenic and radiation among other therapies.[15] The French-American-British system of classification developed in 1976 played a key role in systematically identifying different types of leukaemia based on morphology. The use of antibodies to characterise the phenotype of leukaemia was developed in the following decade.[16] Allogeneic transplantation began in the 1970s and provided the first example of the potential of immunotherapy in acute leukaemia.[17]

Immunotherapy in AML has been limited to date. The initial studies using unconjugated antibodies against AML lacked efficacy.[18] Gemtuzumab Ozogamicin (GO) an ADC was given accelerated approved by the FDA in the year 2000 for the treatment of AML in patients over sixty with relapsed AML who were not candidates for chemotherapy.[19] GO was subsequently withdrawn from the market due to increased mortality and limited efficacy only to be approved again in 2017 by the FDA using a different dosing schedule.[19]

A major factor limiting antibody-based therapy in AML is the lack of a clear target which separates a malignant cell from a healthy haematopoietic stem and progenitor cells.[20] A number of strategies have been devised to validate truly leukaemic specific targets which are expressed frequently in AML or have looked to incorporate transplantation to alleviate haematological toxicity.[21] Further limitations in the development of AML immunotherapy relate to limits of preclinical models to represent a heterogeneous disease.[22] These factors have seen AML therapy significantly lag in the development of effective immunotherapies compared to other disorders such as B cell lymphoma.

Role of Leukaemic Stem Cells in AML

Any effective immunotherapy for AML would have to target Leukaemic Stem Cells (LSC). LSC are a small proportion of AML blasts but are critical for the continued propagation of AML even after effective therapy. Therapeutics derived from Monoclonal antibodies (mAb) have a high target specificity and it is critical that LSC are targeted for long term efficacy. The phenotypes of LSC are continuing to be understood and targeting a significant proportion of this heterogeneous population remains a major challenge for mAb based therapy. Leukaemic stem cells have traditionally been defined as leading to leukaemic engraftment in serial xenotransplantation experiments and giving rise to non LSC progeny.[23] Phenotypic and gene expression studies suggest a myeloid progenitor cell of origin for LSC rather than Haematopoietic Stem Cells (HSC).[24, 25] LSC have been shown to be resistant to traditional chemotherapy and primarily reside in the G0 phase.[25, 26] An intrinsic difficulty in targeting LSC with immunotherapy is their heterogeneity both between and within patients.[25] It has become clear that the bone marrow niche plays a key role in the support of LSC, limiting the effect of conventional cytotoxics but the impact of this environment in antibody based therapy is unclear.[27]

The first population of LSC described reside in the CD34+ CD38- AML population which can repopulate SCID mice.[28] It is now clear that LSC are found in other phenotypic fractions. Newer mouse models have been developed. The most extensively used strain in AML therapy development is the *NOD.Cg-Prkdc<scid> Il2rg<tm1Wjl>/SzJ* (NSG) in which the *IL2RA* gene has been knocked out of the NOD/SCID base to reduce the efficacy NK cell function.[29] NSG mice engraft human AML at a higher rate and populations of LSC that reside in the CD34+ CD38+ compartment are more readily

identified.[30] AML with nucleophosmin 1 (NPM1) mutations, which is often CD34-, has led to discovery of CD34- LSC.[31] The diversity of LSC phenotypes may further be underestimated due to the current models in use. It is likely that AML *in situ* has further populations of LSC that are not currently described as they cannot engraft current mouse models.

Antigen Targets in AML

A number of antigens have been studied as targets in AML (Table 1.1). The ever-expanding list of antigens being investigated demonstrates that no one ideal target has been found. CD33 is the best studied antigen in AML and the only one with an approved therapeutic directed against it, GO. The following is an examination of the preclinical and clinical data to date for each antigen to evaluate their potential as a target in AML. The latest stage of development for different immunotherapy modalities for each target is shown in Table 1.2

Table 1.1 Characteristics of Individual AML Antigens

Target	Expression on AML	Advantages	Disadvantages
CD33	88-99%	not expressed on non haematopoietic cells	HSPC expression low surface density
CD123	45-78%	IL-3 involved in AML proliferation present on LSC	HPSC expression possible expression on endothelial cells
CLL-1 (CD371)	92%	expression in most cases present on LSC	present on myeloid progenitors
CD47	>95%	expression on most cases increased potential for phagocytosis of AML blasts	most cases low expression present on erythrocytes
CD38	>95%	expression on most cases approved anti-CD38 antibodies	CD38- AML subset which is enriched for LSC expressed on myeloid progenitors
CD70	>95%	expression on most cases upregulated with hypomethylating agents	regulates lymphocyte and HSPC activity
CD133	69%		present on HSPC
CXCR4 (CD184)	67%	role in AML migration	present on HSPC
CD44	>95% (all variants included)	expression on most cases CD44 facilities proliferation and limits AML reduced	multiple isoforms
FLT3 (CD135)	>95%	expression in most cases	present on HSPC
IL1-RAP	79%		soluble form may occupy binding sites
LeY	46%	minimal expression in healthy tissue	carbohydrate antigen
NKG2D	75%	not expressed on HSPC	expressed on NK and CD8 T cell
TIM-3 (CD366)	>95%	may act as a checkpoint inhibitor	not expressed on HSPC
WT1	73%	limited expression on healthy tissue	intracellular target
CD300f	72-85%	not expressed on non haematopoietic cells	present on HSPC
CD302	82%		present on HSPC hepatic expression

CD33

CD33(siglec-3) was first discovered in 1983 on myeloid progenitors with monoclonal antibodies and eventually cloned in 1988.[32] It is a member of the sialic acid-binding immunoglobulin like lectin family. CD33 is a 67 KD protein with a structure that contains two Ig-like extracellular domains and two ITIM-like sequences in the cytoplasmic domain.[33] Alternate splicing produces a shorter isoform on the cell surface that lacks the V-set Ig like domain.[33] CD33 is an established myeloid marker and is expressed on myeloid committed cells but not found on HSC initially.[34] There has been reported expression of CD33 on some lymphocyte subsets but the lymphoid expression pattern is not completely understood.[33] CD33 is able to recruit the inhibitory tyrosine kinases SHP-1 and SHP-2 which have roles in development and differentiation.[35] Monoclonal antibodies to CD33 have been shown to trigger apoptosis in normal haematopoietic cells and leukaemic cells, as well as inhibit the differentiation of DCs from myeloid progenitors.[32]

CD33 is expressed on 88-99% of AML.[36, 37] A property of CD33 that has potential therapeutic implications is internalisation when bound by bivalent antibodies.[38] Internalisation allows for ADC to deliver their toxic payload. The AML samples of most patients contain at least a subset of cells that are CD33 positive.[37] Current evidence suggests that AML subtypes derived from more primitive progenitors such as the core binding factor leukaemias may be less likely to express CD33 on their LSC while some more mature LSC do express CD33, but the efficacy of GO in clinical trials of favourable AML calls this into question.[39] CD33 does not appear to be expressed outside of the haematological system.[40] It has been shown that CD33+ cord blood cells are able to engraft and generate multilineage haematopoiesis which does challenge the thought that HSC are primarily CD33-.[41] The lack of expression on HSC and non-haematopoietic organs suggested a reduced toxicity profile with limited off target effects.

Initial therapies targeting CD33 with unconjugated antibodies had limited clinical success. One concern is that the low surface density of CD33 may limit Antibody dependent cell mediated cytotoxicity (ADCC).[42] The murine anti-CD33 antibody M195 demonstrated effective ADCC with guinea pig and rabbit complement but limited activity with human complement or effector cells.[43] Clinical response was limited to a transient reduction in blast counts in a few patients despite adequate antibody saturation.[18] A humanised version of M195, Lintuzumab, was developed with a greater than eight fold increase in affinity for CD33 and demonstrated ADCC with human effector cells.[38] Lintuzumab delivered only very modest responses in AML during the initial trials despite antigen saturation throughout the four week trial period.[38] The limited clinical utility was later confirmed in two randomised trials, the first in salvage therapy and the second in older patients with newly diagnosed disease in combination with low dose Cytarabine.[44, 45] The limited efficacy of unconjugated antibodies has led to the development of other antibody based immunotherapies targeting CD33.

GO is a humanised IgG4 kappa antibody linked to an N-acetyl gamma calicheamicin dimethyl hydrazide toxin. Upon binding GO is internalised to mediate toxin delivery.[46] It is linked via a hydrolysable linker which allows for toxin release in acidic conditions, as found in lysosomes.[47] The antibody component of GO does not demonstrate ADCC.[48] The toxin binds to the minor DNA groove leading to strand breaks which causes cell cycle arrest and in some cases apoptosis via cytochrome c, BCL-2 family proteins and caspases.[42] The efficacy of GO has been variable in both AML cell lines and primary AML samples with some undergoing cell cycle arrest and or apoptosis but others have been unaffected.[49, 50] Initial pharmacokinetic studies demonstrated a maximum tolerated dose of 9mg/m2 with complete receptor saturation; this dose given every two weeks was carried forward with most of the subsequent studies.[51] Haematologic toxicity was a concern from the phase I trial with two

patients having >5 weeks of neutropenia. An open label multicentre single agent study of patients in first relapse demonstrated a Complete Remission (CR) of 16% and a Complete Remission with incomplete platelet recovery (CRp) of 13% for an Overall Response Rate (ORR) of 30%.[52] There was significant haematological toxicity with 97% and 99% of patients suffering from grade 3-4 neutropenia and thrombocytopenia respectively. In addition to the haematological toxicity, 23% of patients had grade 3-4 hyperbilirubinemia with a median duration of 20 days.[53] Phase III studies began on the efficacy Subsequent phase II trials demonstrated an ORR of 20-30% with some lower exceptions.[42] Four subsequent randomised studies showed little benefit with the addition of GO in newly diagnosed AML.[42] GO has demonstrated a significant effect against Acute Promyelocytic Leukaemia (APL) in several settings, presumably due to the increased density of CD33 in that disease.[54] Due to limited activity overall in AML and the toxicity demonstrated by increased deaths before day 30 in the SWOG 106 study GO was withdrawn from the market in June 2010.[54] A major toxicity concern was the development of hepatic Sinusoidal Obstructive Syndrome (SOS). The rate of SOS was low in the absence of all-HSCT but significantly increased the risk (64%) in those exposed to GO in the previous 3.5 months prior to transplantation.[55, 56] Despite its withdrawal from the market there remained ongoing interest in utilising GO in specific roles and questioning over whether its withdrawal was justified. A meta-analysis of four randomised trials totalling 3325 patients demonstrated both a progression free survival and overall survival advantage when GO was added to induction regimens for adults with favourable and intermediate cytogenetic risk AML.[57,58] The ALFA-0701 trial employed a fractionated lower dose regimen during induction (3mg/m^2 on days 1,4 and 7) and demonstrated a progression free survival advantage for GO addition to 7:3 induction but not an overall survival advantage.[59] The event free survival advantage was seen on favourable and intermediate but not adverse risk groups in subgroup analysis. Importantly there was no significant difference in the rates in SOS, possibly due to the lower dose and fractionated method of delivery. These updated findings led to the relisting of GO by the FDA in 2017 in addition to induction therapy for patients with AML and favourable or intermediate cytogenetic risk.

Other ADCs targeting CD33 have also been studied. AVE9633 is an anti CD33-maytansinoid conjugate developed by ImmunoGen Inc. The maytansine toxin is a potent tubular inhibitor that did not progress as a systemic agent due to significant toxicity as sub-therapeutic doses.[60] The maytansine derivative used in AVE9633 is DM4 and the conjugate demonstrated significant preclinical *in vitro* and *in vivo* activity.[61] The phase I trial demonstrated very limited clinical activity and the development of AVE9633 was terminated.[60]

Vadastuximab Talirine (VT) (SGN-33a) is a humanised murine anti-CD33 antibody that is linked to a Pyrrolobenzodiazepine Dimer (PBD) structurally related to anthramycin.[62] Each individual ADC contains 2 molecules of the PBD dimer that is released after internalisation and proteolytic cleavage of the linker.[63] PBD molecules cross link DNA and interrupt cell division leading to cell death.[64] The main advantages of VT over GO are the addition of cysteine residues that allow for uniform PBD loading and a highly stable dipeptide linker that ensure intracellular delivery of PBD molecules.[65] Preclinical studies demonstrated *in vitro* and *in vivo* activity in AML cell lines, including Multidrug Resistant (MDR) protein positive lines.[66] A phase I study of VT monotherapy demonstrated no significant non-haematologic toxicity with haematologic toxicity as the dose limiting factor.[67] Of patients evaluated for response 28% achieved a CR or Complete Remission with incomplete count recovery (Cri) with 50% of those demonstrating Minimal Residual Disease (MRD) negativity on flow cytometry. Another phase I trial exploring the toxicity of VT has been conducted with induction therapy consisting of 7 + 3 (Daunorubicin 60mg/m2 and Cytarabine 100mg/m2) in patients less than 65 years old. A maximum tolerated dose of 20mcg/kg on days 1 and 4 demonstrated grade four haematological toxicity in all patients and a median time of neutrophil recovery at day 33 and

platelet recovery at day 35.[68] The CR + CRi rate was 78% with more than half of those patients being MRD negative. VT has also been trialled in the consolidation and maintenance setting with those on consolidation having 5mcg/kg, 10mcg/kg or 20mcg/kg on day 1 in combination with high dose Cytarabine.[69] The maintenance cohort received single agent VT on day 1 at 5mcg/kg for up to 8 cycles (if no HCT) or four cycles post HSCT starting on day 60—100 in the absence of graft versus host disease. The consolidation cohort demonstrated 100% grade four haematologic toxicity and there were significant delays to subsequent cycles in 4/10 patients due to thrombocytopenia. The maintenance cohort had less haematologic toxicity with 41% and 36% having grade thee or four neutropenia and thrombocytopenia. The remission rate was 79% for the consolidation group and 75% in the maintenance group. Activity was demonstrated in high risk patients, those with adverse cytogenetics and precedent Myelodysplastic Syndrome (MDS), with an overall median relapse free survival of 9.1 months. This early phase data was promising and a global phase III study CASCADE (NCT02785900) utilising hypomethylating agents with or without VT was inititated.[70] This trial was stopped early by the data safety monitoring board due to an excess of deaths, including deaths from fatal infections, in the experimental arm. Given the distribution of CD33 on HSPC it is not surprising that myelosuppression and infections remained a significant limiting toxicity for VT.

AMG 330 is a CD33/CD3 BiTE currently under investigation. Preclinical *ex vivo* experiments with primary AML samples and healthy donor HSCs showed that AMG 330 inhibited AML growth and was not toxic to HSCs.[37] It has been demonstrated that upregulation of T cell inhibitory molecules can reduce the effectiveness of AMG 330 but also that cell lysis can be improved through the blockade of the PD-1/ PD-1L axis, suggesting a possible synergistic effect with check point inhibitors.[37, 71] There is a current phase I clinical trial (NCT02520427) for single agent AMG 330 in relapsed refractory AML. The initial presentation of the outcomes from this trial demonstrate serious adverse events in 66% of patients, including Cytokine Release Syndrome (CRS), cytopenias and infectious complications.[72] 2 of 35 patients achieved a CR. Another bispecific construct targeting CD33 is the bispecific (CD33/CD3) tandem antibody AMV-564. It has demonstrated preclinical activity against primary AML samples and has been chosen for a clinical trial (NCT03144245).[73] Preliminary data from this trial indicates that AMV-564 may be well tolerated with no grade 3-4 adverse events related to the study drug.[74] A reduction in bone marrow blasts was seen in 12 of 18 patients, with one achieving a partial response.

CD33 directed CAR T cells demonstrated cytotoxicity the AML cell line HL-60 and primary AML blasts.[75] A major concern is the possibility of prolonged haematological toxicity due to the long duration of CAR T cells. It has been shown that CD33 directed CAR T cells reduce myeloid progenitors and generate myeloid lineage cytopenia in murine models.[76] The generation of transient CAR T cells and CAR T cells that incorporate a suicide switch may circumvent long term haematological toxicity but none have been tried to date clinically.[77] An alternative method that has been proposed is to incorporate CD33 directed CAR T cells into an HSCT using HSC which have CD33 edited out of the genome.[78] This work demonstrated that CD33 is not required for the development or function of myeloid cells and can be removed from human HSC. While this strategy may be theoretically possible it would be complex and resource intensive. Despite the risk of haematologic toxicity there are currently several CD33 directed CAR T cell clinical trials that are planned or are recruiting (NCT02958397, NCT01864902). Anti-CD33 CAR T cells have been reinfused into a patient without preconditioning chemotherapy, the patient had fevers the day of the infusion and intermittently thereafter as well as a reduction in the neutrophil count.[79] There was a transient reduction in the blast count from 20% to <6% but the blast count later rose at three weeks.

CAR NK cells represent a new line of possible cellular therapy against malignancy. CD33 directed CAR NK cells with a CD28 and 41BB domain have been trailed in a phase I study.[80] Preliminary results in three patients with R/R AML underwent infusion of CAR NK cells after salvage chemotherapy. The infusions were tolerated in all patients, but subsequent relapse was seen in each case.

CD123

After CD33 the most studied target for antibody-based therapy in AML is CD123. CD123 is the Interleukin 3 alpha chain receptor and encoded by the gene *IL3RA*. IL-3 plays a key role in stimulating cell cycle progression and differentiation.[81] IL-3 induces tyrosine phosphorylation which activates the JAK/STAT pathway leading to target gene transcription.[82] Prevention of apoptosis is activated by the action of IL-3 by phosphorylation on pro apoptotic pathways.[83] CD123 is expressed on 45-78% of AML.[36] AML cells have increase DNA synthesis *in vitro* when exposed to IL-3, suggesting an important role in AML proliferation.[84, 85] CD123 is also of significant interest as it has been demonstrated to be to be present on a very high proportion of the CD34+ CD38- LSC with reduced expression on HSCs.[86-89] Despite this finding, when more potent immunotherapies are used such as CAR T cells, HSC and early progenitor toxicity is a major concern. It has been shown that CD123 directed CAR T cells are able to ablate normal human haematopoiesis in a Patient Derived Xenograft (PDX) NSG mouse model.[90]

The murine anti-human CD123 mAb 7G3 demonstrated the ability to reduce engraftment of primary AML in NOD/SCID mice with *ex vivo* treatment prior to engraftment, suggesting targeting of LSC.[91] While there were consistent reductions in the rate of primary and secondary engraftment after treatment by 7G3, a reduction in AML in xenotransplanted mice with established disease occurred in a minority of the cases (2/6).[91] CSL 360, a 7G3 derived recombinant IgG1 mAb, has been trailed in a phase I study that showed limited efficacy; one of 40 patients achieved a CR despite adequate antibody saturation.[92] Given the limited efficacy of CSL 360 an Fc receptor modified humanised anti CD123 antibody, CSL 362 (Talacotuzumab), has been developed. The Fc receptor has been modified to have high affinity to CD16, allowing for increased NK cell mediated ADCC.[93] Preclinical data demonstrated that CSL 362 was able to kill primary AML samples *in vitro* with autologous NK cells and *in vivo* in NOD/SCID mice with established disease.[94] A phase I single agent study in patients who are in CR with a high risk of relapse showed minimal toxicities with 50% of the patients remaining in CR.[95] Of the six patients who were MRD positive on enrolment three converted to MRD negative. Single agent CSL 362 demonstrated limited efficacy but significant toxicity, including infections and cytopenias, in an elderly cohort of patients who have failed hypomethylating agents.[96] A randomised controlled study of decitabine with or without CSL 362 for patients not fit for intensive chemotherapy was stopped early due to a lack of efficacy in the treatment arm.[97]

ADCs are currently being developed targeting CD123. SGN-CD123a is humanised anti-CD123 antibody that utilises a protease cleavable dipeptide linker to conjugate PBD dimers.[98] *In vitro* testing has demonstrated efficacy in 11/12 AML cell lines, including MDR positive ones, as well as 20/23 primary AML samples that ranged from favourable to adverse cytogenetics.[98] *In vivo* mice experiments showed that SGN-123a was able to reduce the burden of established AML in cell lines and primary AML.[99] A phase I study of SGN-123a has been terminated (NCT02848248) for yet unpublished reasons. SL-101 is a novel antibody-toxin conjugate that was in development. SL-101 consists of a scFv that is conjugated to a 38kd region of the Pseudomonas exotoxin A.[100] SL-101 was active against 10/12 cell lines, only HL-60 and U397, which are negative for the CD123 antigen showed resistance.[101] SL-101 also demonstrates an increased cytotoxicity in LSC compared to

leukaemic blasts, as well as increased inhibition of leukaemic colony formation compared to healthy bone marrow (70% vs 6%).[101] Exposing two primary AML samples to SL-101 *ex vivo* prior to transplantation in NSG mice did show an overall reduction in engraftment in the BM.[101]

Flotetuzumab (MDG006) is a Dual Affinity Re-Targeting (DART) scaffold comprised of mAbs to CD123 and CD3. It combines the VL of CD123 and VH of CD3 on one chain with the VL of CD3 and VH of CD123 on the other chain, these chains associate via charged residues that promote "diabody" type associations and are stabilised with a disulphide bond.[102] Flotetuzumab demonstrated efficacy in AML cell lines *in vitro* and *in vivo*. There was a transient decrease in red cell mass of Non-Human Primates (NHP) but no neutropenia or thrombocytopenia, a current phase I trial is recruiting (NCT02152956). The initial results of this trial demonstrate near universal CRS with grade three or higher adverse events in 13% of patients. The overall response rate was 22%.[103]

Anti-CD123 CAR T cells have proven to be effective at killing primary AML samples *in vitro* and *in vivo*.[90, 104] Despite CAR T cell therapy failing to impair colony formation in an *in vitro assay* it ablated normal haematopoiesis in a humanised mouse model.[89, 102] A different group developing CAR T cells targeting CD123 and CD33 have demonstrated reduced toxicity to human HSPCs with the anti-CD123 CAR T cells compared to the anti-CD33 CAR T cell in an NSG mice xenotransplantation model.[104] The conflicting results of toxicity to HSPCs by anti-CD123 CAR T cells suggest individual construct designs may play an important role in discerning AML from HSPC. There is concern for CD123 expression on endothelium and a patient infused with anti-CD123 CAR T cells died of CRS and capillary leak syndrome, though this was not reproduced in a small trial replication deficit anti-CD123 CAR T.[105] There are several current phase I clinical trials in anti-CD123 CAR T cells recruiting (NCT02159495, NCT03796390, NCT04010877). One trial requires a suitable allogeneic donor identified at screening to facilitate allo-HSCT if refractory cytopenias develop (NCT03766126).

CLL-1 (CD371)

C-type lectin like molecule-1 (CCL1/Clec12a/CD371) is a transmembrane protein that is expressed on CD34+ CD38+ and CD34+ CD33+ progenitors but not CD34+ CD38- stem cells.[106] CLL-1 is expressed on 75-92% of primary AML.[107, 108] CD34+CLL-1+ primary AML cells are able to engraft NOD/SCID mice, suggesting presence on LSC.[109] A bispecific antibody for CLL-1 and CD3 with a fully intact Fc region has been developed that is highly efficacious in killing CLL-1+ AML cell lines *in vivo*.[107] A major concern in this construct is the toxicity of targeting myeloid progenitors and CRS; the entire initial cohort of NHP administered a high affinity bispecific antibody were euthanised within 30 hours after developing vascular shock and fever, only lower affinity antibodies could subsequently be tolerated and these exhibited myeloid lineage reductions.[107] Another anti-CLL-01 BITE (MCLA-117) demonstrated effectiveness against AML cell lines and primary AML.[110] It did not reduce mature myeloid cells but did deplete myeloid progenitors. There is a current clinical trial recruiting using MCLA-117 monotherapy for patients with relapsed refractory AML (NCT03038230). An anti-CLL-1 ADC with a PBD toxin , CLT030, demonstrated effectiveness against AML cell lines and primary AML *in vitro* and *in vivo*.[111] CLT030 did not demonstrate toxicity against erythroid precursors but did demonstrate marked toxicity against granulocytic and monocytic precursors. Another anti-CLL-1 PBD based ADC, anti–CLL-1-ds-PBD, again demonstrated effectiveness against AML cell lines and primary AML *in vitro* and *in vivo*.[112] When trialled in NHP there was a transient reduction in granulocytes and monocytes but preservation of haemoglobin and platelets. Anti-CLL-1 CAR T cells have shown activity against AML and heterogeneous results against HSPC. The second generation anti-CLL-1 CAR T cell with the 41BB costimulatory endodomain, CLL-1.BBζ, depleted AML cell lines and primary AML

but was toxic to normal mature myeloid cells while sparing myeloid progenitors.[113] A third generation CAR T cell with both 41BB and CD28 regions was effective against AML cell lines and primary AML but eliminated myeloid progenitors, sparing HSCs.[114] Compound CD33/CLL-1 targeting CAR T cells are been trailed in a phase I study (NCT03795779). Case reports from this study demonstrate that the CAR T cells are effective in depleting AML leading to with bone marrow aplasia, necessitating the need for an allo-HSCT.[115]

CD47

CD47 is an immunoglobulin like molecule that is involved in neutrophil tracking, neuronal regeneration and T cell co-stimulation.[116] Another function is providing a "don't eat me" signal by acting a ligand for the signal regulatory protein α which inhibits phagocytosis by macrophages and some DC.[117] Expression of CD47 is found in almost all AML with 75% of cases having low scores and 25% having high scores by immunohistochemistry.[118] CD47 has been shown to upregulated on both LSC and HSC with overexpression in AML.[119] The distribution of CD47, including on erythrocytes, is concerning for potential haematologic toxicity. Anti-CD47 antibodies have been engineered with a competent Fc backbone and a silent Fc backbone to assess the importance of an active Fc mechanism in potential therapeutic activity.[120] Those antibodies with a competent Fc backbone had significant activity against both AML cell lines and primary AML *in vitro* and *in vivo.* Significant toxicity was seen in NHP studies where the Fc competent antibodies led to a 40% decrease in red cell mass while the Fc silent antibodies had a minimal effect. These findings suggest that while and anti-CD47 antibody may have activity against AML, the activity may not be able to be decoupled from erythroid toxicity. Hu5F9-G4 is a humanised anti CD47 antibody that has demonstrated efficacy in preclinical experiments in NHL and paediatric brain tumours.[121, 122] Initial reports from a phase Ib trial of single agent Hu5F9-G4 in newly diagnosed patients with AML or MDS (NCT02678338) described anaemia and thrombocytopenia being the most frequent toxicities and a CR/CRi rate of 53% [123]

CD38

CD38 is a single chain type two transmembrane ectoenzyme that functions as a dimer.[124] CD38 is a multifunctional protein that contributes to cell proliferation, differentiation, chemotaxis and T cell activation.[125] CD38 was originally defined as a T cell activation molecule but has since been shown to be independent of cell lineage.[126] It is expressed on >95% of AML.[127] Daratumumab is a human antibody against CD38 that can induce cell destruction by ADCC, CDC, antibody dependant phagocytosis and apoptosis. After phase III trials demonstrated efficacy in relapsed multiple myeloma it now has an established role in the treatment of that disease.[128, 129] Daratumumab was effective in depleting primary AML implanted into calcium phosphate scaffolds coated with human BM mesenchymal stromal cells that were imbedded in immunodeficient mice.[127] In addition to efficacy as a single agent Daratumumab has been proposed to be used in combination with all-trans retinoic acid as it has been shown to increase CD38 expression in AML, which may overcome the lack of expression the CD34+ CD38- populations enriched with LSC.[130] The lack of CD38 expression on the initially described and best studied LSC population appears to limit its role as a target for antigen specific therapy in AML. A phase II open label clinical trial of Daratumumab in patients with relapsed or refractory AML is currently recruiting (NCT03067571).

CD70

CD70 is the ligand of CD27 and this interaction helps regulate lymphocyte and HSPC activity.[131] CD70 has minimal expression on healthy HSPC and is expressed on 97% of in AML as well as being upregulated in other malignancies [132, 133]. The blockade of CD70 with a monoclonal antibody inhibits self-renewal of AML and LSC while extending survival in a xenograft model of primary AML.[134] Hypomethylating agents have been shown to induce CD70 expression further on AML, thus suggesting synergistic potential with CD70 antibodies, and a phase II trial using this combination in previously untreated AML is underway (NCT04023526) [135]. What remains unclear is if blocking the CD70/CD27 axis will affect haematopoiesis even if the HSPC themselves do not express CD70.

CD133

CD133 is a 5-transmembrane molecule that is present on 30-70% of human CD34+ BM cells.[136] CD34+ CD133+ cells are capable of sustaining haematopoiesis while CD34+ CD133- are not.[136] CD133+ correlates with the presence of CD34+, with 69% of CD34+ AMLs expressing CD133. The presence of CD133 made no significant difference in the ability for primary AML cells to engraft NOD/SCID mice.[136] 293C3-SDIE is a chimeric antibody to CD133 that has been optimised to bind with high affinity to CD16. A single preclinical trial has shown an increase in NK activation (increased CD69 and CD107a expression) with 293C3-SDIE in the presence of primary AML *in vitro*, but *in vivo* cytotoxicity was not significantly increased.[137] While 293C3-SDIE does bind CD34+ HSC there was no increase in NK cell activation markers. PDX experiments from a single donor with AML plus healthy donor NK cells demonstrated that 293C3-SDIE could prevent AML from reaching a detectable level despite a 6-month incubation. The requirement of healthy effector cells would likely make 293C3-SDIE most promising as a consolidative therapy, perhaps in the setting of MRD positivity.

CXCR4 (CD184)

Chemokine C-X-C motif receptor 4 (CXCR4) is a chemokine G protein coupled receptor, that with its ligand chemokine C-X-C motif ligand 12 (CXCL12), mediates migration to bone marrow, cell adhesion, migration and invasion in haematopoietic cells.[138] CXCR4 is expressed on haematopoietic progenitor cells and variably on 67% of AML blasts and facilitates migration.[139] PF-06747143 is a humanised anti CXCR4 antibody that was able to induce rapid mobilisation of PDX AML cells as well as prolong survival mice engrafted by AML cell lines.[140] Ulocuplumab (BMS-936564/MDX-1338) is a human anti CXCR4 antibody that blocks the binding of CXCL12, preclinical data shows activity via apoptosis in AML both *in vitro* and *in vivo*.[141] Ulocuplumab has completed a phase I trial in relapsed/refractory AML in combination with Mitoxantrone, Etoposide and Cytarabine chemotherapy or as a single agent. There was no dose limiting toxicities and only mild/moderate thrombocytopenia occurred in monotherapy, the overall CR/CRi rate in the combination group was 51% which is similar to historical comparisons.[142] In the Ulocuplumab monotherapy efficacy was limited with 4/30 patients achieved a CR/CRi.

CD44

CD44 is a transmembrane glycoprotein expressed on AML and healthy cells, it is extensively alternatively spliced leading to multiple variants. Almost all AML significantly express some of the

CD44 variants, ranging from a maximum of 67% of exon v4 to a minimum of 0% of exon v10 in a series of 27 cases.[143, 144] CD44 is involved in cell proliferation, maturation and bone marrow homing.[143] Pan CD44 has increased expression on AML compared to CD34+ CD38- cord blood cells, and blocking CD44 with a mAb reduces bone marrow engraftment of AML in a mouse *in vivo* model with no significant change on cord blood cells.[145] A functionally active mAb to CD44 has been shown to promote AML differentiation and inhibit the PI3K and AKT pathways that lead to proliferation.[146] The increased expression on AML and key biological functions make CD44 a promising molecule for further investigation, though the large number of isoforms make epitope selection of potential mAb therapies critical.

FLT3 (CD135)

The Fms-Like Tyrosine kinase 3 (FLT3) receptor is a tyrosine kinase receptor. It and its ligand play an important part in the malignant transformation of primitive haematopoietic cells. FLT3 is present in nearly all AML and FLT3-internal tandem duplication (ITD) as well as tyrosine kinase domain mutations have recently been targetable with small molecule inhibitors.[147] A randomised study showed a PFS and OS advantage with Midostaurin for patients with FLT-ITD positive AML.[148] IMC-EB10 is a neutralising antibody that is binds to FLT3 and initiates ADCC on FLT3 expressing cells.[149] It was able to reduce engraftment and prolong survival of NOD/SCID mice inoculated with the AML cell line MOLM14 while sparing healthy human haematopoiesis in CD34+ xenografted mice. A phase I trial of IMC-EB10 (NCT00887926) in relapsed or refractory AML was terminated for lack of efficacy. 4G8SDIEM is an Fc optimised antibody against FLT3 and has demonstrated ADCC against AML cell lines and primary AML samples, no cytotoxicity was demonstrated against haematopoietic cell precursors.[150]

IL-1RAP

Interleukin 1 receptor accessory protein (IL1RAP/IL1R13) is a co receptor for IL-1 and required for its function.[151] IL1RAP has high or intermediate expression on 79% of AML and an anti-IL1RAP chimeric antibody, 81.2, demonstrated ADCC against CD34+ CD38- AML cells. 81.2 prevented engraftment of AML in immunodeficient mice.[152] 81.2 increased the overall survival of PDX NOD/SCID mice as well as reducing leukaemic burden.[153] A concern with utilising IL1RAP is that there is significant secretion of a soluble form which may occupy mAb binding sites.[154]

LeY

The Lewis Y antigen (LeY) is a difucosylated carbohydrate antigen that under physiologic conditions is primarily expressed during embryogenesis and has limited expression on normal tissue.[155, 156] LeY is expressed on many tumour associated antigens and was present on 46% of primary AML.[157, 158] Second generation CAR T cells have been developed from a Single-Chain Variable Fragment (scFv) of a humanised anti-LeY antibody. These CAR T cells could induce cell lysis in cell lines expressing LeY and not significantly affect normal cells with low LeY expression. OS was prolonged in mice engrafted with human ovarian cancer exposed to the anti-LeY CAR T cells.[159] A Phase I study of five patients was conducted, four of the patients received CAR T cells, three with cytogenetic MRD and one in relapse. The relapsed patient had a transient reduction in blasts but then progressed, all three of the patients in CR progressed with the final patient relapsing at 23 months post infusion.[160] LeY

expression was maintained on patients at the time of progression suggesting loss of antigen was not the cause of treatment failure. There is currently a recruiting clinical trial for anti LeY CAR T cells in myeloid malignancies (NCT02958384). A potential limiting factor in the development of anti-LeY immunotherapy is that high affinity antibodies against carbohydrate antigens can be especially difficult to develop.[161]

NKG2D

Natural Killer Group 2D (NKG2D) is a surface receptor that is expressed normally on NK and $CD8^+$ T cells, but is also expressed on stressed and malignant cells, including the majority of AML.[162, 163] Its limited expression on resting healthy tissues makes it an attractive target. There is currently a phase I/II trial (NCT03018405) with anti- NKG2D CAR T cells recruiting. A case report from this trial of a patient with who had relapsed $FLT3^+$ AML demonstrates initial but transient activity.[164] The patient achieved a complete morphological response but had evidence of clonal evolution with a new *IDH2* mutation. Despite this finding, he had normal trilineage haematopoiesis, providing some evidence to the limited NKG2D expression on HSPC. He subsequently underwent allo-HSCT leading to CR with normal molecular studies. It is unclear if AML subclones may have variable NKG2D expression.

TIM-3 (CD366)

T-cell Immunoglobulin- and Mucin-domain-containing molecule (TIM-3) is a type 1 membrane protein with an immunoglobulin variable like region that is expressed on differentiated TH1 cells and inhibits inflammation.[165] TIM-3 is normally expressed on Th1 and Th17 T cells and acts as an immunoregulatory checkpoint.[166, 167] It is also expressed on the majority of AML blasts but not on HSC or the majority of progenitors.[168] An anti-TIM-3 mAb was able to prevent primary and secondary engraftment of mice in a PDX model.[168] The combination of targeting AML cell lines directly via ADCC and possibly acting as an immune checkpoint receptor makes TIM-3 an attractive target in AML. There is a current clinical trial for an anti-TIM-3 mAb in combination with a PD-1 inhibitor for patients with AML or high risk MDS (NCT03066648). [169] A dual target CAR T cell has been developed that selectively kills targets expressing both CD13 and TIM-3.[170] This strategy was effective *in vitro* and *in vivo* against AML while only marginally depleting HSPC.

WT1

The Wilms Tumour 1 gene is critical for renal tract development but is also overexpressed in many malignancies including AML.[171] Specific *WT-1* RNA transcripts can be found in up to 73% of AML patients.[172] Numerous vaccination studies using *WT-1* peptides have been carried out in AML, together these studies suggest a modest effect.[173] The WT-1 protein is intracellular and therefore not conventionally targetable by antibody derived immunotherapies. One way to target this antigen targeting a peptide component bound to a HLA class one molecule, and one group has developed T cells with an engineered T cell receptor that target the WT1/HLA-A*02:01complex.[174] While the preclinical results are promising this therapy is restricted only to individuals with HLA-A*02:01 expression. A recent clinical trial of engineered T cells against the WT1/HLA-A*02:01 complex has been completed (NCT02550535) with results pending.

Novel Antigens Examined in This Thesis

CD300F

CD300F (IREM-1) is a type 1 transmembrane protein with an extracellular region containing an Ig domain and an intracellular domain containing three tyrosine signalling motifs.[175] Its expressed in myeloid cells with high expression monocytes and dendritic cells with lower expression on granulocytes.[176] CD300f has a number of isoforms which bind with varying specificities to different antibodies.[177, 178] The understanding of CD300f's role is expanding. In murine mast cells (where it is also known as LMIR3) it is a receptor for ceramide, a key regulator of inflammation, and acts in an inhibitory manner to suppress cytokine production and degranulation.[179, 180] CD300f presence on mast cells reduces neutrophil migration to the peritoneal cavity in mice post caecal puncture, thus providing further evidence of an inhibitory role in the innate immune system.[181] The role of CD300f on neutrophils has been further characterised, showing it to be a negative regulator of response to sepsis, by reducing their response to *pseudomonas aeruginosa*.[182] Alternatively CD300f also recognises phosphatidylserine on apoptotic cells and mediates their clearance by phagocytosis.[183] CD300f as more recently been associated in function of the nervous system through its presence on macrophages and microglial cells. Schwann cell CD300f is associated with the number of macrophage present post peripheral neve injury as well as the rate of nerve recovery.[184] A single nucleotide polymorphism found in CD300f is associated with major depressive disorder in women, in addition CD300f deficient female mice demonstrated depressive behaviours, suggesting that CD300f is critical to microglial fitness.[185]

CD300f is expressed in AML but its biological role in that context has yet to be determined. CD300f was detected on 72% of primary AML (38/52) and 54% (7/13) of blasts in myelodysplastic syndrome.[176] CD300f was also present on 54% (13/24) of CD34+ CD38- AML fraction. There was no reported expression on CD34+ CD38- HSC from healthy BM.[176] mAb targeting CD300F are internalised within two hours of binding allowing for potential development of ADC therapeutics.[176] Unconjugated mAb demonstrated ADCC and complement dependant cytotoxicity (CDC) *in vitro* as well reducing the amount of established disease in both a cell line xenotransplantation and PDX model.[176] CD300f has a number of isoforms and specific isoforms have been explored as specific targets in AML. The exon four encoded region is expressed extracellularly at significantly higher levels on AML with monocytic differentiation compared to HSPC.[186] A specific conformational change could also be induced with an anti-CD300f antibody, DCR-2, which allows for increased binding of another anti-CD300f antibody in AML with monocytic differentiation compared to HSPC. These findings may allow for the development of potential therapeutics which are effective against AML with monocytic differentiation but lack the haematopoietic toxicity seen in many AML antibody-based therapies. An alternative approach was to use an anti-CD300f ADC a conditioning agent.[187] The ADC can kill AML and HSPC *in vitro* and *in vivo*, demonstrating potential as a future conditioning agent.

CD302

CD302 (also known as DEC 205 associated c-type lectin 1 (DCL1) or Clec13A is a c type lectin receptor (CLR) originally identified from the Hodgkin lymphoma cell line L428.[188] CD302 is the simplest type-1 CLR and the highest expression of transcripts are found in the liver, followed by the lungs, PBMC and the spleen.[188] The ligand and a definitive function are yet to be discovered but the co-localisation of

CD302 on migratory structures (f-actin rich filopodia, lamellopodia and podosomes) in Mφ and transfected cell lines suggests a role in migration.[189, 190] Within the haematopoietic system CD302 is expressed on myeloid cells, primarily Mφ, mDC and granulocytes. CD302 is present on 88% of AML blasts and 80% of the CD34+ CD38- fraction.[191] We demonstrated that an anti-human CD302 antibody has significant ADCC in the AML cell line HL-60. Hepatic transcript expression is a concern but functional and staining studies suggested significantly extracellular expression on hepatic compared to AML cell lines. CD302 is expressed on BM and cord blood HSPC with a similar distribution of CD33.

Table 1.2 AML Targets and Current Stage of Development					
Target	Naked Antibodies	Antibody Drug Conjugates	BiTEs/DARTs	CAR T Cells	Engineered T Cells
CD33	Clinical Trials Completed	Approved (GO)	Clinical Trials Recruiting	Clinical Trials Recruiting	
CD123	Clinical Trials Completed	Clinical Trials Recruiting	Preclinical Data	Clinical Trials Recruiting	
CLL-1 (CD371)		Preclinical Data	Preclinical Data	Clinical Trials Recruiting	
CD47	Clinical Trials Completed				
CD38	Clinical Trials Recruiting				
CD70	Clinical Trials Recruiting				
CD133	Preclinical Data				
CXCR4 (CD184)	Clinical Trials Completed				
CD44	Clinical Trials Completed				
FLT3 (CD135)	Clinical Trials Completed				
IL1-RAP	Preclinical Data				
LeY				Clinical Trials Completed	
NKG2D	Clinical Trials Recruiting				
TIM-3 (CD366)	Clinical Trials Recruiting				
WT1					Clinical Trials Completed
CD300f	Preclinical Data	Preclinical Data			
CD302	Preclinical Data				

Conclusions

Immunotherapy has significantly advanced in the treatment of B cell malignancies but less so in AML. The target selection in AML may have to be less stringent given limited leukaemia specific markers. The prevalence of so many AML targets with none coming to the fore suggests that no antigen studied has all the ideal characteristics in a tumour target. The development of AML immunotherapy may not only depend on searching for new targets but using the current ones in different ways compared to those in B cell malignancies.

AML has several unique challenges compared to B cell malignancies when developing surface molecule-based therapeutics. The population of patients and the severity of the disease is a principle challenge in the development of any AML treatment. Most patients are older and may have a compromised immune system at the time of diagnosis. The speed of onset from the disease often does not allow for screening periods employed by many clinical trials.

A principle difficulty in selecting an AML antigen is the presence or absence on normal HSPC. The implications of this are discussed extensively in Chapter 4. Another concern is if the paradigm of single antigen targeting is feasible in AML given the inherent plasticity and sub-clonal escape causing relapse.[192, 193] Requiring multiple targets for cytotoxicity may reduce off target effects, as with anti-TIM-3 and CD13 CAR T cells, but this will likely exacerbate the problem of antigen escape.[170]

Even if an ideal antigen target was found, the ideal effector remains unclear. GO has demonstrated the potential of ADC but has limited overall effect size and small therapeutic window. VT confirmed that judging the therapeutic window with ADC will be difficult in AML given the excess mortality in the treatment arm of the CASCADE study. Multiple clinical trials of unconjugated antibodies and BiTEs have now failed to show a strong efficacy signal. CAR T cells and other cellular therapies may hold promise but to date there has not been a strong clinical signal of efficacy. The improved outcomes of patients with intermediate to high risk AML with allo-HSCT reminds us that immunotherapy can be effective in AML and refining a treatment beyond transplant is worthy of ongoing study.

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Chapter 2: Generation of mouse xenograft models of acute myeloid leukaemia and healthy human haematopoiesis

Introduction

Mouse models using human xenografts are critical for the evaluation of anti-AML therapeutics *in vivo*. The advent of immune deficient mice led to the first reports of serial engraftment of human AML xenografts into NOD/SCID mice.[1] AML with myelomonocytic differentiation best engrafted mice and cells from the CD34+ CD38- fraction, termed leukaemia initiating cells or leukaemia stem cells (LSC), led to the highest percentage of engraftment.[1] A greater degree of immunosuppression was achieved from the NOD/SCID parent with the deletion of the interleukin-2 receptor gamma chain, termed *NOD.Cg-Prkdc<scid>Il2rg<tm1Wjl>/SzJ or NOD/SCIDγ* (NSG) mice, which lack NK cells and allow for increased engraftment of leukaemic cells.[2] CD34+ CD38- LSC were especially of interest as they are noted to be quiescent and chemotherapy resistant in NSG mouse models.[3] It is now apparent that LSC originate outside of the CD34+ fraction, especially in AML where the majority of cells are CD34-.[4] The limited ability of cells outside of the CD34+ CD38- fraction to engraft mice may be an inherent deficiency in xenograft models as opposed to a true representation of AML biology. While transgenic mouse models of AML are useful in the understanding of AML biology, they are less useful tools in the testing and development of AML therapeutics, necessitating the need for xenografts.

Humanised mouse models have developed in parallel to AML xenograft models. The first experiments to successfully engraft human HSPC used SCID mice.[5] As with AML xenografts, the field of humanised mouse models benefited greatly from the generation of the NSG strain which allows for increased engraftment and lineage development.[6] Further enhancement of human innate immune cells can be accomplished by incorporating human cytokine genes into the NSG parent.[7] Another method to humanise mice is to inject humanised ossicles into the subcutaneous space of NSG mice which can act as the human bone marrow niche.[8] Each model has strengths and weaknesses and should be chosen based on the experimental question.

AML xenografts are required to assess potential efficacy of therapeutics while humanised mouse models can predict toxicity. More recently humanised mouse models have been used to assess the potential for antibody based allo-HSCT conditioning approaches in diseases such as MDS.[9] We therefore developed the following models to assess the potential of CD300f based ADC. This chapter reviews the development of the mouse models required for this work. A schema of the models used is presented in Figure 2.1.

Figure 2.1

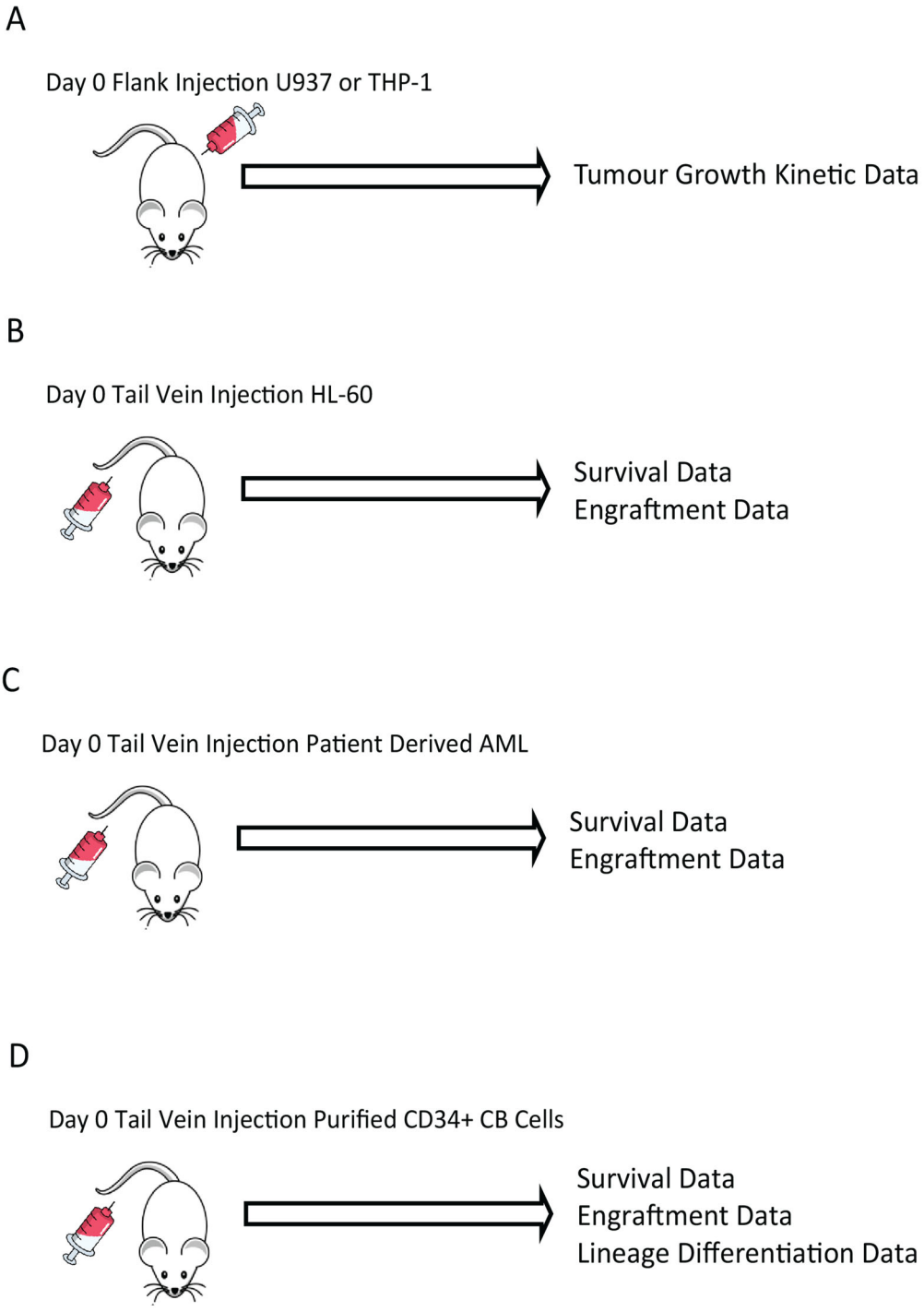

Figure 2.1 Mouse xenograft model overview. Mouse xenograft models developed include (A) subcutaneous cell line, (B) systemic HL-60 cell line, (C) systemic patient derived AML and (D) healthy humanised mouse model.

Methods

Human Samples: The AML cell lines HL-60 and THP-1 were obtained by Derek Hart at the Christchurch School of Medicine, University of Otago. The U973 cell line was obtained from the American Type Culture Collection. All cell lines were maintained in complete RPMI 1640 (complete RPMI) containing 10% fetal calf serum, 2 mM Gluta-MAX, 100 U/mL penicillin, and 100 μg/mL streptomycin (ThermoFisher). Blood from patients with AML were collected at Concord Repatriation General Hospital (CRGH) or Royal Prince Alfred Hospital both in Sydney, Australia. Clinical and pathological characteristics, including karyotype, NPM1 and FLT3 internal tandem duplication (FLT3-ITD) mutations were recorded. Cord blood (CB) samples were collected by the Sydney Cord Blood Bank. Mononuclear cells were isolated from samples using density gradient centrifugation through Ficoll-Paque Plus (GE Healthcare) according to the manufacturer's protocols from all primary samples. Donors provided informed consent under ethics protocols approved by the Sydney Local Health District Human Research Ethics Committee (HREC/12/CRGH/59, HREC/11/CRGH/61 and 118).

Mice: NSG mice (Australian BioResources) were housed in micro-isolator cages under specific-pathogen-free conditions. All xenograft experiments used NSG or NOD/SCID mice (Animal Resource Centre). Animal experiments were performed under the ethics protocol number, 2018/025, approved by the Sydney Local Health District Animal Welfare Committee. All animals were allowed a minimum of 1 week without intervention following importation to the experimental facility. Mice were monitored weekly unless post intervention (irradiation, inoculation with human derived cells or administration of treatment) after which they would be monitored daily for a minimum of 14 days. Monitoring was carried out by assessing the animal weight, and clinical score (fur texture, posture and activity). If a total score of >4 was met at any point during the experiments the animals were euthanised.

Subcutaneous Cell Line Models: NSG mice had their flank shaved on Day -1. On Day 0 they were injected subcutaneously with AML cell lines, either U937 or THP-1. Cell dose was $1x10^6$, $2.5x10^6$ or $5x10^6$. Subcutaneous tumours were measured with electronic callipers. Weights, clinical scores and tumour volume were measured daily from Day -1 onwards. Mice were euthanised when the tumours reached a size of >1000mm^3 or the clinical score was >4. Tumours were analysed by flow cytometry to confirm establishment of AML cell lines.

Intravenous HL-60 Model: NSG mice were irradiated (200cGy) on Day -1. They were injected with $5x10^6$ HL-60 cells via the tail vein on Day 0. Clinical scores were monitored from Day 0 onwards and mice were euthanised if the score was >4. Bone marrow, peripheral blood and spleen were collected for engraftment analysis. A concurrent cohort of NOD/SCID mice were treated identically to compare engraftment with NSG mice.

Primary AML Model: All samples chosen were from peripheral blood with greater than 90% AML blasts. Mice were irradiated (150cGy) on Day -1. They were injected with a set amount of primary AML cells per donor via the tail vein on Day 0. Clinical scores were monitored from Day 0 onwards and mice were euthanised if the score was >4. Peripheral blood collected from the tail vein on Day +40, +56, and +70. All remaining mice were euthanised on Day +90 where bone marrow, peripheral blood and spleen were collected for engraftment analysis.

Healthy Humanised Mouse Model: CD34+ cells were isolated from CB by positive selection (Miltenyi 130-046-702) using an AutoMACS Pro (Miltenyi Biotec) according to the manufacturer's instructions under sterile conditions. Purity of CD34+ cells was >90% in all experiments. Mice were irradiated (150cGy) on Day 0 and injected with either 50,000 or 100,000 cells four hours after

irradiation. Clinical scores were monitored from Day 0 onwards and mice were euthanised if the score was >4. Analysis of engraftment by peripheral blood from the tail vein was performed on weeks 8 and 12. Mice were euthanised on Week 12 where bone marrow, peripheral blood and spleen were collected for engraftment analysis.

Flow Cytometry: Blood samples from mice were resuspended in Phosphate Buffered Saline (PBS) containing 1% Bovine Serum Albumin (BSA), prior to red cell lysis. Cells from AML xenografts were mechanically dissociated from tumour, bone marrow or spleen prior to suspension in PBS containing 1% BSA, prior to red cell lysis. Antibody staining for flow cytometry and subsequent analysis was performed in PBS containing 1% BSA. Flow cytometry was performed on an Accuri flow cytometer (BD Biosciences) for AML xenografts. Analysis of healthy humanised mice was performed on a Fortessa LSR flow cytometer (BD Biosciences).

Antibodies: The following panel was used for the AML cell line and primary xenografts; anti-human CD45-AF488 (HI30), CD33- PE (WM53), and anti-mouse CD45-PerCP Cy5.5 (30-F11) (all form BD Biosciences). Isotype-PE (CMRF-81) and CD300f-PE (DCR-2) were both made in house. The following antibodies were used for the healthy humanised mice; CD3-AF700 (UCHT1), CD19-V450 (HIB19), CD56-PeCy7 (NCAM16.2), CD45-AF488, CD34-PE-CF594 (581), CD33-PE, CD38-APC (HIT2), CD90-AF700 (5E10) and anti-mouse CD45-PerCP Cy5.5 (all from BD Biosciences). CD34+ CB purity was confirmed with CD45-AF488 and CD34-PE-CF594.

Statistical Analysis: Between group analysis was compared using T tests using Graphpad Prism (GraphPad Software, Inc). Error bars represent standard error of the mean.

Results

Subcutaneous Cell Line Models: Both THP-1 (Figure 2.2A) and U937 (Figure 2.2B) successfully engrafted NSG Mice. There was a lower standard deviation between dose levels in U937 mice (74mm^3) compared to THP-1 (138mm^3) when all time points are compared. Combining all dose groups U937 achieved a more rapid engraftment with tumours reaching 500mm^3 by Day 11 on average compared to Day 17 for THP-1.

Figure 2.2

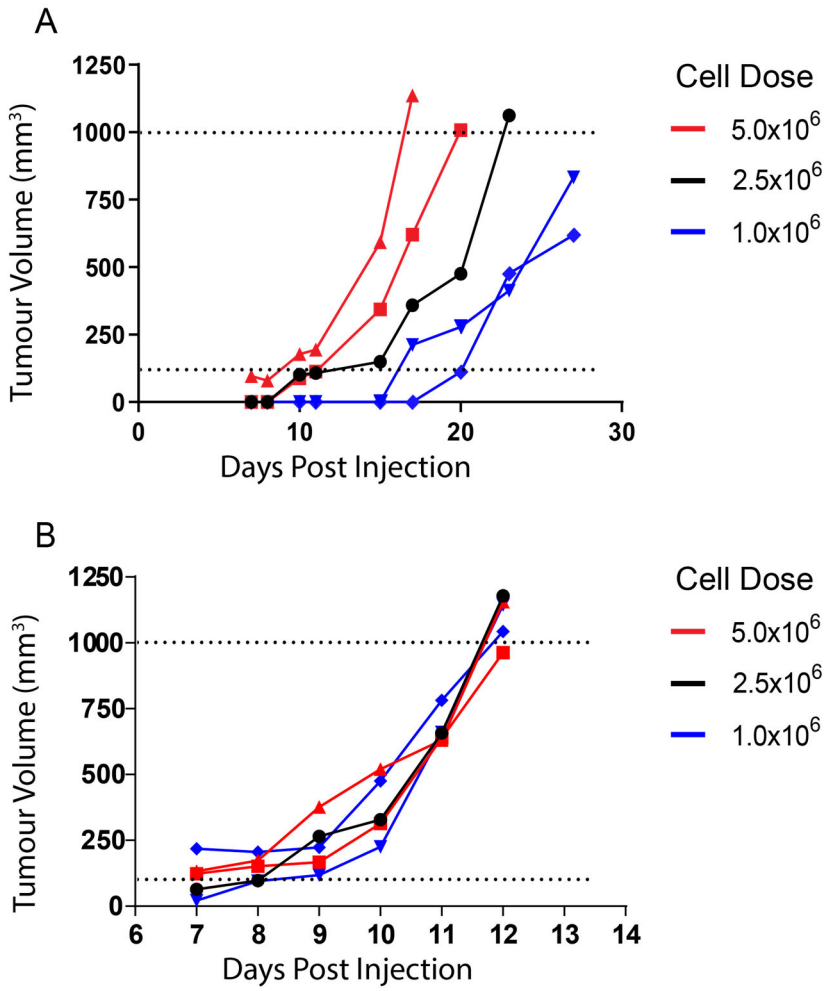

Figure 2.2 Subcutaneous Cell Line Model Growth Kinetics. Growth kinetics of flank tumours of THP-1 (A) or U937 (B) injected subcutaneously into the flanks of NSG mice. Results from individual mice, n = 1-2 mice per cell dose level.

Our previous work with mantle cell lymphoma tumours demonstrated that antigen expression may change in intensity for subcutaneous models. We therefore assessed CD300f expression in U937 and THP-1 when grown subcutaneously. Tumours harvested form THP-1 (Figure 2.3) and U937 (Figure 2.4) displayed the expected phenotype of mouse CD45-, human CD45+ and CD33+. The Median Fluorescent Index (MFI) ratio (MFI of test antibody/ MFI of isotype antibody) of CD300f was preserved in both cell lines. The MFI for CD300f was higher on U937 (5.6) tumours compared to THP-1 (2.5) tumours. No mice in either cohort demonstrated an increase in clinical score.

Figure 2.3

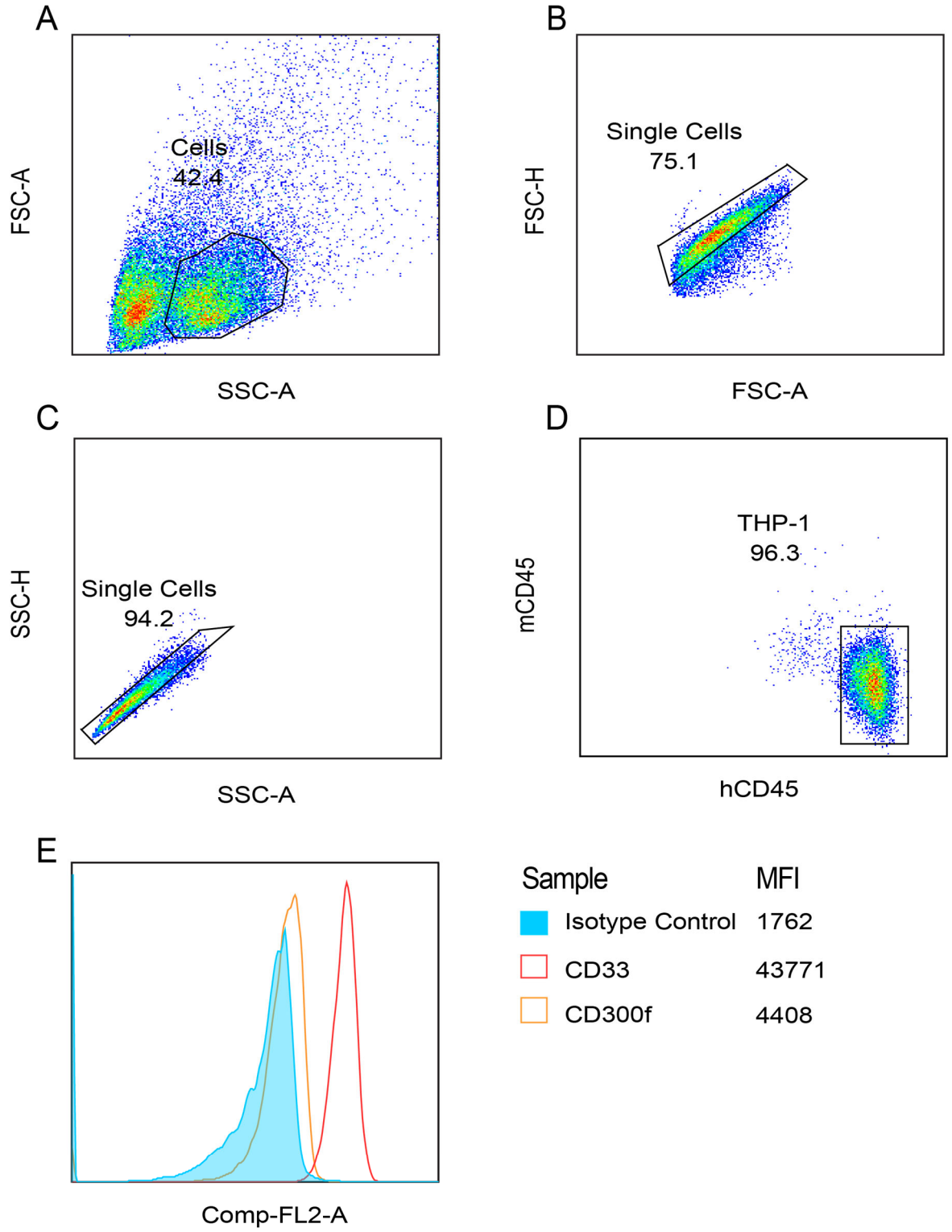

Figure 2.3 THP-1 tumour analysis and gating strategy by flow cytometry. The intact cellular population (A) was examined with single cell exclusion (B,C). The human CD45 positive fraction (D) was examined for CD33 and CD300f expression (E) compared to an isotype control.

Figure 2.4

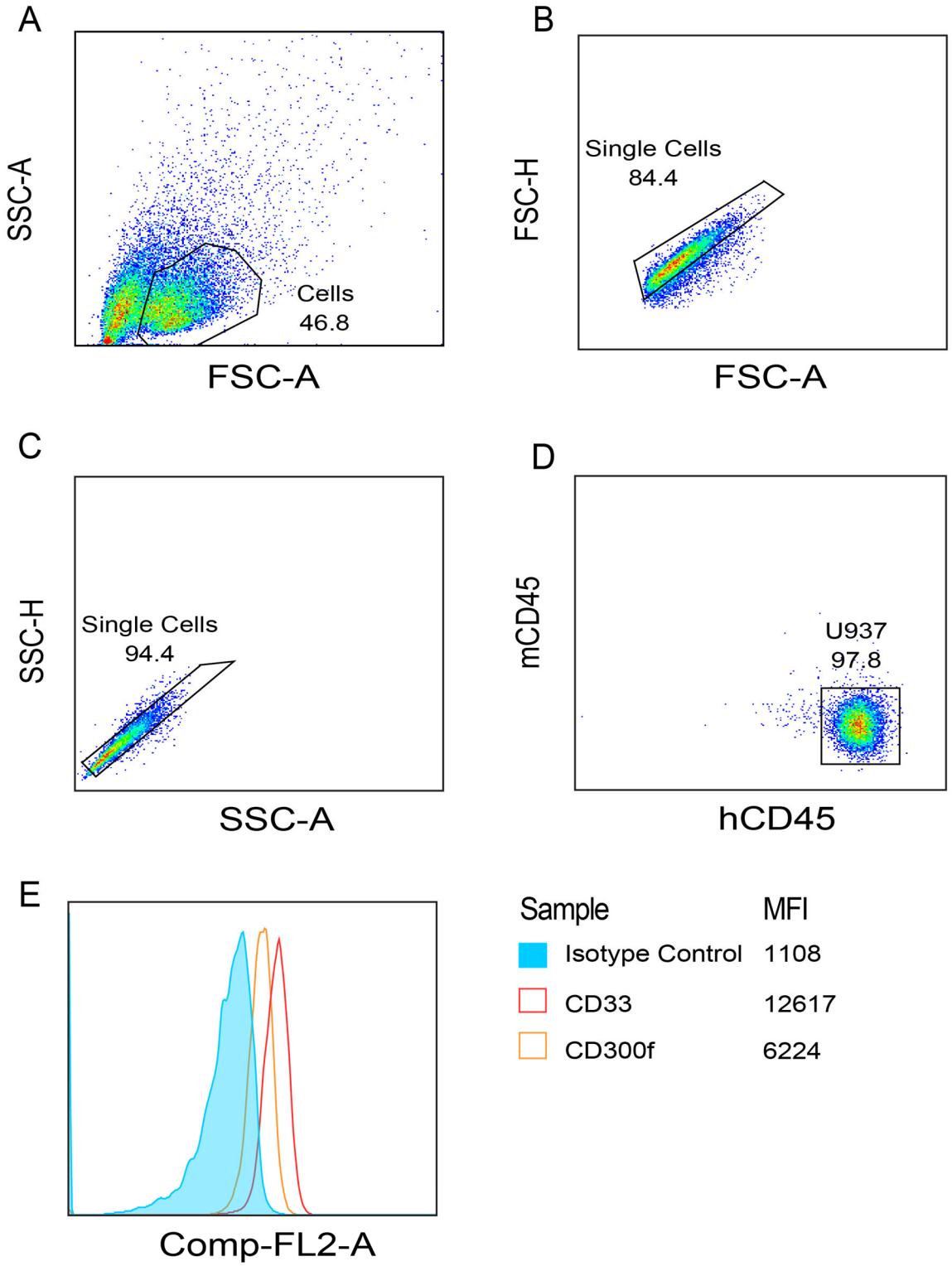

Figure 2.4 U937 tumour analysis and gating strategy by flow cytometry. The intact cellular population (A) was examined with single cell exclusion (B,C). The human CD45 positive fraction (D) was examined for CD33 and CD300f expression (E) compared to an isotype control.

Intravenous HL-60 Model: The Dendritic Cell Research Group had an existing protocol of established AML xenografts via tail vein injection of NOD/SCID mice with HL-60. To help enhance the limited bone marrow engraftment a comparative NSG model was established. Using NSG mice significantly enhanced engraftment of the bone marrow (Figure 2.5). The median bone marrow percentage of hCD45 cells was 80.62% in NSG compared 0.34% in NOD/SCID. Median survival post inoculation of NSG mice was reduced (22 days) compared to NOD/SCID mice (34 days).

Figure 2.5

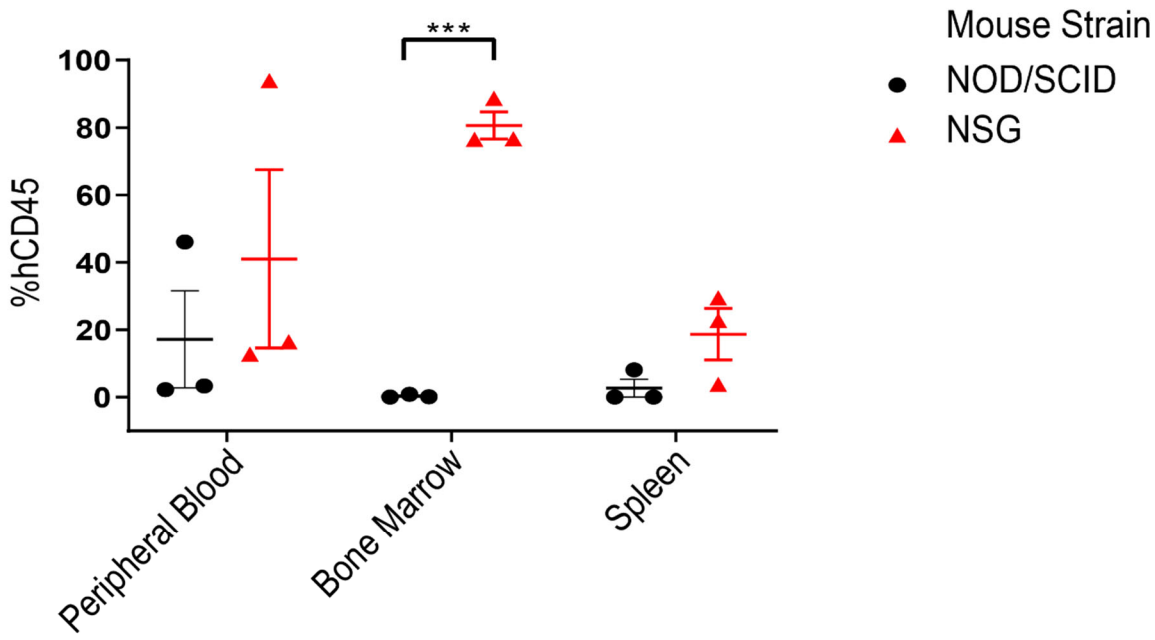

Figure 2.5 HL-60 engraftment by mouse strain. Human CD45+ cells per target organ at time of euthanasia when clinical score reached >4. Error bars correspond to SEM. *** p <0.001

Primary AML Model: The patient derived AML local repository was examined for suitable samples to establish primary AML xenografts. The following characteristics were required; > $75x10^6$ total cells available per donor, samples containing >90% blasts and collections form a single time point per donor. Four suitable samples were identified (Table 2.1).

Table 2.1 Characteristics of Primary AML samples used for xenografts.

Sample	Age	Sex	WHO Diagnosis	FAB	WCC	Karyotype	NPM1	FLT3-ITD
CRGH11	59	M	AML with MDS related changes	M4	54	Normal	Negative	Detected
CRGH14	95	F	AML with MDS related changes	M5	63	NA	NA	NA
CRGH18	60	M	AML NOS	M4	10	Normal	Negative	Negative
CRGH22	77	M	AML with MDS related changes	M5	150	t(8:19) +20	NA	NA

NOS, not otherwise specified. NA, not available

Engraftment trials were limited by donor material available. The number of cells injected was varied due to post thaw cell numbers from different donors and varied from $8x10^5$ to $5x10^6$. The engraftment kinetics varied between donors (Figure 2.6), with the most rapid establishment seen in samples CRGH11 and CRGH22. There was no increase in clinical score among the mice, one mouse inoculated with CRGH22 was euthanised early to ensure that engraftment data could be collected. Bone marrow engraftment was highest with CRGH11 and CRGH18 (Figure 2.7). Representative bone marrow plots are shown in Figure 2.8.

Figure 2.6

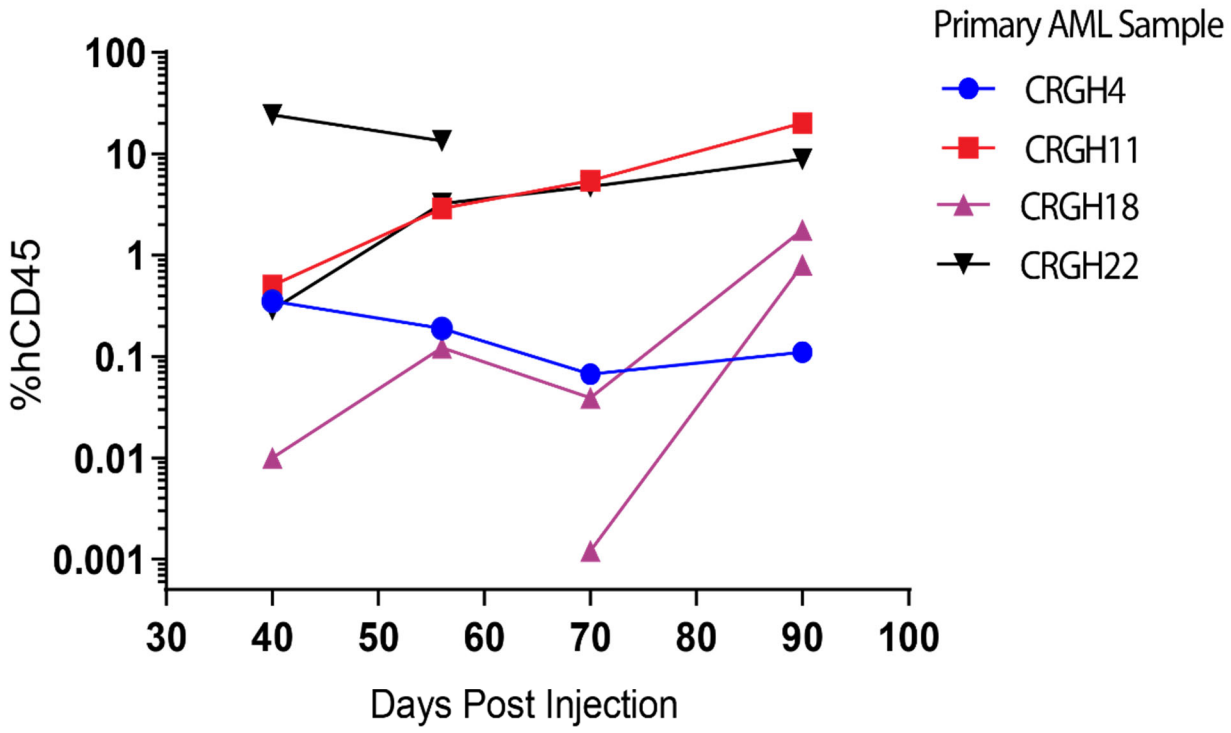

Figure 2.6 Primary AML engraftment kinetics. Human CD45+ cells in peripheral blood post injection. Results of individual mice by AML sample. n = 1-2 per primary AML sample.

Figure 2.7

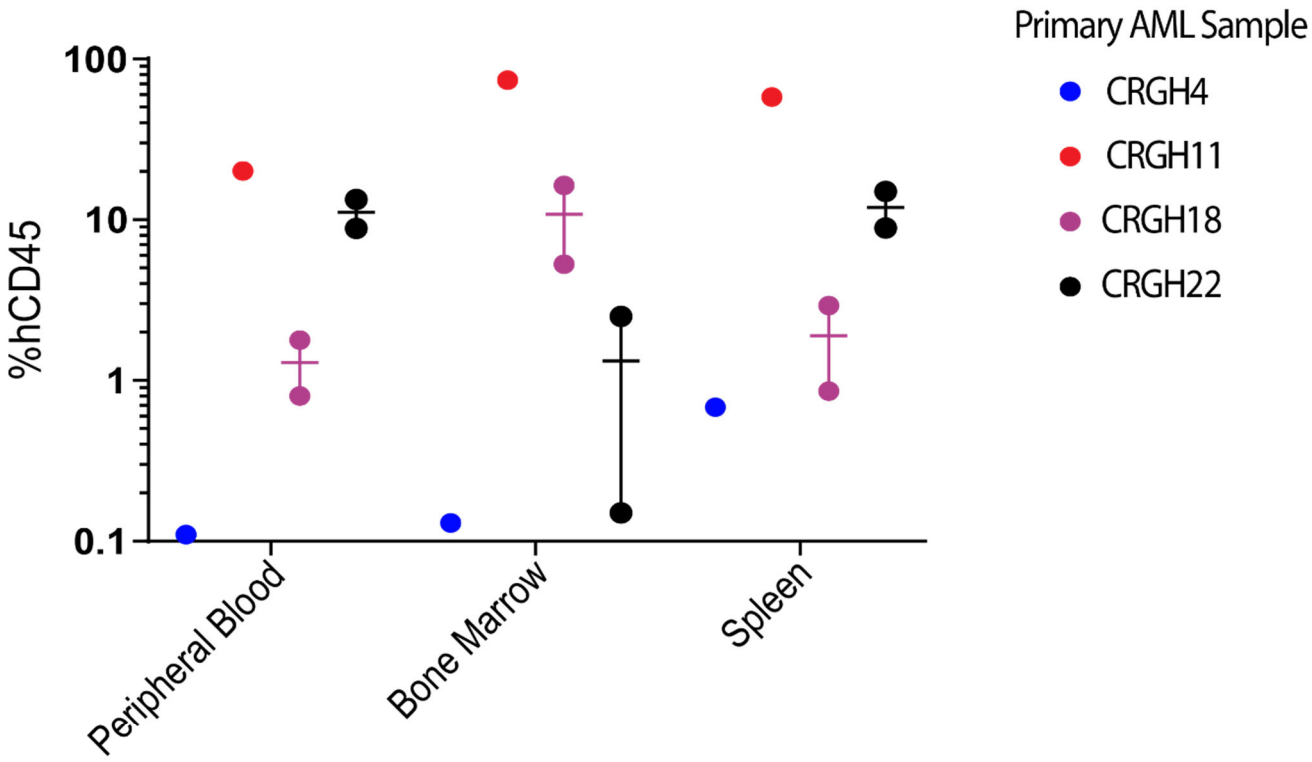

Figure 2.7 Primary AML organ engraftment. Human CD45+ cells post primary AML injection. Results of individual mice by AML sample. n = 1-2 per sample.

Figure 2.8

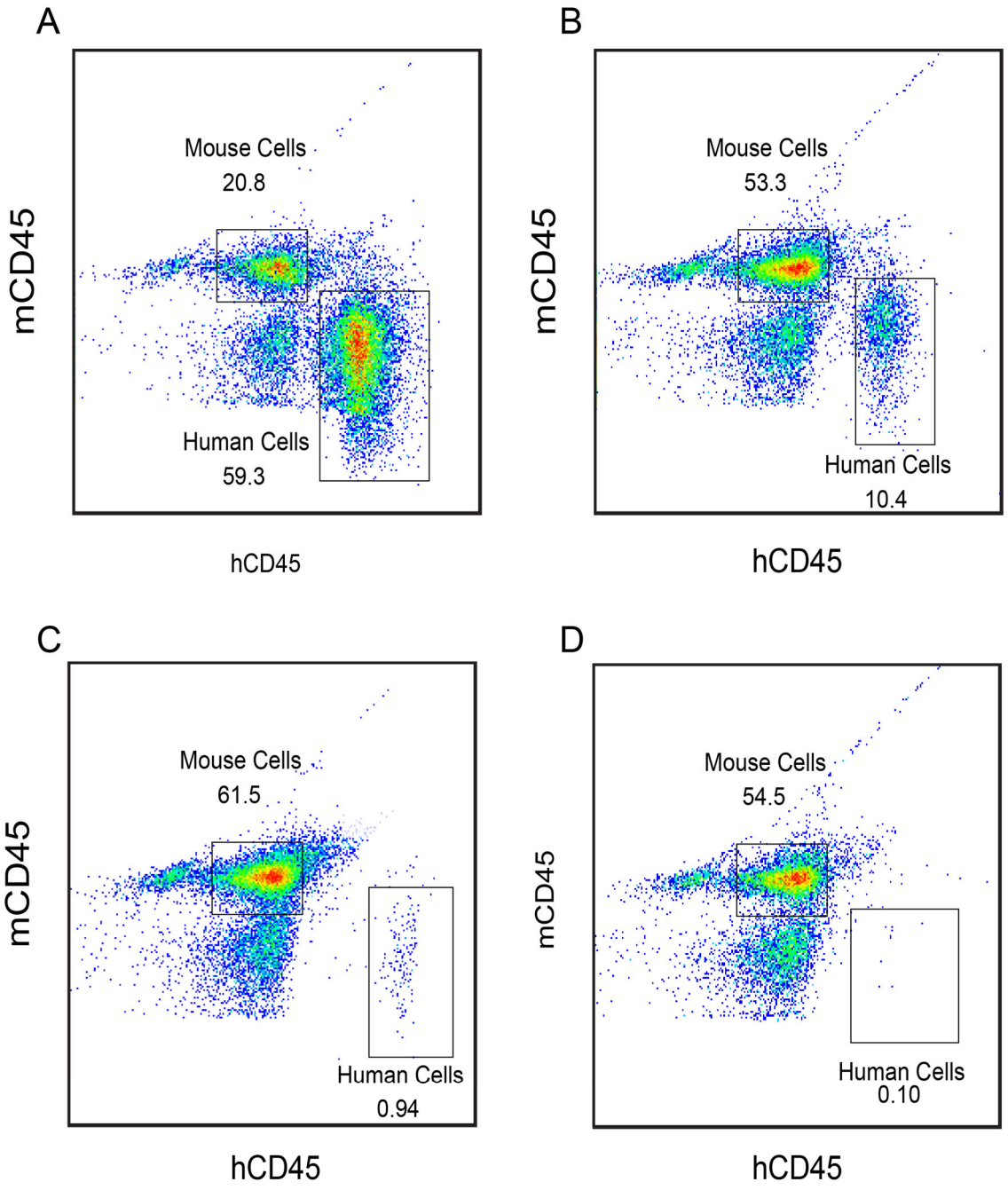

Figure 2.8 Primay AML bone marrow engraftment. Representative plots from samples CRGH11 (A), CRGH18 (B), CRGH22 (C) and CRGH14 (D).

Healthy Humanised Mouse Model: CD34+ cells were isolated at > 90% purity. Representative plots of CB (Figure 2.9) pre (A) and post (B) CD34+ selection are shown. HSPC were confirmed by the expected phenotype of being CD34+ and CD45 dim. Engraftment kinetics were examined in mice according to CD34+ cell dose from individual cords (Figure 2.10). The $1x10^5$ produced superior engraftment compared with $0.5x10^5$ cell dose.

Figure 2.9

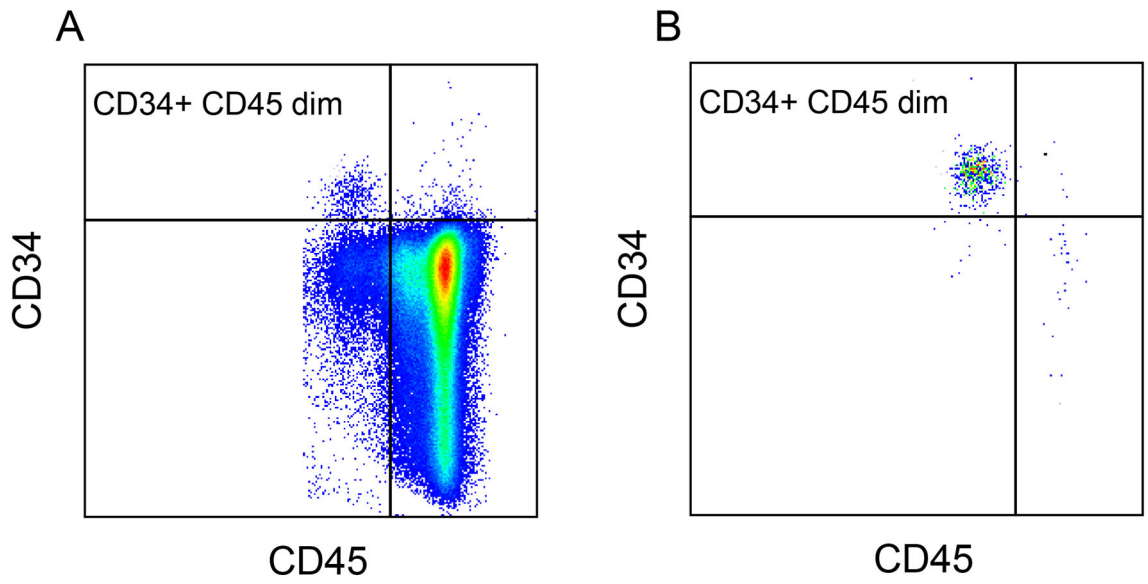

Figure 2.9 Isolation of CD34+ cells from cord blood. Representative plots of CB pre-selection (A) and post positive selection (B).

Figure 2.10

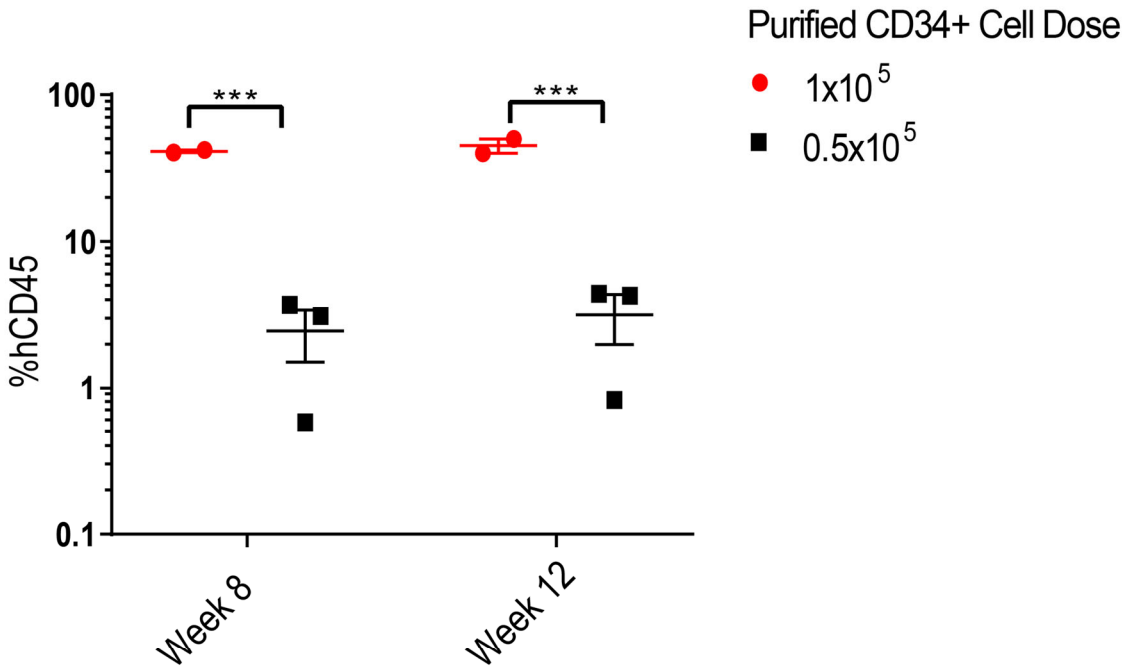

Figure 2.10 Humanised mouse engraftment kinetics. Human CD45+ cells at designated time points. n = 2-3 mice per group. Error bars correspond to SEM. *** p <0.001

Humanised mice generated CD19+, CD3+, CD33+ and CD34+ populations by 12 weeks with representative plots from blood, bone marrow and spleen are shown in Figure 2.11. Human HSC (CD34+, CD38, CD90+, CD45RA-) and multipotent progenitor (MPP) (CD34+, CD38, CD90-, CD45RA-) cells could be found in bone marrow at week 12 (Figure 2.12).

Figure 2.11

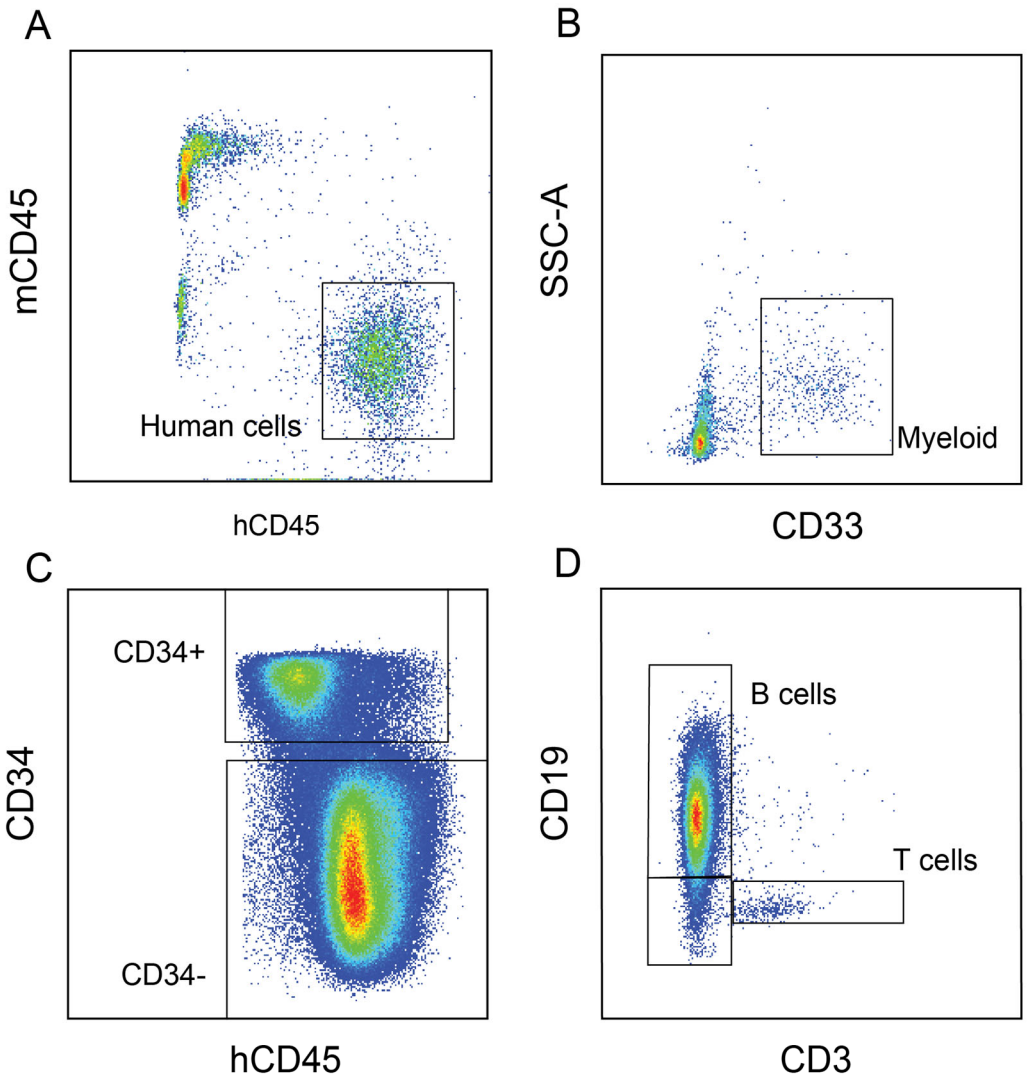

Figure 2.11 Human cell populations in humanised mouse xenografts at week 12. Representative plots. All cells in blood (A). Human CD45+ cells in blood (B), bone marrow (C) and spleen (D).

Figure 2.12

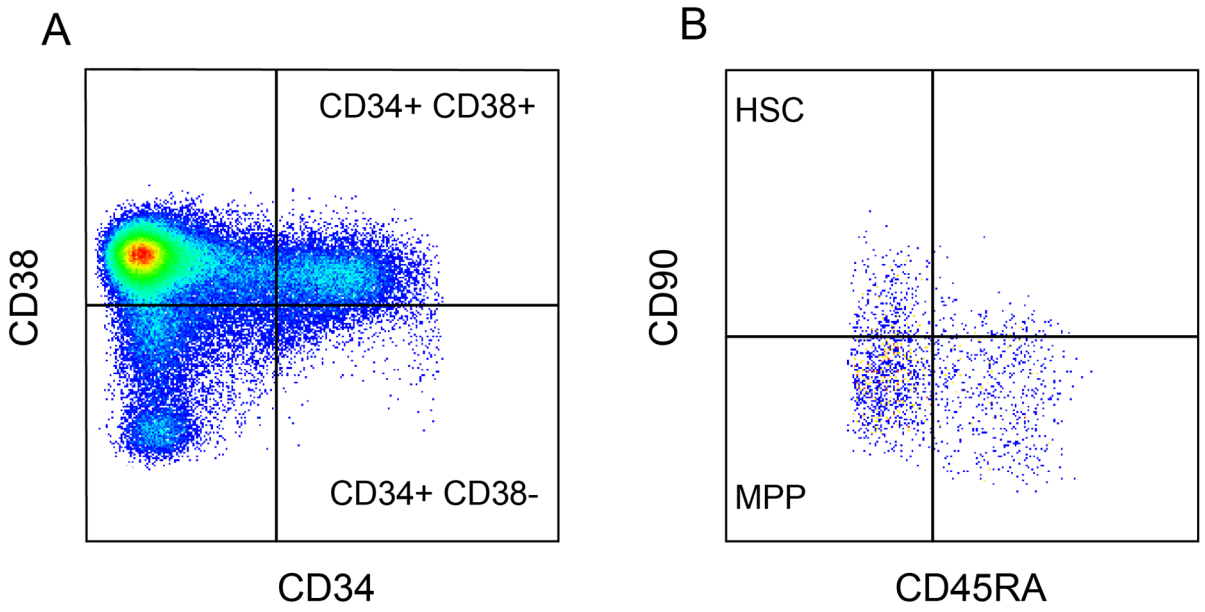

Figure 2.12 Human HSPC cell populations in humanised mouse xenograft bone marrow at week 12. Total human CD45+ cells (A) and primitive human HSPC (B) from the CD34+, CD38- fraction.

Conclusion

This work generated mouse xenografts to test the potential efficacy of an anti-CD300f antibody-based therapies. Our THP-1 and U937 growth kinetics from our subcutaneous models was similar to the results from other groups using the same cell lines.[10, 11] The U937 subcutaneous model was chosen to take forward for future therapeutic experiments as it had a rapid consistent growth rate as well as higher expression of CD300f. It has been established that NSG mice have a superior engraftment of HL-60 compared to NOD/SCID.[12] Our systemic HL-60 experiments confirmed these findings and the NSG model was preferred over the NOD/SCID due to the significantly increased engraftment of the bone marrow, more closely resembling the true microenvironment of AML. The shorter duration of the NSG experiment was also preferential over the NOD/SCID.

Developing models from primary samples was far more difficult. A sustained generation of primary AML xenografts requires thought to be placed into sample collection, documentation and storage. We had some limitations in sample numbers as well as collection and storage practices that had to be overcome. It was expected that some primary AML samples would not engraft well. CRGH11 provided adequate engraftment of the BM to test future therapies. It was the only known sample with a FLT3-ITD mutation, which are associated with superior NSG engraftment.[13]

The humanised mouse model was also somewhat restricted by cell numbers but not as severely as the primary AMLs. Achieving high levels of CD34+ cell purity was accomplished relatively quickly and the models showed robust engraftment with the dose of $1x10^5$ cells. At this dose most major lineages could be identified and there was significant CD34+ cell engraftment in the bone marrow, which included primitive subsets. T cell development is known to limited in the model that we used and this can be overcome with newer techniques.[14] Given the primary focus of our work was on early haematopoiesis the limited T cell development was acceptable. These models allowed the publication of Chapter 6 “Targeting CD300f to enhance hematopoietic stem cell transplantation in acute myeloid leukaemia”.

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Chapter 3: Examination of CD302 as a potential therapeutic target for acute myeloid leukemia

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RESEARCH ARTICLE

Examination of CD302 as a potential therapeutic target for acute myeloid leukemia

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Abstract

Acute myeloid leukemia (AML) is the most common form of adult acute leukemia with ~20,000 new cases yearly. The disease develops in people of all ages, but is more prominent in the elderly, who due to limited treatment options, have poor overall survival rates. Monoclonal antibodies (mAb) targeting specific cell surface molecules have proven to be safe and effective in different haematological malignancies. However, AML target molecules are currently limited so discovery of new targets would be highly beneficial to patients. We examined the C-type lectin receptor CD302 as a potential therapeutic target for AML due to its selective expression in myeloid immune populations. In a cohort of 33 AML patients with varied morphological and karyotypic classifications, 88% were found to express CD302 on the surface of blasts and 80% on the surface of $CD34^+$ $CD38^-$ population enriched with leukemic stem cells. A mAb targeting human CD302 was effective in mediating antibody dependent cell cytotoxicity and was internalised, making it amenable to toxin conjugation. Targeting CD302 with antibody limited *in vivo* engraftment of the leukemic cell line HL-60 in NOD/SCID mice. While CD302 was expressed in a hepatic cell line, HepG2, this molecule was not detected on the surface of HepG2, nor could HepG2 be killed using a CD302 antibody-drug conjugate. Expression was however found on the surface of haematopoietic stem cells suggesting that targeting CD302 would be most effective prior to haematopoietic transplantation. These studies provide the foundation for examining CD302 as a potential therapeutic target for AML.

Introduction

Monoclonal antibodies (mAb) and their derivatives such as antibody drug conjugates (ADC), bispecific T Cell engagers and chimeric antigen receptor T cells, are rapidly being developed as

Competing interests: GJC is a Director of DendroCyte which has intellectual property associated with CD302 targeting of AML. We have provided the full name (DendroCyte Biotech Pty Ltd) and number (2019900018) of the provisional patent awarded for CD302 targeting of AML (lines 21-3). This does not alter our adherence to PLOS ONE policies on sharing data or materials with the exception of the MMRI-20 (anti-CD302) monoclonal antibody which was obtained under a Material Transfer Agreement with Mater Medical Research Institute (Raymond Terrace, QLD Australia).

the next generation of anti-cancer treatments [1]. These therapeutic agents offer the advantage of high specificity and potency with the potential of limited toxicity due to their ability to recognise molecular targets on tumours [2]. Whilst advances have been made in the development of mAb based therapy in other haematological diseases such as B cell lymphoma [3] and multiple myeloma [4], progress in acute myeloid leukemia (AML) has remained unsatisfactory. An ideal AML target should be highly expressed on the surface of leukemic blasts with limited expression on healthy cells [5]. AML arises from haemopoietic stem cell (HSC) and multipotent progenitor populations (MPP) resulting in substantial overlap in surface molecule expression [6]. Additional properties including internalisation, induction of antibody dependent cell mediated cytotoxicity (ADCC) or functional repression are favourable for designing mAb therapeutic strategies. Despite ongoing work, no ideal AML target has been identified [5, 6]. Approximately 70% of patients under the age of 60 achieve complete remission following conventional treatment, but many relapse causing a 40% overall survival rate [7]. This is believed to be due to the persistence of leukemic stem cells (LSC), which are not eliminated efficiently with current treatments and re-populate over time [8–10]. Therefore, it is critical that an AML target molecule be expressed by LSC and blasts. Given the heterogeneous nature of AML, different targets could be required for the treatment of malignant cells represented by this disease.

We propose CD302 as a potential therapeutic target for AML. CD302 is the simplest type I transmembrane C-type lectin receptor (CLR) described [11]. The protein consists of 232 amino acids containing a single C-type lectin like domain. Amongst human leukocytes, CD302 is restricted to myeloid derived populations including monocytes, macrophages, dendritic cells and granulocytes. This expression profile led us to explore CD302 as a potential target for myeloid malignancies. CD302 colocalizes with f-actin rich filopodia, lamellopodia and podosomes in macrophage and transfected cell lines, indicative of a role in attachment or migration [11], a function subsequently confirmed by CD302 knockout mouse studies [12].

In the current study, we examined CD302 expression on leukemic cell lines and primary AML in comparison to HSC and monocytes from healthy donors. We explored the ability of anti-CD302 mAb to mediate ADCC and affect leukemic cell migration using *in vitro* and *in vivo* models. We have further characterised differences in CD302 distribution between AML and hepatocyte cell lines and demonstrated a proof of principal *in vitro* ADC model.

Material and methods

Preparation of tissue samples

Patient blood or bone marrow (BM) samples from patients with AML were collected at the Concord Repatriation General Hospital (CRGH) or Royal Prince Alfred Hospital (Sydney, Australia). Patients ranged from 16–95 years of age, and had blast percentages in sample that ranged from 10–95% at the time of collection (S1 Table). Healthy donor blood samples were collected, with informed consent, from a donor panel maintained by the Department of Haematology, CRGH. Blood was obtained by venesection and PMBC isolated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare) with the manufacturer's protocols. Cord blood (CB) samples were obtained from the Sydney CB Bank with mononuclear cells collected as above. BM aspirates were collected from the posterior iliac crest of patients and healthy volunteers. Samples were then passed through a 22G needle to disrupt BM fragments before proceeding to isolation of mononuclear cells as above. To purify human monocytes, healthy donor peripheral blood mononuclear cells (PBMC) were labelled with CD14 Microbeads (Miltenyi Biotec) and positively selected using an AutoMACS Pro (Miltenyi Biotec). Ethical approval for human studies was obtained from the Sydney Local District Human Research Ethics Committee (HREC/12/CRGH/59, HREC/11/CRGH/61 & 118).

Cell lines

HL-60 and HEL cell lines were previously obtained by Professor Derek Hart at the Haematology & Immunology Research Group, Christchurch School of Medicine, University of Otago, New Zealand. The U937 and HS-5 cell lines was sourced from the American Type Culture Collection (ATCC). These lines were maintained in complete RPMI 1640 (supplemented with 10% FCS, 2mM Gluta-MAX, 100U/ml penicillin and 100μg/ml streptomycin; ThermoFisher). The HepG2 cell line was purchased from ATCC and grown in DMEM with 1g/l D-glucose, 10% FCS, 2mM Gluta-MAX, 100U/ml penicillin and 100μg/ml streptomycin (ThermoFisher).

Gene expression

AML gene expression data was retrieved from the Gene Expression Omnibus microarray dataset GSE14468 [13]. The series matrix files were parsed in R and the probe ID and signal value corresponding to *CD302* (*203799_AT*) and *CD33* (*206120_AT*) extracted. Only samples containing French American British (FAB) AML subtype classification were analysed. For quantitative PCR (qPCR), total RNA from tissues or cells was extracted using TRIzol reagent and synthesized into cDNA using the SuperScript III kit (ThermoFisher) as per manufacturer's instructions. Method and primers for human CD302 and HPRT qPCR are described in [12].

Flow cytometry

AML and healthy PBMC samples were phenotyped with CD45-V500 (HI30), CD34-PE-CY7 (581), CD38-V450 (HB7) and CD33-PE (WM53), CD90-AF700 (5E10), CD45RA-APC-H7 (SH9), CD11c-APC-AF700 (B-Ly6) and HLA-DR-APC-H7 (L243) mAbs from BD Biosciences or BioLegend. The lineage (Lin) cocktail consisted of CD235a (GA-R2), CD14 (MφP9), CD20 (2H7), CD19 (HIB19), CD56 (NCAM16.2) and CD3 (SK7; BD Biosciences). The mouse anti-human CD302 IgG1mAb antibody (MMRI-20) was used unlabelled or as a PE conjugate [11]. The mAb CMRF-81, specific for tetanus toxoid, was used as the mouse IgG1 isotype control [14]. DAPI (3μM; ThermoFisher) staining was used to exclude dead cells. Data were collected on Accuri C6, Canto, Fortessa LSR or Influx flow cytometers (BD Biosciences) and analysed with FlowJo 10 software (Treestar). The gating strategy for identifying BM/CB HSC and MPP are shown in panel A of S1 Fig. Binding was displayed as a geometric mean fluorescence intensity (geoMFI) ratio which was calculated by the formula: geoMFI test antibody/geoMFI isotype control. A ratio of $\geq$3 was considered positive. T-distributed stochastic neighbour embedding (t-SNE) visualisation was performed on FlowJo 10.

Western blot

Cell suspensions ($2.5x10^7$ cells/ml) were solubilised in modified RIPA buffer (1% Triton X-100, 0.25% sodium deoxycholate, 0.15M NaCl, 50mM Tris-HCl, 5mM EDTA containing Protease Inhibitor (Roche, Basel, Switzerland)). Protein content was determined by bicinchoninic acid assay (ThermoFisher). Lysates (5μg) were fractionated on a 4–12% Bis-Tris gel (Bolt, ThermoFisher) under reducing conditions. Proteins were transferred to nitrocellulose (Novex Miniblot; ThermoFisher) using an iBlot. Membranes were stained with Ponceau before overnight incubation in 5%BSA in TBST. Membranes were incubated with 1μg/ml rabbit anti-human CD302 polyclonal antibody (LS-C119435; LS Bio) followed by 1:1000 HRP conjugated goat anti-rabbit IgG Fc antibody (A6154 Sigma-Aldrich). Protein bands were detected by chemiluminescence using Clarity Western ECL substrate (BioRad) and visualised on a BioRad GelDoc. Molecular weights (MW) of proteins were determined by comparison with Precision Plus standards (BioRad).

Immunohistology

HepG2 or HL-60 cells ($4x10^4$ cells/well) were adhered to Lab-Tek II Chamber Slide (ThermoFisher) for 30 min or overnight in 5% CO_2 at 37˚C, respectively. Cells were fixed with 4% paraformaldehyde and rehydrated using 1% BSA/PBS. Cells were blocked with 10% goat serum (Invitrogen) and stained with either MMRI-20 or isotype control (10μg/ml) for 30 min at 37˚C. Goat anti-mouse (GAM) IgG-AF488 antibody (ThermoFisher) was used to detect primary antibodies. Phalloidin-AF594 and 18μM DAPI (ThermoFisher) were used to identify f-actin at the cell surface and the nucleus, respectively. Slides were imaged using a 3i VIVO Spinning Disc Microscope (Intelligent Imaging Innovations, Inc.) and analysed with Image J (NIH) software.

Antibody internalisation assay

HL-60 cells were incubated with MMRI-20-PE or isotype control-PE (10μg/ml) on ice for 20 min. Antibody coated cells were then incubated at 37˚C/5% CO_2 for the indicated times to allow internalisation. After incubation, a secondary GAM IgG-AF488 antibody was applied to the samples for 20 min on ice to detect remaining surface antibody and compared to the total (surface and internalised) PE staining. Cells were fixed in 1% paraformaldehyde/PBS followed by flow cytometry analysis. Relative MFI was calculated as a percentage of staining at 0 min.

Colony forming units (CFU)

Frozen CB cells were incubated with MMRI-20 mAb followed by GAM IgG-AF488 (Invitrogen). Subsequent staining with a Lin stain was performed. $DAPI^-$ Lin^- $CD302^+$ or $CD302^-$ fractions were FACS isolated and resuspended in IMDM media (Stemcell Technologies). Equal numbers of each sorted fraction were plated at $1.5–2.5x10^4$ cells/plate in semi-solid methylcellulose medium (MethoCult Classic, Stemcell Technologies). Plates were cultured at 37˚C and 5% CO_2 for 12–14 days prior to counting of multi-lineage, myeloid and erythroid colonies in wells with a light microscope.

ADCC

HL-60 or U937 target (T) cells labelled with 2.5μM Calcein-AM (ThermoFisher) as per manufacturer's protocol and resuspended in complete RPMI 1640. Target cells ($5x10^3$) were mixed with $5x10^4$ C57BL/6J female mouse (Animal Resources Centre, Perth, Australia) spleen effectors (E), 1000U of human IL-2 (Invitrogen) and the indicated concentrations of MMRI-20 or isotype control in triplicate. Plates were incubated for 18h at 37˚C and 5% CO_2. Cells were subsequently labelled with 3μM DAPI for 20 min to detect death of Calcein-AM^+ target cells via flow cytometry. Spontaneous and maximal death was determined by culturing target cells alone or with 2% Triton-X, respectively. Cytotoxicity was calculated with the formula: E+T (antibody)–E+T(no antibody)/ T(max)–T(spontaneous).

Migration assays

HL-60 and U937 were incubated with MMRI-20 or isotype control mAb (10 μg/ml) in 1% BSA/RPMI at 37˚C for 1 hr and washed twice before layering 1 x 10^5 cells onto 5μm transwell filters coated with 0.1 mg/ml fibronectin or a confluent layer of HS-5 cells. CXCL12 (160 ng/ml) or 1% BSA/RPMI media alone was added to the lower chamber. After 4 h incubation at 37˚C and 5% CO2, cells migrating into lower chamber were enumerated using flow cytometry. Results were reported as the chemotaxis index: migration with chemokine divided by migration with media only.

Xenogeneic NOD/SCID AML mouse model

NOD.CB17-*Prkdc*^{scid}/J (NOD/SCID) female mice were purchased from the ARC. All mice were housed at the ANZAC Research Institute under specific pathogen free conditions. NOD/SCID mice were irradiated with 250cGy from an X-ray source (XRAD 320, Precision X-Ray; Connecticut, USA) one day before cell transfer. HL-60 cells were resuspended in X-VIVO at 2x10^6 cells/ml and incubated with 10μg/ml MMRI-20 or isotype control mAb for 1 h. Antibody coated cells were washed three times with PBS and 5x10^6 cells transferred intravenously (iv) into irradiated NOD/SCID mice. Mice were euthanised at pre-defined time point (d21) or at humane endpoint determined by disease score (maximum d28). To minimise suffering, disease scores were assessed daily for 14d post-transfer and then weekly if disease score $\leq$1, which derived from combined scores of 0–2 for weight loss, posture, activity and fur texture. A disease score of $\geq$4 led to euthanasia within an hour. No animal died prior to meeting defined endpoint. BM, spleen and blood were collected for flow cytometry analysis after euthanasia as described [12]. HL-60 cells were identified by flow cytometry using mouse CD45-PerCP/Cy5.5 (30-F11), human CD45-FITC (HI-30) and CD33-PE (WM53) mAbs with the gating strategy in panel B of S1 Fig. Engraftment was assessed as: number of human CD45 cells/ number of human plus mouse CD45 cells. All animal procedures and staff were approved by the Sydney Local Health District Animal Ethics Committee (#2015/026).

Pyrrolobenzodiazepine (PBD) cytotoxicity assay

HL-60 and HepG2 cells (1x10^4) or human PBMC (1x10^5) were plated in quadruplicate with serial dilutions of MMRI-20 or isotype control and equimolar concentrations of GAM IgG secondary antibody attached via cleavable linker to PBD (Moradec). Cells were incubated at 37˚C and 5% CO_2 for 96h. Viability was then measured with CellTiter-Glo luminescent assay (Promega) for cell lines or flow cytometry for dendritic cells (DAPI$^-$Lin$^-$HLA-DR$^+$CD11c$^+$) and monocytes (DAPI$^-$SSC^{hi}Lineage$^+$HLA-DR$^+$CD11c$^+$) in PBMC and compared to untreated samples.

Statistics

Statistical analyses were performed with Prism 7 (GraphPad Software). Mean values with SEM are shown in graphs. Pearson's coefficient (r) was used to determine correlation. Non-parametric paired or unpaired t-tests or ANOVA (with multiple test correction) were used to determine statistical differences between groups with $p<0.05$ deemed significant.

Results

CD302 is expressed on blasts and LSCs of most AML patients

CD302 has a restricted expression profile on myeloid cells within haematopoietic populations [11] leading us to hypothesise that this CLR could be expressed by AML that arise from the myeloid lineage. A comparison of *CD302* to *CD33* gene expression in a published cohort of 460 AML patients [13] demonstrated high expression of the former across FAB disease subtypes (highest on M4-M5) and a moderate positive correlation of expression between the markers (r = 0.4749, $p<0.0001$; panels A-B of S2 Fig).

We examined a panel of primary AML blood samples of 33 patients with varied morphological and karyotypic classifications (S1 Table) for cell surface protein expression of CD302 by flow cytometry (Fig 1A) using MMRI-20. The geoMFI ratio of CD302 staining relative to a mouse IgG1 isotype control demonstrated that CD302 was expressed 3-fold higher than background on the surface of AML blasts in 88% of patients (29/33) and in LSC enriched CD34$^+$CD38$^-$ cells in 80% of patients (16/20; Fig 1B). This was similar to the proportion of

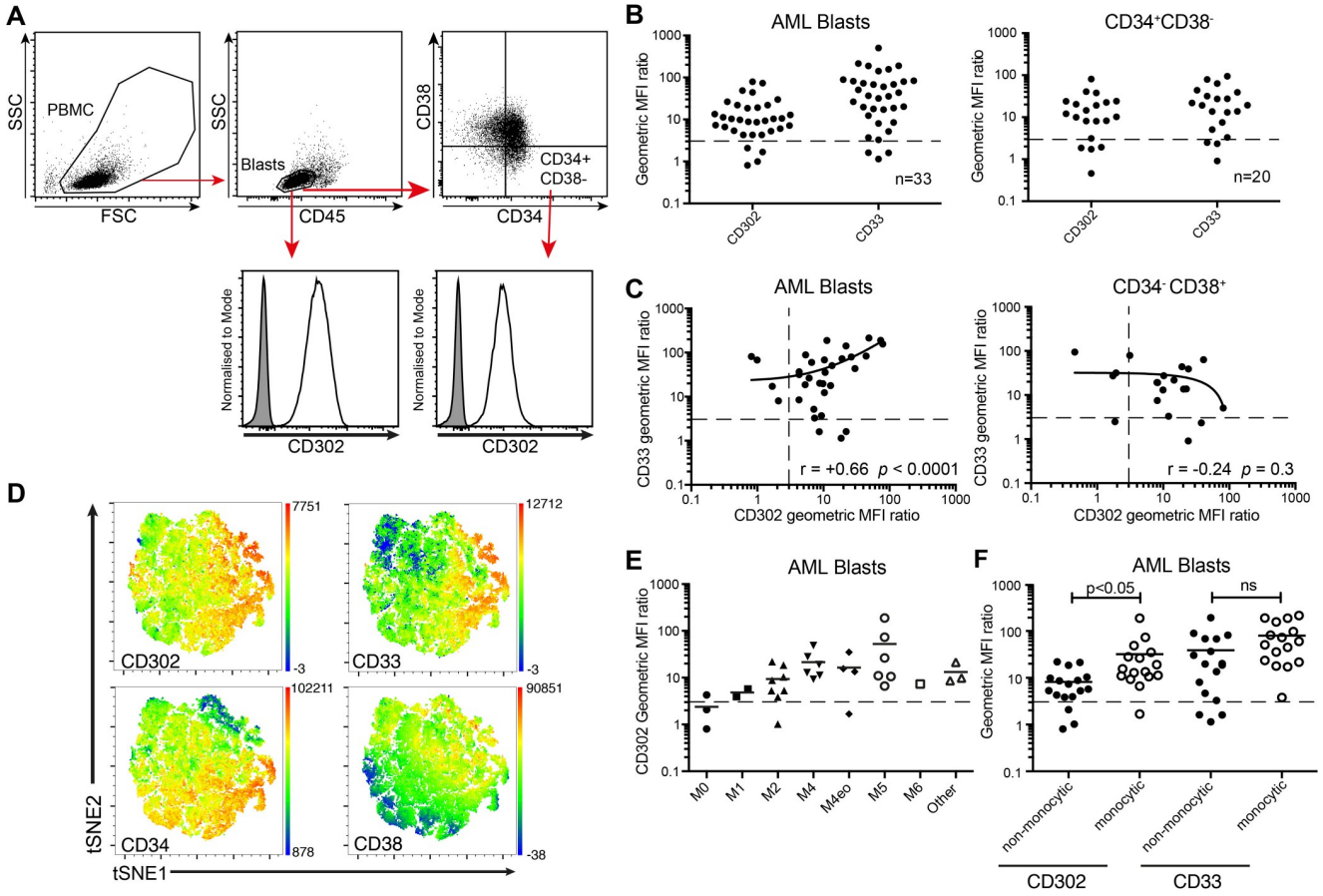

Fig 1. Expression of CD302 on leukemic blasts, CD34$^+$CD38- LSC and HSC. (A) Gating strategy used to identify CD45^{lo}SSC^{lo} AML blasts and CD34$^+$CD38$^-$ LSC. (B) Scatter dot plots showing the CD33 and CD302 expression on AML blasts (n = 33) and LSC fraction (n = 20). Samples were stained with MMRI-20 and CD33 mAb. Populations with a geoMFI ratio ≥3, shown above the dotted line, were considered to be positive. (C) Relationship between mean CD33 and CD302 expression on AML blasts and on AML LSC from patients. The solid lines were generated by linear regression. (D) Six parameter data (including CD117, CD34, CD33, CD38, CD45 and CD302) from five concatenated AML patient samples was converted into two t-SNE dimensions and overlayed with heatmaps of the indicated marker's MFI. (E) Summary of CD302 expression by AML blasts across FAB subtypes based on their morphology and immunophenotypic characteristics, as outlined in the 2008 WHO classification. (F) CD302 and CD33 expression on AML samples with monocytic differentiation, FAB subtype M4 and M5, were compared to other subtypes of AML.

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AML cases showing surface staining of CD33 with the WM53 mAb clone (91% of blast and 85% of CD34$^+$CD38$^-$ cells). No significant difference was observed between the geometric MFI ratios of CD302 and CD33 in blasts or CD34$^+$ CD38- AML in the patient cohort. Consistent with the gene expression analysis, correlation analysis revealed that mean CD302 expression correlated positively with mean CD33 expression on AML blasts in patient samples (r = 0.66, $p<0.0001$; Fig 1C). However, no significant correlation between CD302 and CD33 was observed in the CD34$^+$CD38$^-$ populations. T-SNE is an algorithm that reduces multi-parameter flow cytometry data into two t-SNE parameters, allowing visualization of cellular organisation in two dimensions. Transforming the six parameter data (including CD33, CD34, CD38, CD45, CD117 and CD302 expression) from five concatenated AML samples into a two dimensional t-SNE plot and highlighting areas of high CD302 and CD33 expression illustrated the correlation between these markers at the cellular level (Fig 1D). As CD302 is highly expressed by human blood monocytes [12], we examined if AML with monocytic differentiation would express a higher amount of CD302. Indeed, CD302 was expressed significantly higher in AML from these patients than those with non-monocytic subtype cases (Fig 1E and 1F).

MMRI-20 is internalised and mediates ADCC against leukemic cell lines

We next investigated whether targeting CD302 with a mAb could be therapeutic against AML. Three potential target leukemic cells lines, HL-60, HEL and U937 were examined for their surface CD302 expression by flow cytometry. MMRI-20 staining was highest on HL-60, followed by HEL and lowest on U937 (geoMFI ratios of 5.4, 3.3 and 1.6, Fig 2A), consistent with previously described transcript levels [15].

To determine whether targeting surface CD302 with mAb could mediate ADCC towards leukemic cells by immune effector cells, HL-60 were co-cultured with mouse splenocytes as effector cells in the presence of different concentrations of MMRI-20 or an isotype control mAb. After 20 h of culture, addition of MMRI-20 increased the killing of target cells in a dose dependent manner, while culturing with the isotype control resulted in minimal killing (Fig 2B). We compared ADCC mediated against surface $CD302^{hi}$ HL-60 to that of $CD302^{lo}$ U937 with optimal levels of antibody. As anticipated, target killing was greater for HL-60 than U937 (Fig 2C). However, despite low surface CD302 levels, ADCC against U937 induced by MMRI-20 was still significantly higher than that achieved by the isotype control.

The internalising capacity of MMRI-20 was tested on the $CD302^{hi}$ HL-60 cell line using a flow cytometry based assay (Fig 2D). The assay showed that MMRI-20 bound to the surface of cells was reduced to ~75% of its starting level after 30 min of culture at 37˚C (Fig 2D). In contrast, the total level of MMRI-20 (surface and intracellular) remained constant through the course of the experiment, indicating that the antibody had not dissociated from the surface but was internalised into cells with CD302 (Fig 2D).

Given that we have previously shown CD302 to contribute to migration [12], we explored whether MMRI-20 could alter this function when bound to AML cells. We performed an *in vitro* assay where the chemotactic ability of HL-60 to the BM homing chemokine CXCL12 was compared between cells pre-incubated with MMRI-20 or an isotype control antibody (Fig 2D). Transwells were coated with either fibronectin or HS-5 stromal cells, forcing cells to utilise cellular protrusion (e.g. podosomes) to transmigrate through the barrier. Regardless, HL-60 migrated in a similar fashion towards CXCL12 in all conditions tested, suggesting MMRI-20 did not alter the chemotactic ability of the leukemic cells, at least in this *in vitro* assay.

Anti-CD302 mAb reduces engraftment of AML in NOD/SCID mice but does not alter survival

We established a xenogeneic model of AML for testing antibody targeting. HL-60 cells were injected iv into irradiated NOD/SCID mice, allowing leukemic cells to engraft and disseminate causing illness requiring euthanasia between d21 to 28 post cell transfer. To investigate whether anti-CD302 antibodies affected AML engraftment, we pre-coated HL-60 cells with MMRI-20 or an isotype control *ex vivo* prior to transferring them into NOD/SCID mice. Both cohorts were euthanised on d21 and the presence of AML in their BM, spleen and blood was examined by flow cytometry. Coating with MMRI-20 significantly reduced HL-60 burden in BM and spleens and a trend towards reduction in the blood (Fig 3A). However, this difference did not lead to a lower disease score nor an extended survival time for mice receiving MMRI-20 versus isotype control coated AML (Fig 3B and 3C).

CD302 is expressed by healthy HSC in BM and CB

We obtained three BM and five cord blood samples from healthy donors and examined CD302 expression on HSC and progenitors via flow cytometry (Fig 4A). MMRI-20 was found to bind to the vast majority of HSC, MPP and $CD34^{+}CD38^{+}$ from both BM and cord blood in

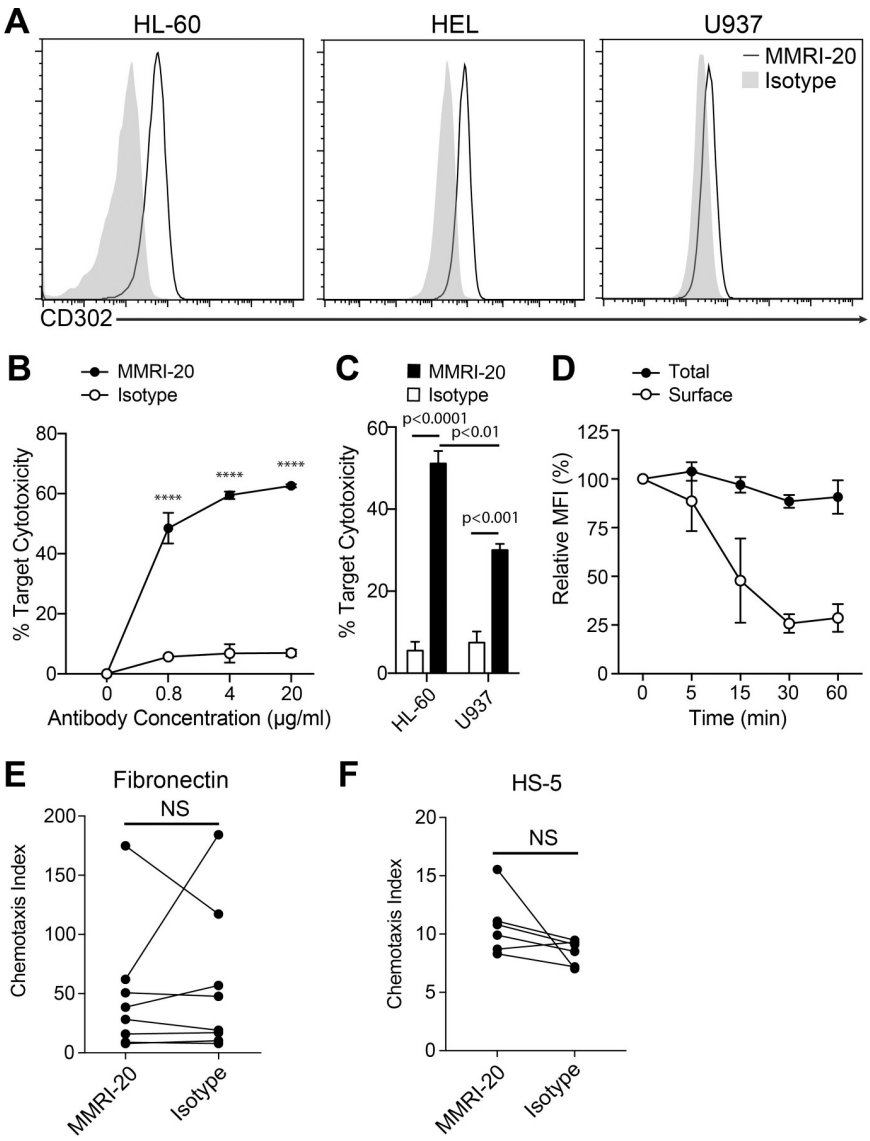

Fig 2. Antibodies targeting CD302 are able to be internalised and mediate ADCC of target cells. (A) Flow cytometry histograms showing the surface expression of CD302 on leukemic target cell lines as determined by staining with the MMRI-20 compared to a mouse IgG1 isotype control. (B) CD302 internalisation by HL-60 cells determined by flow cytometry. Total staining determined by MMRI-20-FITC 37˚C incubation for the indicated times after which residual surface CD302 was measured with anti-mouse IgG-PE at 4˚C. MFI of antibody staining is reported relative to pre-incubation levels. (C) MMRI-20 induced ADCC against HL-60 target cells. Calcein-AM labelled HL-60 were incubated for 18h with mouse spleen effectors at a 1:10 ratio, together with1000U IL-2 and the indicated concentrations of MMRI-20 or isotype control mAb. Target cell killing was measured as 7-AAD^{+} Calcein-AM^{+} cells by flow cytometry and presented relative to death in target alone (0%) or with 2% Triton X solution (100%). **** $p<0.0001$, two-way ANOVA. (D) ADCC elicited against HL-60 (CD302^{hi}) and U937 (CD302^{lo}) leukemic targets using 20μg/ml MMRI-20 or isotype mAb control. Experiments representative of three experiments. Differences tested by two-way ANOVA. (E-F) HL-60 cells were incubated with either MMRI-20 or isotype control mAb for 30 mins at 37˚C and tested for their ability to migrate across 5 μm transwells coated with (E) fibronectin or (F) HS-5 cells towards 160 ng/ml CXCL12 or media alone. Cells in bottom chamber were enumerated after 4h by using flow cytometry and migration presented as the chemotaxis index. Circles connected by lines represent individual paired experiments. No significant difference (NS) between MMRI-20 and isotype group (paired t-test).

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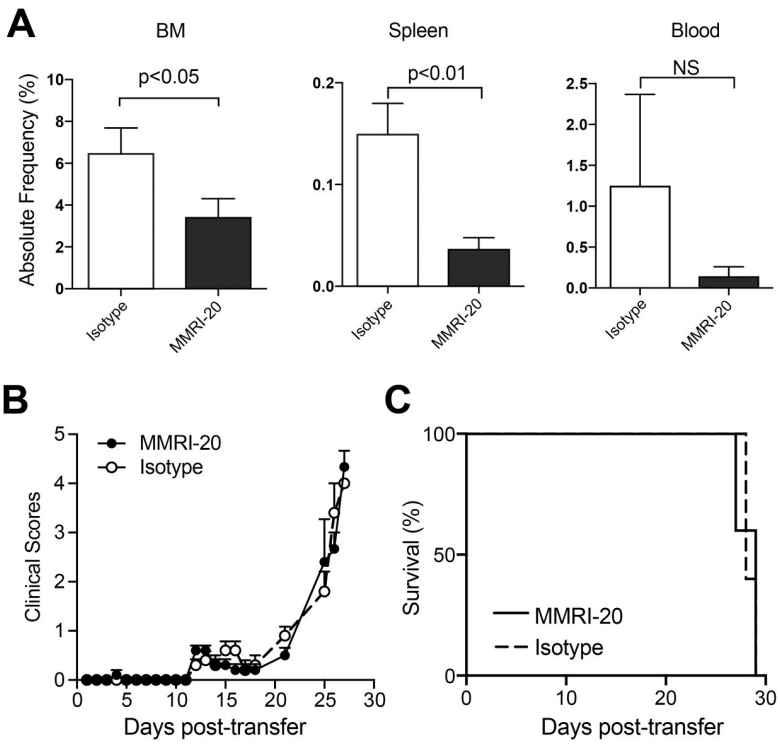

Fig 3. *Ex vivo* MMRI-20 binding reduces engraftment of leukemic cell lines HL-60 in NOD/SCID mice. (A) Bar graphs showing absolute frequency of HL-60 cells coated with MMRI-20 or isotype control mAb in BM, spleen and blood of NOD/SCID mice 21 days after iv injection (n = 6/group). HL-60 cells were identified as human $CD33^+$, human $CD45^+$, mouse $CD45^-$ cells in tissue cell suspensions by flow cytometry (see panel B of S1 Fig). (B) Disease scores and (C) survival curves of five NOD/SCID mice injected with MMRI-20 or isotype control mAb coated HL-60 cells.

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all samples (Fig 4A and 4B). To determine whether $CD302^-$ progenitor populations could maintain normal haematopoiesis, we compared the CFU potential of $CD302^+$ or $CD302^-$ populations. The two progenitor fractions were isolated by FACS based on MMRI-20 staining and CFU of various blood developmental lineages deriving from each set were compared. Generation of CFU of all lineages were completely abrogated in the $CD302^-$ fraction (Fig 4C and 4D), suggesting that the majority of HSC progenitors were contained in the $CD302^+$ fraction.

Characterisation of CD302 expression by liver cells

Expression of CD302 in liver was previously reported [11, 12]. We compared the transcriptional expression of *CD302* in human liver, $CD14^+$ monocytes, HepG2, HL-60, and U937 using quantitative PCR (panel A of S3 Fig). Consistent with our previously studies [11, 12], liver showed the highest expression of *CD302* amongst the tested samples. We then investigated if the MW of human CD302 protein in liver was different to that of leukemic cells by Western blot (panel B of S3 Fig). In contrast to the mouse studies, CD302 in the human liver cell line HepG2 showed a similar MW to that in the HL-60 leukemic cell line. When HepG2 was labelled with MMRI-20 and examined by flow cytometry or immunohistology, we observed that that CD302 protein was primarily intracellular with minimal expression on the cell surface (panel C-D of S3 Fig). This was in contrast to the MMRI-20 staining pattern of the leukemic HL-60 cell line, where surface CD302 staining was detected by both techniques.

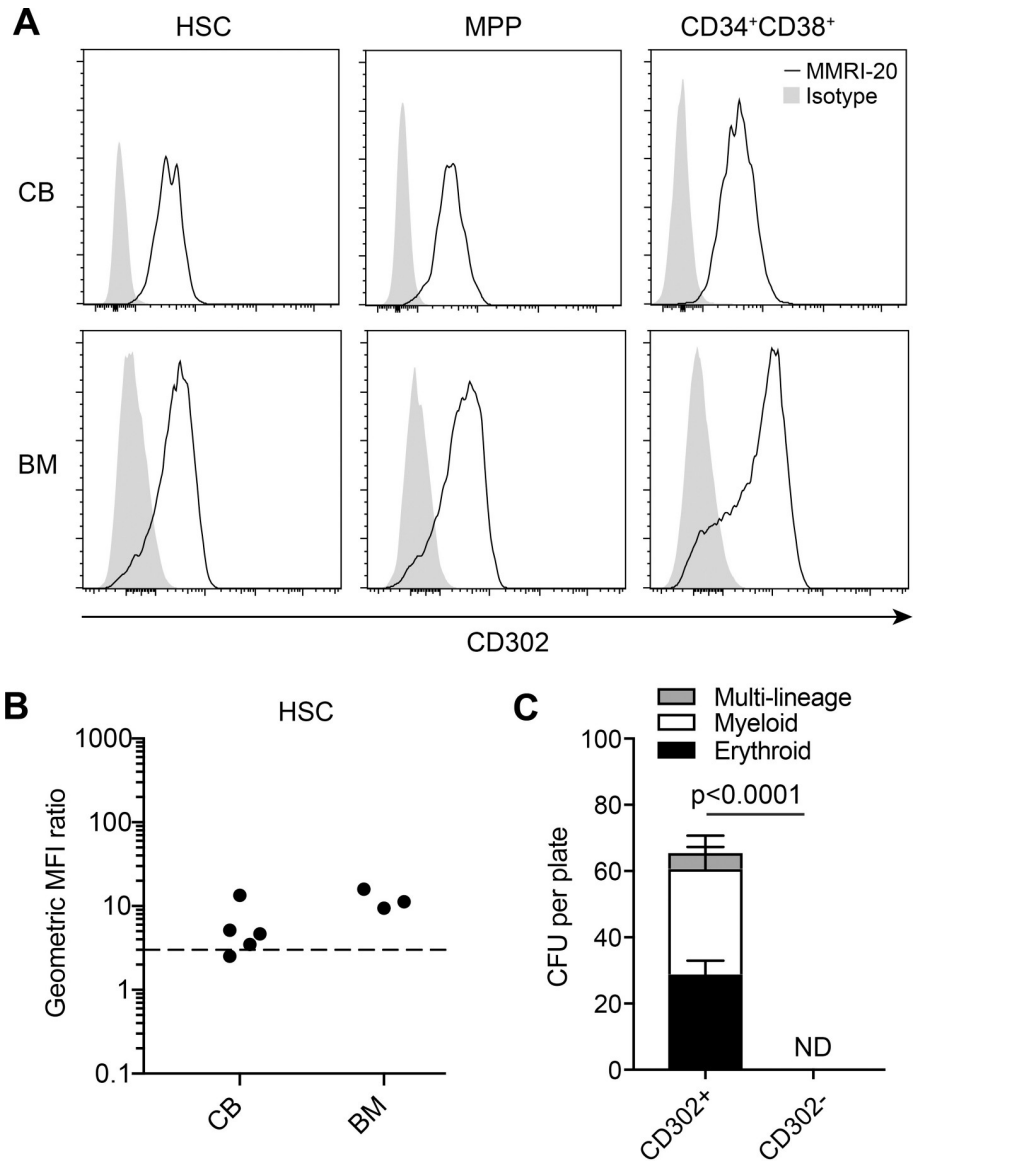

Fig 4. CD302 is expressed on healthy BM and cord blood. (A) Representative flow cytometry histograms showing the expression of CD302 on healthy BM (n = 3) and CB (n = 5) HSC, MPP and $CD34^{+}CD38^{-}$ populations. Gating strategy shown in panel A of S1 Fig. (B) geoMFI ratios of CD302 expression on HSC in all BM and CB samples. Dotted line indicates a geoMFI of 3 above which was considered positive. (C) Graphs showing CFU of multi-lineage, myeloid and erythroid lineages counted 12–14 days after seeding wells with equal numbers of $Lin^{-}CD302^{+}$ and $CD302^{-}$ sorted CB populations (n = 6). ND, no detection of CFU colonies.

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PBD toxin delivery through CD302 mAb mediates killing of leukemic but not hepatic cell lines

To examine the potential of CD302 as an ADC target, HL-60 or HepG2 cells were co-cultured with either MMRI-20 or isotype control antibody in the presence of GAM IgG mAb bound to PBD (GAM-PBD). PBD delivered high toxicity towards HL-60 via MMRI-20 in a dose dependent manner when compared to the isotype control (Fig 5A). In contrast, HepG2 co-cultured with either MMRI-20 or isotype antibody plus GAM-PBD both showed equivalent, minimal toxicity, suggesting that the killing of HepG2, was not CD302 specific (Fig 5A). Similarly, we

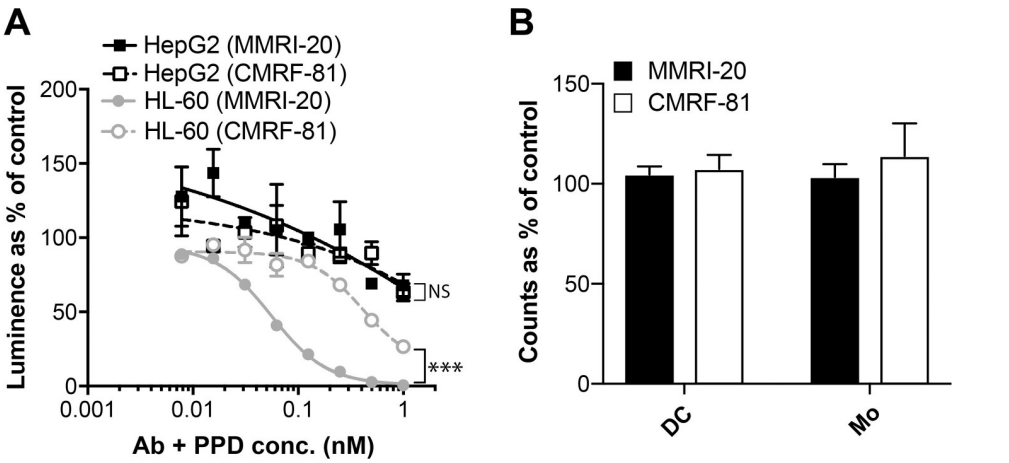

Fig 5. PBD delivery through CD302 mAb mediates killing of leukemic but not hepatic cell lines. (A) Comparison of HepG2 or HL-60 killing after 96 h culture with the indicated concentrations of MMRI-20 or isotype control mAb together with equimolar concentrations of a GAM IgG secondary antibody conjugated with PBD toxin. Cell viability measured with the CellTiter-Glo luminescent assay and compared as a % to untreated controls. One of three representative experiments shown. $^{***}p<0.001$, 2-way ANOVA. (B) PBMC were cultured in quadruplicate for 96h with 1nM MMRI-20 or isotype control mAb together with equimolar concentrations of a GAM IgG secondary antibody conjugated with PBD toxin. Flow cytometry was used to count viable ($DAPI^-$) dendritic cells (Lin^- $HLA\text{-}DR^+CD11c^+$) and monocyte ($SSC^{hi}Lineage^+HLA\text{-}DR^+CD11c^+$) in wells after culture and compared as a % to untreated controls. One of two representative experiments shown.

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found no toxicity towards dendritic cells or monocytes when PBMC were cultured with MMRI-20 or isotype antibody plus GAM-PBD for 96h (Fig 5B).

Discussion

Immunotherapies involving mAbs and their derivatives have proven to be encouraging strategies for treating haematological malignancies. There is a pressing need for alternative targets for AML treatment, especially those expressed by LSC that cause disease relapse. We have identified the CLR CD302 as a possible AML target. Using the mouse anti-human CD302 mAb MMRI-20, we showed expression of CD302 on primary AML blast and various leukemic cell lines. The vast majority of primary AML samples expressed CD302 (88%) with significant correlation to CD33 (a clinically established target for AML) at the transcript, cellular and patient level. Importantly, MMRI-20 bound to $CD34^+CD38^-$ blasts, a population enriched in LSC, in 80% of patient samples.

There are various therapeutic mechanisms of an anti-AML mAb including ADCC through recruitment of immune effector cells or through inhibition of critical functions of AML [5]. MMRI-20 could mediate the ADCC of leukemic cell lines expressing high or low levels of CD302. In spite of the role for CD302 in migration [11, 12], binding of MMRI-20 to HL-60 leukemic cells did not alter their mobilisation towards the BM-homing chemokine CXCL12 in fibronectin or stromal cell transmigration assays. However, we cannot rule out that the antibody could inhibit migration in other experimental settings. Future identification of the ligand for CD302 will provide insight into whether antibodies can block ligand interaction and how this alters AML migration.

Further investigation into the therapeutic capability of the MMRI-20 mAb for AML was performed in an *in vivo* xenogeneic NOD/SCID model. Binding of CD302 with MMRI-20 reduced engraftment of the HL-60 leukemic cell line in BM, spleen and blood. However, the ADCC function of the naked mouse antibody in this model was likely hampered by the low

natural killer and macrophage activity in NOD/SCID mice [16], which may explain why HL-60 was not completely eliminated from hosts and why this did not lead to a survival advantage.

A common strategy for improving the therapeutic activity of anti-cancer antibodies is to attach a toxin or radioisotope for delivery to target cells (i.e. ADC). Like other CLR, CD302 is endocytic and was previously shown to take up MMRI-20 antibodies in CD302 transfected Chinese hamster ovary cells and healthy blood myeloid cells [11]. The rapid internalisation of MMRI-20 by the HL-60 leukemic cell line denoted CD302's potential as a target for ADC. Evidence for the likely efficacy of a CD302 ADC was provided with the efficient killing (sub-nanomolar IC50) of HL-60 cells using a secondary mAb to deliver PBD via MMRI-20 internalisation.

Target molecules for AML to date have unavoidably shown wider expression on healthy myeloid populations [5]. This is also the case with CD302, which can be found on monocytes, macrophages, granulocytes and dendritic cells [11, 12]. MMRI-20 was also shown to bind HSC in healthy BM and cord blood samples. Although the presence of CD302 on healthy haematopoietic cells might cause potential toxicity against these populations, studies have shown that ADC targeting markers (e.g. gemtuzumab ozogamicin with CD33) expressed by HSC progenitors and myeloid populations [17] can still provide clinical benefits for patients [18]. The presence of CD302 on HSC would therefore not necessarily exclude it as a potential therapeutic target. Haematological toxicity could be managed by adding CD302 mAb or ADC treatment to conditioning therapies given prior to HSCT.

Another consideration for utilising CD302 as an AML target is its expression in liver, raising concerns of hepatotoxicity [11]. Mouse studies have shown that CD302 transcript is expressed by hepatocytes and liver sinusoidal endothelial cells [12], although the liver protein appeared to exhibit a different MW to that in myeloid cells. Western blot analysis of the human liver cell line HepG2 and leukemic cell line HL-60 showed a similar MW for CD302. However, in contrast to the abundant surface CD302 found on HL-60, we predominantly detected intracellular distribution on the HepG2 cell line with undetectable surface expression by flow cytometry or immunohistology. Consistent with this difference, delivery of PBD via CD302 could kill HL-60 but not HepG2, raising the possibility that a CD302 ADC could deliver a therapeutic effect against AML cells with minimal liver toxicity. It is noted that this observation needs confirmation in primary liver cells and further study into CD302 function in this organ is required.

Conclusion

The heterogeneous nature of AML and the urgent need for new therapeutics makes characterising all potential target markers a necessity. CD302 is expressed highly on blasts and LSC enriched $CD34^+$ $CD38^-$ populations in the majority of AML patients thereby showing potential as a therapeutic AML target. Further studies are required to investigate the potential toxicity of a CD302 mAb and derivatives in healthy human tissue and establish the ideal therapeutic window for their use.

Supporting information

S1 Table. Clinical and pathological characteristics of AML patient samples tested in the current study.
(DOCX)

S1 Fig. Gating strategies. (A) Gating used to identify progenitor cells in human BM and CB samples (CB shown). Gating strategies to identify HL-60 leukemic cells in NOD/SCID BM,

spleen and blood (BM shown).
(TIF)

S2 Fig. Microarray analysis of transcript levels of CD302 and CD33 in AML patients. (A) Probes specific for *CD302* and *CD33* transcripts were compared in a cohort of 460 AML patients over various FAB subtypes. Statistics shown in table below. (B) Correlation of CD302 and CD33 gene expression in all patients.
(TIF)

S3 Fig. CD302 is expressed minimally on the surface of human liver cells. (A) Transcript expression of CD302 relative to the HPRT housekeeping gene was determined by qPCR in three cDNA samples derived from human liver, monocytes or the indicated cell lines. Expression shown as fold changes relative to the U937. (B) Western blot comparing the size of CD302 protein band in HepG2 and HL-60 cells. (C) Comparison of flow cytometry CD302 surface staining of HepG2 and HL-60 cell lines with MMRI-20 compared to an isotype control. (D) Immunohistology staining of CD302 (green) with MMRI-20 in HepG2 or HL-60 cells. Phalloidin staining (red) was used to highlight the cellular surface while DAPI (blue) staining reveals the nucleus. A composite of phalloidin and DAPI with MMRI-20 or isotype control antibody staining is shown for comparison. Scale bar marks 20μm.
(TIF)

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Writing – review & editing: Pablo A. Silveira, Georgina J. Clark.

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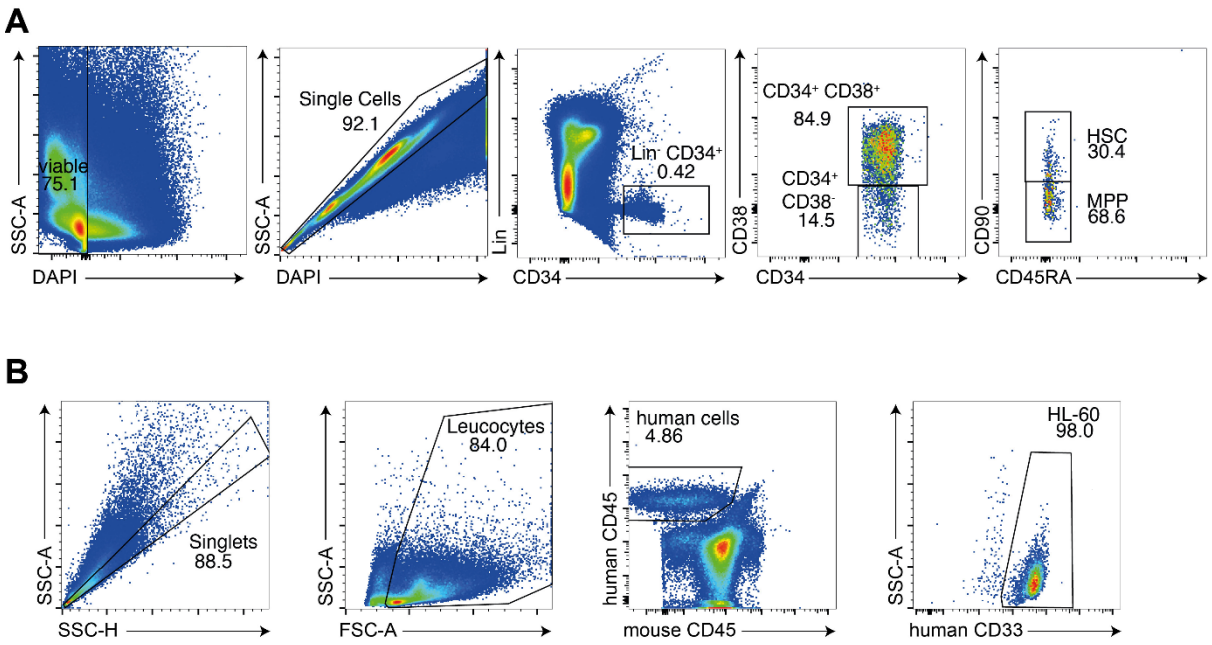
Supplementary Table 1

Age	Sex	Sample	WHO Diagnosis	FAB	WCC	BM blasts %	Karyotype	NPM1	FLT3-ITD
23	M	BM	AML with inversion 16	M4Eo	160	60	46,XY,inv(16)(p13;q22)	Negative	Negative
44	F	PB	AML with inversion 16	M4Eo	35	50	Inversion 16	Negative	Negative
51	UD	BM	AML with inversion 16	M4Eo	UD	UD	Inversion 16	NT	NT
16	M	BM	AML with inversion 16	M4Eo	111	45	Inversion 16	NT	NT
76	F	BM	AML not otherwise specified	M4	14	70	Normal	NT	NT
74	M	PB	AML not otherwise specified	M2	16	43	+8	NT	NT
59	M	PB	AML not otherwise specified	M5	146	95	Normal	NT	NT
51	M	PB	AML not otherwise specified	M0	2.5	73	No metaphases	Negative	Negative
86	F	PB	AML not otherwise specified	M1	23	NT	unknown	NT	NT
61	F	BM	AML not otherwise specified	M6	0.7	26	complex	NT	NT
41	F	BM	AML not otherwise specified	M5	5.2	78	complex	NT	Negative
59	M	BM	AML not otherwise specified	M0	14	60	Normal	Negative	Negative
24	M	PB	AML not otherwise specified	M5	64	90	t(11;17), +8	Negative	Negative
73	F	PB	AML not otherwise specified	M0	8	85	Normal	Detected	Negative
79	M	BM	AML not otherwise specified	M2	55	60	+6	Negative	Negative
61	M	BM	AML not otherwise specified	M2	7	25	No metaphases	NT	NT
44	UD	BM	AML not otherwise specified	M1	UD	UD	t(9:11)	NT	NT
86	UD	BM	AML not otherwise specified	M4	UD	UD	+8	NT	NT
19	UD	BM	AML not otherwise specified	M2	UD	UD	monosomy 7	NT	NT
61	UD	BM	AML not otherwise specified	M4	UD	UD	Normal	NT	NT
77	F	PB	AML with myelodysplasia-related changes	M2	128	55	Normal	NT	NT
89	M	PB	AML with myelodysplasia-related changes	M2	30	16	Normal	NT	NT
59	M	PB	AML with myelodysplasia-related changes	M4	54	56	Normal	Negative	Detected
95	F	PB	AML with myelodysplasia-related changes	M5	63	NT	unknown	NT	NT
82	M	PB	AML with myelodysplasia-related changes	M5	3.8	53	+19	NT	NT
75	M	PB	AML with myelodysplasia-related changes	M2	6.5	60	Normal	NT	NT
72	F	PB	AML with myelodysplasia-related changes	M5	30	80	Complex;del(12)(p12),del(13)(q21), add(17)(p11.2)[1]46,XX[1]	NT	NT
70	M	BM	Therapy related myeloid neoplasm	M4	16	82	Normal	Detected	Detected
79	M	PB	Therapy related myeloid neoplasm	M4	150	67	46, XY, add(12)(q24.3)	NT	NT
87	M	PB	Therapy related myeloid neoplasm	M2	2.2	62	47,XY,t(8;16)(p11;p13), +8	NT	NT
75	M	PB	Myelodysplastic syndrome with excess blasts	N/A	3.5	15	+8	NT	NT
81	M	PB	Chronic myelomonocytic leukemia	N/A	5.7	10	Normal	NT	NT
55	M	BM+PB	Blastic plasmacytoid dendritic cell neoplasm	N/A	4.3	85	Normal	Negative	Negative

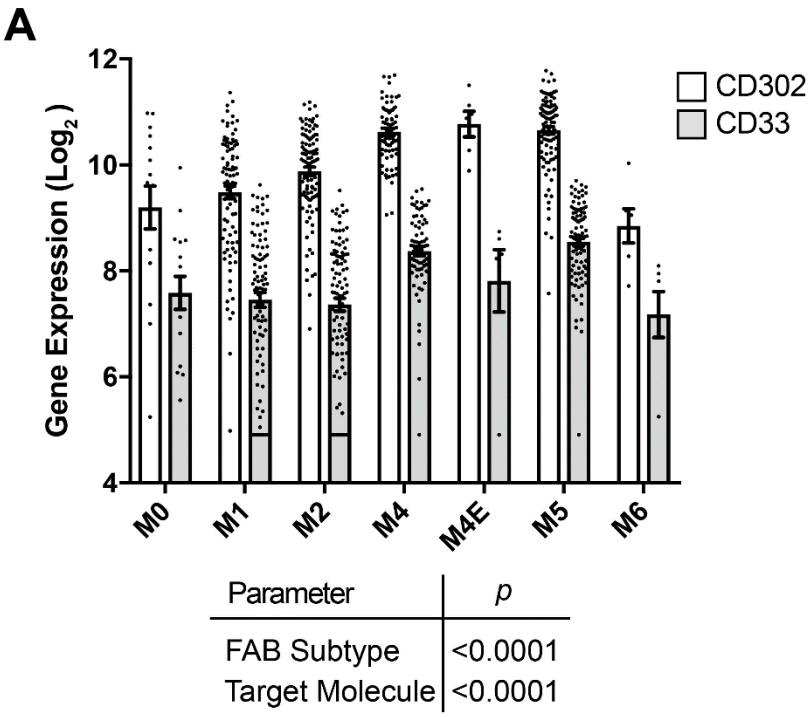
Clinical and Pathological Characteristics of AML Patient Samples Tested in the Current Study.

Supplementary Figure 1

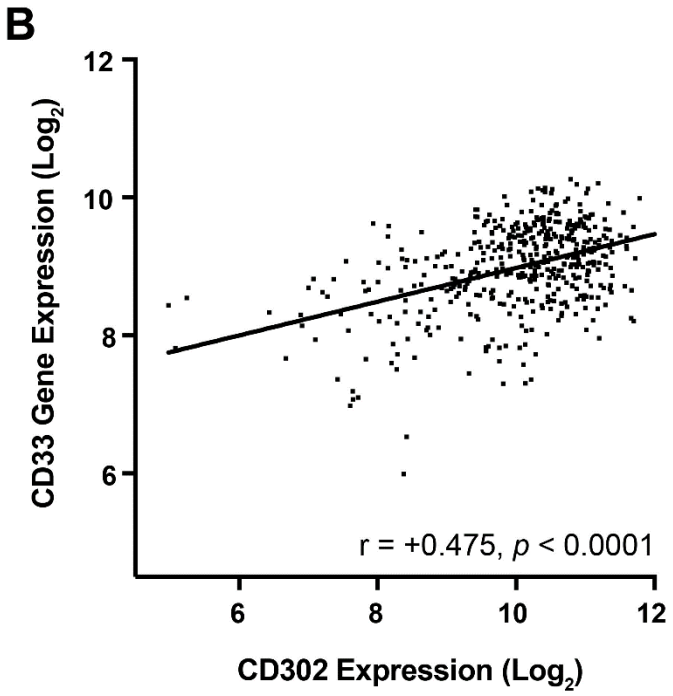


(A) Gating used to identify progenitor cells in human BM and CB samples (CB shown). Gating strategies to identify HL-60 leukemic cells in NOD/SCID BM, spleen and blood (BM shown).

Supplementary Figure 2

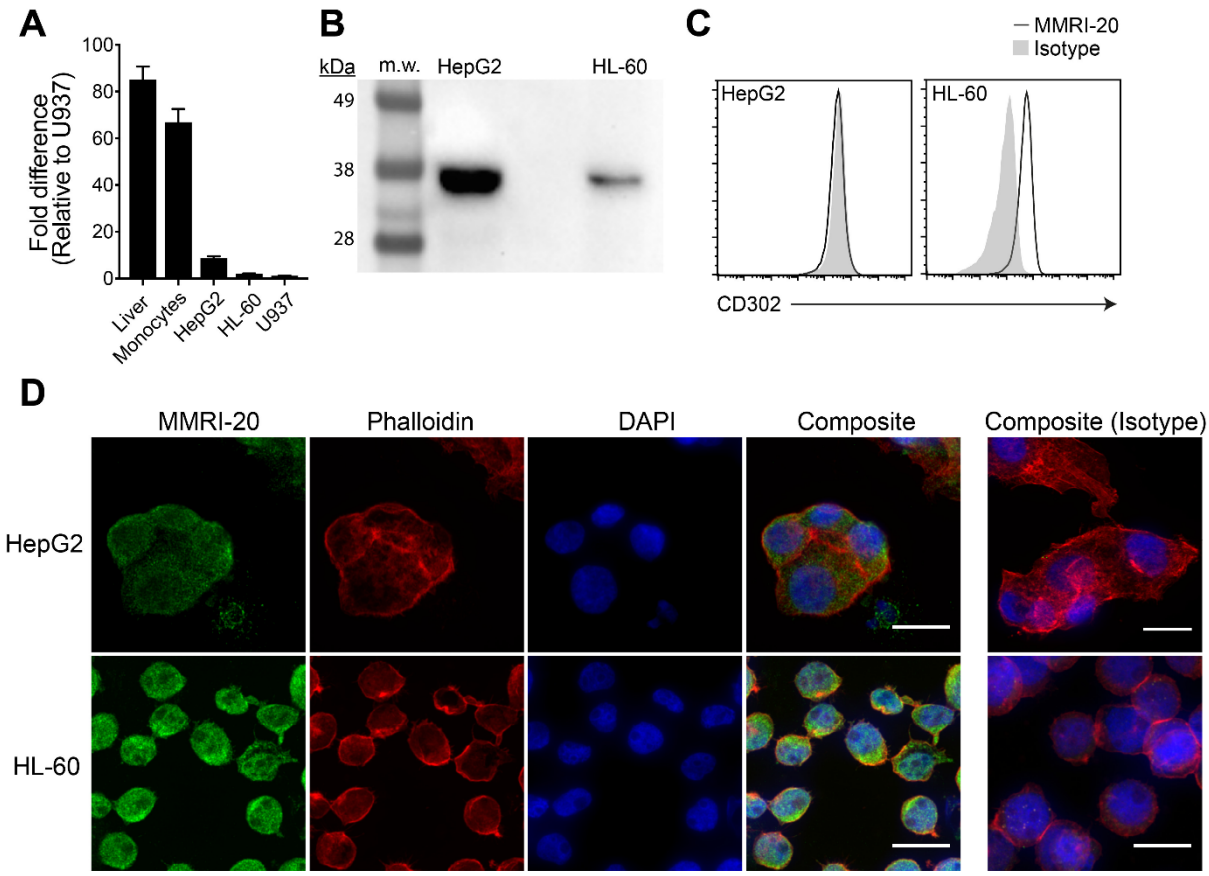


Parameter	*p*
FAB Subtype	<0.0001
Target Molecule	<0.0001



(A) Probes specific for *CD302* and *CD33* transcripts were compared in a cohort of 460 AML patients over various FAB subtypes. Statistics shown in table below. (B) Correlation of CD302 and CD33 gene expression in all patients.

Supplementary Figure 3



(A) Transcript expression of CD302 relative to the HPRT housekeeping gene was determined by qPCR in three cDNA samples derived from human liver, monocytes or the indicated cell lines. Expression shown as fold changes relative to the U937. (B) Western blot comparing the size of CD302 protein band in HepG2 and HL-60 cells. (C) Comparison of flow cytometry CD302 surface staining of HepG2 and HL-60 cell lines with MMRI-20 compared to an isotype control. (D) Immunohistology staining of CD302 (green) with MMRI-20 in HepG2 or HL-60 cells. Phalloidin staining (red) was used to highlight the cellular surface while DAPI (blue) staining reveals the nucleus. A composite of phalloidin and DAPI with MMRI-20 or isotype control antibody staining is shown for comparison. Scale bar marks 20µm.

Chapter 4: Is hematopoietic stem cell transplantation required to unleash the full potential of immunotherapy in acute myeloid leukemia?

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Review

Is Hematopoietic Stem Cell Transplantation Required to Unleash the Full Potential of Immunotherapy in Acute Myeloid Leukemia?

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Abstract: From monoclonal antibodies (mAbs) to Chimeric Antigen Receptor (CAR) T cells, immunotherapies have enhanced the efficacy of treatments against B cell malignancies. The same has not been true for Acute Myeloid Leukemia (AML). Hematologic toxicity has limited the potential of modern immunotherapies for AML at preclinical and clinical levels. Gemtuzumab Ozogamicin has demonstrated hematologic toxicity, but the challenge of preserving normal hematopoiesis has become more apparent with the development of increasingly potent immunotherapies. To date, no single surface molecule has been identified that is able to differentiate AML from Hematopoietic Stem and Progenitor Cells (HSPC). Attempts have been made to spare hematopoiesis by targeting molecules expressed only on later myeloid progenitors as well as AML or using toxins that selectively kill AML over HSPC. Other strategies include targeting aberrantly expressed lymphoid molecules or only targeting monocyte-associated proteins in AML with monocytic differentiation. Recently, some groups have accepted that stem cell transplantation is required to access potent AML immunotherapy and envision it as a rescue to avoid severe hematologic toxicity. Whether it will ever be possible to differentiate AML from HSPC using surface molecules is unclear. Unless true specific AML surface targets are discovered, stem cell transplantation could be required to harness the true potential of immunotherapy in AML.

Keywords: AML immunotherapy; hematopoietic stem cell toxicity; Chimeric Antigen Receptor (CAR) T cells; antibody drug conjugates

1. Introduction

Until recently, therapeutic options for Acute Myeloid Leukemia (AML) had changed very little. In the last decade, there has been a substantial increase in knowledge of the molecular landscape of AML, which has led to several new targeted therapies [1]. However, many of these molecular therapies have limited duration of action when not combined with conventional chemotherapy [2]. Since the FDA approval of Rituximab in 1997, immunotherapy has become an increasingly important part of the management of hematologic malignancies [3]. Unconjugated antibodies, Antibody Drug Conjugates (ADC), Bispecific T Cell Engagers (BiTEs), and Chimeric Antigen Receptor (CAR) T cells are all now part of accepted therapy.

More potent forms of immunotherapy, such as Chimeric Antigen Receptor (CAR) T cells, lead to target cell aplasia, which is tolerable in B cell malignancies [4–6]. Indeed, nearly all the benefits of targeted immunotherapy are in the setting of B cell malignancies. Aside from Gemtuzumab Ozogamicin (GO), no immunotherapies have been accepted for hematologic malignancies of myeloid origin [7]. The limited efficacy in AML to date highlights the challenges presented.

Most surface molecules expressed on AML are also expressed on Hematopoietic Stem and Progenitor Cells (HSPC), and potent immunotherapies against traditional AML molecules (CD33 and CD123) have led to hematologic toxicity, both in preclinical models and in clinical trials [8–10]. An additional issue is that the complex subclonal architecture and plasticity of surface molecules makes tumor escape a significant concern [11–14]. Despite these challenges, new immunotherapies against AML continue to be tested. Allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT) is an established cellular therapy in AML, but the toxicity and limited efficacy, especially in high risk patients, allows for potential improvement. In this procedure, patients undergo conditioning chemotherapy with or without radiotherapy followed by transfusion of donor hematopoietic cells. Immunosuppression is required after the transplant to reduce the chance of Graft Versus Host Disease (GVHD). Allo-HSCT has been shown to reduce the relapse rate of AML and is the only potentially curative therapy in those with refractory disease [15,16]. The decision on whether to offer an allo-HSCT is complex and considers patient fitness, risk of AML relapse, and availability of donors. Generally, if a suitable well-matched donor is available allo-HSCT is recommended for those with intermediate or adverse risk disease [15]. The major limitation is that most patients are not fit for the procedure due to their age at diagnosis and other comorbidities. Autologous HSCT is an alternative to allo-HSCT that is associated with reduced toxicity, but is only effective in patients without high risk disease and is not widely utilized in all jurisdictions [17].

While the majority of the emerging anti-AML immunotherapies seek to control disease without allo-HSCT by sparing HSPC (Figure 1), others look to targeted immunotherapy to act as a component of allo-HSCT and accept that depleting HSPC is required to control AML (Figure 2).

Graft Versus Leukemia (GVL) in allo-HSCT provides evidence that immune mechanisms can reduce relapse risk in AML. The evidence of GVL was seen in preclinical models and confirmed clinically with the observations of reduced relapse rates in patients who develop GVHD, and an increased relapse rate in patients who receive T cell depleted grafts [18,19]. The immune mechanism of GVL is complex; incompletely understood; and involves T, NK, and antigen-presenting cells with likely multiple leukemia antigens [20]. This contrasts to current immunotherapies, which primarily target a single molecule. Immunotherapy can often play a complementary role with allo-HSCT, acting as bridge to transplant. This is seen in B-cell Acute Lymphoblastic Leukemia (B-ALL), where the ADC, Inotuzumab Ozogamicin, and Bispecific T Cell Engagers (BiTE), Blinatumomab, have been shown to eliminate leukemic cells capable of achieving Minimal Residual Disease (MRD) negativity prior to allo-HSCT [21,22]. The emergence of immunotherapies altering the treatment landscape in regard to allo-HSCT can be seen in the field of B-ALL. CD19 CAR T cells are capable of inducing sustained Complete Remission (CR), thus potentially minimizing the role of allo-HSCT in children and young adults with relapsed ALL [23]. Despite the emergence of CAR T cells in B-ALL, it is unclear if the need for allo-HSCT will be reduced over time or if these therapies will play complementary roles. Given that relapse occurs post CD19 CAR T cell therapy, the role of consolidation with an allo-HSCT is not currently established [24,25].

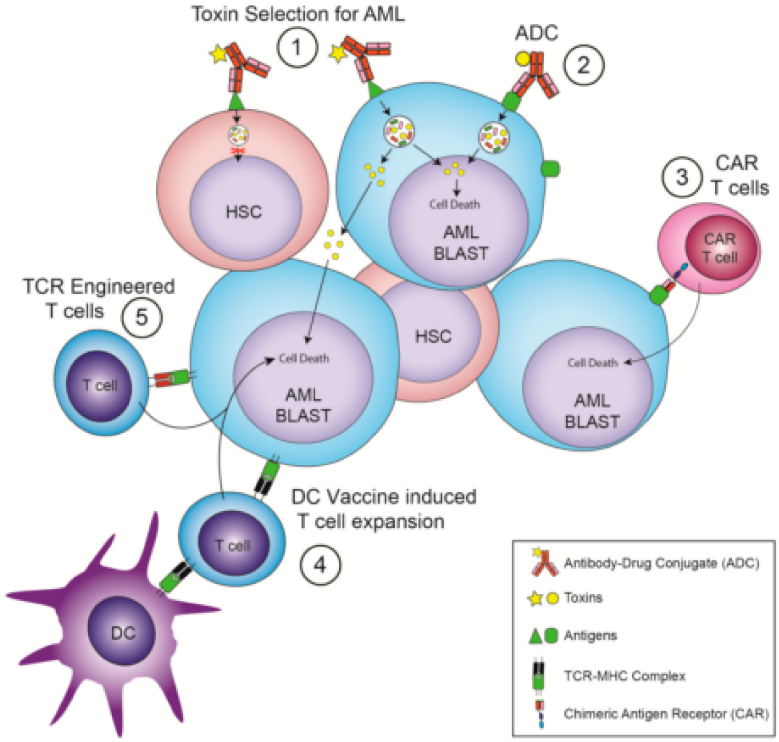

Figure 1. Immunotherapies that avoid the need for hematopoietic stem cell transplantation. (1) Antibody Drug Conjugates (ADC) that recognize Hematopoietic Stem Cells (HSC) and Acute Myeloid Leukemia (AML) but whose toxin selectively kills AML cells. (2) ADC that recognize AML-specific surface targets. (3) Chimeric Antigen Receptor (CAR) T cells that recognize AML-specific surface targets. (4) Dendritic cell (DC) vaccination leading to a T cell response against AML-specific intracellular targets. (5) T cells expressing T-cell receptors (TCRs) engineered to recognize AML-specific intracellular antigens displayed by a Major Histocompatibility Complex (MHC) molecule.

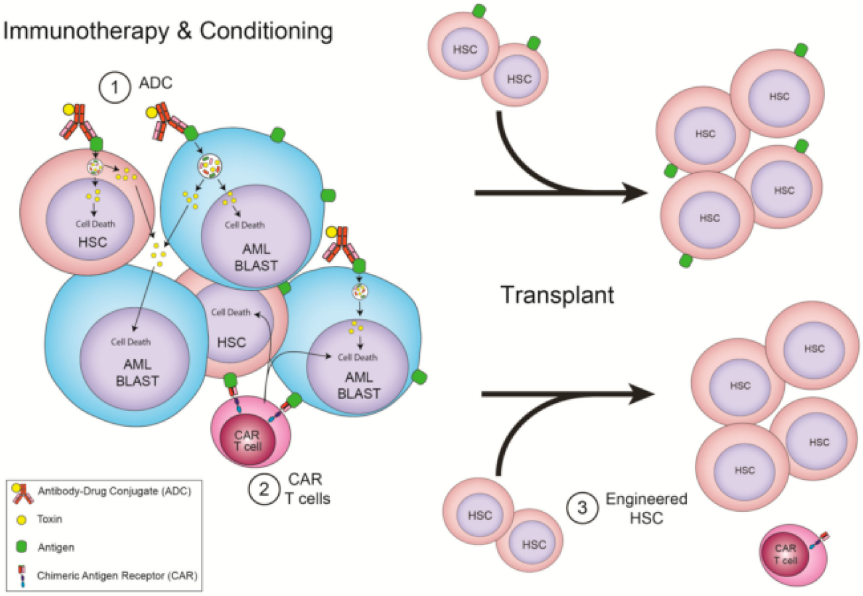

Figure 2. Immunotherapies that require allo-HSCT. (1) ADC that recognize HSC and AML shared antigens as conditioning for a transplant. (2) CAR T cells that recognize HSC and AML shared antigens and are depleted with conditioning for a transplant. (3) CAR T cells that recognize HSC and AML shared antigens as conditioning for a transplant, but whose donor cells have the target removed, allowing for CAR T cell persistence.

Optimal disease control prior to allo-HSCT in AML is an important part of reducing relapse risk. Patients with refractory disease going into to transplant have a much higher risk of relapse compared to those in CR [26,27]. Those who are MRD-positive at the time of transplant have worse outcomes [28–30]. Even though MRD positivity is associated with a high relapse rate post allo-HSCT, these patients may still benefit from transplant [31]. Immunotherapy in AML may contribute as a bridge to transplant, as has been established in B-ALL [21,22]. There are already emerging strategies reported of adding targeted AML agents to conventional conditioning regimens, which can reduce the relapse risk post-transplant in high-risk patients with AML [32]. As AML and HSPC share antigens, it is possible that the step of disease depletion with immunotherapy prior to allo-HSCT can also act as a form of conditioning. This application would have the advantage of depleting residual disease and reducing nonhematologic toxicity by potentially replacing nonspecific conditioning agents that eliminate host HSPC. The disadvantage of this strategy is the requirement of an allo-HSCT.

Allo-HSCT carries significant limitations. The median age of diagnosis of AML is 72, which prevents a majority of patients from accessing allo-HSCT due to treatment related toxicity [33]. Allo-HSCT requires chemotherapeutic agents as conditioning to suppress the host's immune system while removing residual AML and HSPC. These factors are critical to allow for the safe engraftment of donor cells. The development of nonmyeloablative and Reduced Intensity Conditioning (RIC) regimens has allowed more patients to benefit from allo-HSCT by reducing the treatment-related mortality [34]. These developments have made transplantation in selected patients over the age of 65 and increasingly over the age of 70 possible, but not without significant morbidity and mortality [35,36]. In attempts to broaden the benefits of allo-HSCT, there is an emerging effort to reduce treatment related mortality from allo-HSCT by replacing traditional conditioning immunotherapy [37]. Clinical trials (NCT02963064) have been established in children with severe combined immunodeficiency receiving chemotherapy and radiation free conditioning with a CD117 antibody, with early reports demonstrating partial engraftment [38]. An antibody-based conditioning strategy against CD117 has been shown to be effective in eliminating myelodysplastic syndrome HSPC and allowing for engraftment of healthy HSPC preclinically [39]. This emerging field will likely converge with AML immunotherapy due to shared targets.

Is Disease Control Linked to Hematologic Toxicity? Implications from Targeting CD33

CD33/Siglec-3 is a protein from the immunoglobulin superfamily, with expression that ranges from myeloid progenitors to mature myeloid cells [40]. CD33 is expressed in 90–95% of AML [41,42]. Positivity is often defined by a specific median fluorescent intensity ratio by flow cytometry, using either an external control or an internal negative control such as lymphocytes [42–44]. Despite the vast majority of cases meeting positive median fluorescent intensity ratio criteria, a substantial proportion of cases have less than 70% AML cells expressing CD33 when examined on an individual cell level and this impacts the efficacy of immunotherapy [44]. It is accepted that CD33 is expressed on myeloid progenitors but there have been conflicting reports on the expression on Hematopoietic Stem Cells (HSC) [45,46]. Functional studies demonstrating CD33+ cells are capable of serial engraftment in immunodeficient mice suggests its expression on HSC [45].

Gemtuzumab Ozogamicin (GO) is an ADC using a calicheamicin warhead and the only currently listed form of immunotherapy for AML. Its history with regulatory authorities is long and complex. GO was given accelerated approval in 2000 by the FDA only for it to be withdrawn in 2010 [47,48]. The results of the ALFA-0701 study and a meta-analysis demonstrating improved survival in low to intermediate risk patients led to a subsequent GO approval by the FDA in 2017 for the treatment of AML in the upfront setting with a fractionated dosing schedule [48–51]. While the increased rates of sinusoidal obstruction syndrome (SOS), especially with higher doses, are the best known adverse effect of GO, hematological toxicity has been a feature of GO across several studies [48]. The results from the ALFA-0701 study using the recommended fractionated dosing schedule during induction showed prolonged neutropenia after consolidation phases as well as prolonged and persistent thrombocytopenia

after induction [50]. When used outside of intensive chemotherapy, GO was associated with universal pancytopenia but did increase survival compared to best supportive care [52]. While GO has tolerable hematologic toxicity and is capable of benefiting some patients with AML, there is still a very large proportion of patients who do not benefit from it. More potent forms of immunotherapy have been targeted against CD33, and while these may have theoretical advantages in efficacy, they come at the price of increased hematologic toxicity.

Vadastuximab talirine (VT), SGN33a, is an anti-CD33 ADC utilizing a PyrroloBenzodiazepine Dimer (PBD) toxin. VT has a number of theoretical advantages over GO, including uniform drug loading, effectiveness in multidrug resistance positive cell lines, and activity against AML with adverse cytogenetics [53]. VT has displayed impressive CR rates, including MRD negativity when used in the upfront setting as well as a higher rate of response when combined with hypomethylating agents compared to historical controls [54,55]. The potential for significant myelosuppression by targeting CD33 was supported by a dose limiting toxicity (60 ug/kg) of hypocellular marrow in a phase I trial for VT [10]. The outcomes of older patients with newly diagnosed AML treated with VT monotherapy revealed that prolonged neutropenia (median 6.1 weeks) and thrombocytopenia (median 5.1 weeks) were common in those who achieved CR or CR with incomplete recovery [56]. A phase 3 trial of hypomethylating agents +/- VT was suspended after excess deaths due to infection were observed in the VT arm [57]. With increasingly potent therapeutics available, avoiding hematologic toxicity while remaining effective against AML may require an allo-HSCT as a rescue.

2. Strategies Trying to Mitigate Hematologic Toxicity

2.1. Alternate Surface Targets

While there remains several ongoing strategies to target CD33 in AML, the challenges in the development of VT highlight some of the difficulties with this target [58]. Alternate targets with reduced expression across HSPC have become appealing given the potential for reduced hematologic toxicity.

C-Type Lectin-Like Molecule 1 (CLL-1, CD371) is a surface molecule found on AML and committed myeloid progenitors [59]. A CLL-1xCD3 BiTE was broadly effective against a range of AML cell lines [60]. It induced neutropenia that recovered by day 22 in cynomolgus monkeys. This finding confirms the potential for neutropenia, but the recovery suggests a potential to spare HSC. An ADC targeting CLL-1 was effective against in AML cell lines in vitro and in vivo [61]. The ADC significantly reduces granulocytic Colony Forming Units (CFU) while sparing erythroid CFUs. This ADC does not reduce the number of human $CD45^+$ cells in a humanized mouse model. A different ADC against CLL-1 with a PBD toxin depleted AML cell lines and primary AML in vitro and prolonged survival of mice xenografted with an AML cell line [62]. Limited preclinical hematologic toxicity was demonstrated, as the ADC caused reversible neutropenia in cynomolgus monkeys without a reduction in platelets or lymphocytes. These results suggest that an anti-CLL-1 immunotherapy may lead to transient neutropenia with a predictable recovery. While neutropenia is a significant toxicity, it is common amongst AML therapeutics and the consistent recovery across different therapeutics suggests that HSC and early progenitors are unlikely to be targeted by an anti-CLL-1 therapeutic.

T cell immunoglobulin mucin-3 (TIM-3, CD366) has emerged as a potential selective target against AML while sparing normal hematopoiesis. TIM-3 is expressed on AML blasts and is upregulated on T cells of AML patients [63,64]. TIM-3 appears to play a role in Leukemic Stem Cell (LSC) establishment, as TIM-3 antibodies can block engraftment of AML in immunocompromised mice [65]. Within HSPC, only a subset of Granulocyte/Macrophage Progenitors express TIM-3 when assessed by flow cytometry [65]. A TIM-3 antibody was able to deplete $TIM\text{-}3^+$ monocytes in a humanized mouse model but did not impair the development of B or myeloid cells [65]. An early report of a TIM-3 antibody in combination with decitabine in hypomethylating agent naive patients has demonstrated limited toxicity but also limited efficacy to date with a CR rate of 14% (2/14) [66]. A CAR T cell has been developed that selectively kills targets expressing CD13 and TIM-3 [67]. This strategy still

depleted HSPC but not as much as targeting CAR T cells directed against only CD13. Weather a limited long-term depletion of HSPC has clinically significant impacts is unknown.

Natural Killer Group 2D (NKG2D) is a surface receptor that is expressed normally on NK and $CD8^+$ T cells, but is also expressed on stressed and malignant cells, including the majority of AML [68,69]. Its absence on resting healthy tissues makes it an attractive target. A case report from a Phase I/II trial (NCT03018405) with a patient who had relapsed $FLT3^+$ AML who underwent NKG2D CAR T cell therapy demonstrates initial promise [70]. The patient achieved a complete morphological response but had evidence of clonal evolution with a new *IDH2* mutation. Despite this finding, he had normal trilineage hematopoiesis, providing evidence of the lack of HSPC expression of NKG2D. He subsequently underwent allo-HSCT, leading to CR with normal molecular studies. It is unclear if AML subclones may have variable NKG2D expression; this case highlights the challenges in targeting a heterogeneous disease like AML with a single surface molecule. More data are required to determine if NKG2D targeted therapy can be a standalone therapy or a bridge to transplant.

CD70 is the ligand of CD27, and this interaction helps to regulate lymphocyte and HSPC activity [71]. CD70 has minimal expression on healthy HSPC and is substantially upregulated in AML as well as other malignancies [72,73]. The blockade of CD70 with a monoclonal antibody inhibits self-renewal of AML and LSC while extending survival in a xenograft model of primary AML [72]. Hypomethylating agents have been shown to induce CD70 expression further on AML, thus suggesting synergistic potential with CD70 antibodies, and a phase II trial using this combination in previously untreated AML is underway (NCT04023526) [74]. What remains unclear is if blocking the CD70/CD27 axis will affect hematopoiesis even if the HSPC themselves do not express CD70.

While many of the targets to date have been expressed on a majority of AML, there have also been efforts to target surface proteins that are aberrantly expressed on AML even if they constitute a minority of cases. CD7 is a cell surface glycoprotein that is normally expressed on T and NK cells as well as their progenitors [75]. CD7 is expressed in 30% of AML cases [76]. The lack of CD7 expression on HSPC reduces the chance for severe hematologic toxicity resulting from targeting CD7. A CAR T cell directed against CD7 depleted AML cell lines in vitro and in vivo but did not reduce CFU formation from normal cord blood [77]. The possibility of CAR T cell fratricide is reduced by editing the CD7 gene on the CAR T cells so they do not display their target [78]. This strategy would only be suitable for a minority of patients with AML, and the impact of immunosuppression from potentially long-term healthy T cell depletion is unclear.

Another method of target selection that may reduce hematologic toxicity is to selectively target markers specific for monocytic differentiation. Leukocyte immunoglobulin-like receptor-B 4 (LILRB4, CD85k, ILT3) is expressed from promonocytes to mature monocytes [79]. LILRB4 is also expressed on AML with monocytic differentiation i.e., M4 and M5 by FAB classification [80]. A CAR T cell directed against LILRB4 demonstrated efficacy against M5 AML cell lines and primary M5 AML in vitro as well as a M5 AML cell line in vivo [81]. It did not reduce CFU numbers and in a humanized mouse model did not deplete $CD34^+$, $CD33^+$, or $CD19^+$ cells. M5 AML was chosen as the target, as M4 AML did not uniformly express LILRB4. The targeting of M5 AML limits the number of potential patients who may benefit as AML with monocytic differentiation accounts for only 5-10% overall, though the proportion is up to 40% in children [82,83].

CD300f is another surface target expressed across AML, mature myeloid cells, and HSPC [84,85]. There are seven isoforms of CD300f described, and it has been shown that exon 4 expression of the protein is selectively upregulated in AML with monocytic differentiation compared to healthy HSPC [85]. In addition, a monoclonal antibody can enact a conformational change of CD300f allowing a second antibody to bind to AML with monocytic differentiation at high affinity, but not to HSPC. CD300f is a promising target in AML with monocytic differentiation, but more work remains to develop a comprehensive strategy to utilize the selective expression and spare HSPC from future therapeutics.

2.2. Intracellular Targets

While AML-specific surface targets have been difficult to validate, intracellular targets have been described that are substantially upregulated in AML compared to normal populations. The best known upregulated AML antigens are WT1 and PRAME, which are found in the majority of AML samples [86]. Despite their suitability for targeting, the difficulty accessing intracellular antigens has limited their development in immunotherapy.

Dendritic cell (DC) vaccines have been the most common strategy to target AML intracellular antigens, with a wide range of manufacturing strategies and targets explored [87]. DCs loaded with AML antigens are able to stimulate T and NK cells to induce an antileukemia effect [88]. The main advantage of DC vaccination is the demonstrated favorable safety profile, which is especially important given the advanced average age at presentation [88]. The main barriers to entering routine clinical practice are the variability across different strategies and limited efficacy [87]. The type of DC used may be one of the limiting factors in vaccination efficacy. Most studies have used monocyte-derived DCs, which are less efficient at antigen presentation and the generation of T cell responses [89,90]. Using blood DC has the potential to overcome some of the limitations in DC vaccine efficacy [91].

An alternate way to target intracellular antigens and possibly increase the efficacy of treatment over DC vaccination is to use T-cell receptor (TCR)-engineered T cells. Engineered T cells can be designed to recognize intracellular antigens expressed by MHC on AML [92]. $CD8^+$ T cells reactive against WT1 on HLA-A*02:01 are functional in vitro, are home to the bone marrow of patients, and persist after reinfusion [93]. Persistence of engineered T cells against WT1 expressed on HLA-A*24:02 has been demonstrated in an MDS setting [94]. The limited patient numbers published make the assessment of clinical efficacy difficult. A central limitation with this approach is that engineered T cells must be made to recognize a specific HLA allele, thereby restricting utility to only those patients who express the targeted HLA.

2.3. Alternate Effector

A separate strategy to reduce hematologic toxicity is to use a toxin as part of an ADC that demonstrates increased efficacy against AML compared to HSPC. It may be possible to find an effective therapeutic window where an ADC can deplete AML while sparing HSPC. IMGN632 is an ADC that targets CD123 and uses an indolinobenzodiazepine pseudodimer alkylator payload that demonstrates activity against AML cell lines and primary AML in vitro as well as AML cell lines in vivo [95]. IMGN632 had an increased therapeutic window when compared to GO with myeloid progenitor and AML CFU assays. The antibody clone of IMGN632, G4723A, was also conjugated to an alternate more potent toxin that had similar IC90 values between AML and myeloid progenitors. The alternate ADC was >40x more toxic to myeloid progenitors than IMGN632, which demonstrates the importance of the toxin over the target in providing an extended therapeutic window.

3. Strategies Incorporating Allogeneic Hematopoietic Stem Cell Transplantation

Due to the difficulties in avoiding HSPC depletion with anti-AML immunotherapies, an alternative strategy would be to incorporate these therapies into allo-HSCT, which is an established therapy for AML. Attempts to target CD123 with CAR T cells demonstrate how immunotherapies against AML may be incorporated into a transplant model. CAR T cells have shown preclinical in vitro and in vivo efficacy against AML, but there are conflicting reports on the potential for toxicity against HSPC. Some studies demonstrated no effect on myeloid progenitors, while others displayed complete myeloablation in humanized mouse models [8,96]. Depleting CAR T cells after a fixed period is a potential strategy to circumvent destruction of donor HSPC during allo-HSCT. CAR T cells can be depleted by targeting their natural or engineered surface molecules. CAR T elimination has been demonstrated with alemtuzumab, or rituximab when CD20 is introduced during CAR T cell manufacturing [97]. A functional proof of CAR T potential in allo-HSCT was demonstrated by using CD123 CAR T cells to deplete human

cells in a mouse sequentially engrafted with human T cell deplete bone marrow and a human AML cell line [97]. This was followed by rituximab administration to remove the CAR T cells before a subsequent sex mismatched human donor graft [97]. "Bio-degradable" CD123 CAR T cells, which lack the ability for indefinite self-perpetuation, were administered to patients as part of a Phase I trial to exclude toxicity [92]. These CAR T cells were well tolerated but did not display efficacy against AML [92]. The correlation between persistence of CD19 CAR T cells and continued response in B-ALL suggests that a prolonged presence of CAR T cells may be required for efficacy; this may limit the effectiveness of short-term CAR T cells. There are currently clinical trials using CD123 CAR T cells in AML that do (NCT03766126) and do not (NCT04109482) require a nominated transplant donor prior to enrolling, as a precaution in the event of bone marrow aplasia.

An innovative way to allow anti-AML CAR T cells to persist is to remove a common target from HSPC. This strategy has been explored by developing CAR T cells against CD33 and then removing the CD33 from an allo-HSCT donor graft [98]. CD33-depleted myeloid cells did not display any significant developmental or functional differences, and they were able to be manufactured from both human and nonhuman primates HSPC [98]. The CD33 CAR T cells were able to deplete an AML cell line in CD33 KO humanized mice without reducing normal myelopoiesis. While this strategy may circumvent many theoretical problems with immunotherapy in AML, practical issues including cost may become limiting. CAR T persistence would likely increase efficacy but the possibility of antigen escape remains, as seen with $CD19^{-}$ relapses in ALL [99]. To combat this issue, immunotherapies targeting multiple antigens have been developed.

A compound CAR T cell targeting CD33 and CD123 has been developed that demonstrates independent activity against cells expressing either molecule [100]. These CAR T cells were effective in vitro against AML cells lines and were able to prolong survival of xenografted mice with AML cell lines displaying CD33, CD123, or both CD33 and CD123. To enhance safety and reduce the chance of prolonged bone marrow aplasia, the compound CAR T cells were able to be depleted in vivo using alemtuzumab. Results from Phase 1 trial (NCT03795779) of a compound CLL-1 CD33 CAR T cell have been presented. The first patient reported conversion from active disease to MRD negative with pancytopenia; this was followed by a successful matched sibling transplant [101]. A second case presentation with this construct demonstrated ablation of AML and pancytopenia as a result of CAR T cell administration followed by a allo-HSCT [101]. Given the limited efficacy detailed in anti-AML CAR T cell case reports and early phase trial results, compound targeting may represent a significant improvement on sustained efficacy against AML [92]. By incorporating an increasing number of shared targets, this strategy makes it even more likely to have significant hematological toxicity without allo-HSCT rescue.

In addition to CAR T cells, there have been reports of ADCs against CD45 and CD117 to reduce AML and conditions for an allo-HSCT [102]. These antibodies use the ribosomal inhibiting toxin amanitin and are effective at prolonging survival in mice bearing AML xenografts. Both the CD45 and CD117 amanitin ADC depleted human and nonhuman primate HSPC to confirm the potential utility in conditioning [103,104]. These results are still preliminary but offer a possible pathway to AML immunotherapy incorporating allo-HSCT that avoids the complexities of CAR T cell generation.

4. Conclusions

Effective immunotherapy for most patients with AML remains elusive. The experience with GO suggests that immunotherapy may be able to play a role in AML treatment, but the clinical trials with VT illustrate the difficulty in managing more potent therapeutics against AML with the associated increase in hematologic toxicity. Unless a true leukemia specific epitope is found that is consistently expressed across at an individual cell level, then targeting single molecules may continue to show limited efficacy. Multiantigen targeting increases the risks of hematologic toxicity but would likely reduce the chance of tumor escape.

Requiring an allo-HSCT is a major disadvantage of any potential future therapy, as most patients with AML are ineligible for allo-HSCT. The concept of bridging to allo-HSCT may change with AML immunotherapy. In other hematologic malignancies, bridging therapy occurs before conditioning; therefore, the treatment is naturally longer. In AML therapy, it may be possible to combine bridging with conditioning, which may reduce the toxicity of the individual elements combined. Another potential benefit of incorporating immunotherapy in allo-HSCT conditioning for AML is that these therapies may replace traditional conditioning agents. The consistent presentation of AML targets on HSPC may allow for an extension immunotherapy to conditioning in other diseases, including myelodysplastic syndromes as well as inherited disorders of hematopoiesis and immunity that respond poorly to traditional conditioning agents.

The design and trials of emerging AML immunotherapy must be carefully considered to have a chance of success, given the high morbidity and mortality associated with the disease itself. Tolerable and effective immunotherapy that can reduce the need for allo-HSCT would constitute a major change in the treatment of AML, but to date this has been elusive. Finally, the alternative of enhancing the efficacy while reducing the toxicity of allo-HSCT with immunotherapy would constitute substantial progress in finding better outcomes for patients with AML.

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Chapter 5: CD300f epitopes are specific targets for acute myeloid leukemia with monocytic differentiation

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CD300f epitopes are specific targets for acute myeloid leukemia with monocytic differentiation

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Antibody-based therapy in acute myeloid leukemia (AML) has been marred by significant hematologic toxicity due to targeting of both hematopoietic stem and progenitor cells (HSPCs). Achieving greater success with therapeutic antibodies requires careful characterization of the potential target molecules on AML. One potential target is CD300f, which is an immunoregulatory molecule expressed predominantly on myeloid lineage cells. To confirm the value of CD300f as a leukemic target, we showed that CD300f antibodies bind to AML from 85% of patient samples. While one CD300f monoclonal antibody (mAb) reportedly did not bind healthy hematopoietic stem cells, transcriptomic analysis found that CD300f transcripts are expressed by healthy HSPC. Several CD300f protein isoforms exist as a result of alternative splicing. Importantly for antibody targeting, the extracellular region of CD300f can be present with or without the exon 4-encoded sequence. This results in CD300f isoforms that are differentially bound by CD300f-specific antibodies. Furthermore, binding of one mAb, DCR-2, to CD300f exposes a structural epitope recognized by a second CD300f mAb, UP-D2. Detailed analysis of publicly available transcriptomic data indicated that $CD34^+$ HSPC expressed fewer CD300f transcripts that lacked exon 4 compared to AML with monocytic differentiation. Analysis of a small cohort of AML cells revealed that the UP-D2 conformational binding site could be induced in cells from AML patients with monocytic differentiation but not those from other AML or HSPC. This provides the opportunity to develop an antibody-based strategy to target AMLs with monocytic differentiation but not healthy $CD34^+$ HSPCs. This would be a major step forward in developing effective anti-AML therapeutic antibodies with reduced hematologic toxicity.

Abbreviations
ADC, antibody–drug conjugate; AML, acute myeloid leukemia; BM, bone marrow; CHO, Chinese hamster ovary; ECL, enhanced chemiluminescence; HSC, hematopoietic stem cell; HSPCs, hematopoietic stem and progenitor cells; mAb, monoclonal antibody; MFI, median fluorescence intensity; PBMCs, peripheral blood mononuclear cells; TCGA, The Cancer Genome Atlas.

1. Introduction

Intensive chemotherapy with or without allogeneic hematopoietic cell transplant cures a proportion of younger patients diagnosed with AML; however, these therapies are too toxic for the majority of patients (Juliusson *et al.*, 2009). Antibody-based therapies have significantly improved outcomes in other hematologic malignancies, but their development in AML has been limited in comparison (Cuesta-Mateos *et al.*, 2018). Current classification systems of AML depend on recurrent genetic abnormalities to predict prognosis and inform treatment, but the older French-American-British (FAB) classification system is still important in predicting surface molecule expression on AML subpopulations (Vardiman *et al.*, 2009). The AMLs with monocytic phenotypes are acute myelomonocytic leukemia and acute monocytic/monoblastic leukemia. Acute myelomonocytic leukemia AMLs account for 5–10% of cases across all age groups with a median age of 50 years, while acute monocytic/monoblastic leukemia AMLs occur in any age range but are most common in children (Swerdlow *et al.*, 2008). Together, these AML subtypes account for 50% of all AMLs in infants (Masetti *et al.*, 2015). A new targeted therapy with reduced hematologic toxicity in AML with monocytic differentiation, including acute myelomonocytic leukemia as well as acute monoblastic and monocytic leukemia, would be a significant development.

The CD33 antibody–drug conjugate (ADC), gemtuzumab ozogamicin, is the only current antibody-based therapy for AML, after it was reapproved by the FDA in 2017 (Jen *et al.*, 2018). More recent ADCs or chimeric antigen receptor (CAR) T cells targeting CD33 have not been successful, primarily due to significant hematologic toxicity in preclinical models and clinical trials (Pizzitola *et al.*, 2014; Stein *et al.*, 2018). Hematologic toxicity is a difficult challenge to overcome, as many surface molecules found on AML are present on hematopoietic stem and progenitor cells (HSPCs) (Taussig *et al.*, 2005). Other well-described AML targets such as CD123 and CLL-1 have also been associated with significant hematologic toxicity in preclinical development (Gill *et al.*, 2014; Leong *et al.*, 2017). Despite extensive genomic and proteomic analyses of AML and HSPC, no ideal surface target for AML has been found (Perna *et al.*, 2017).

CD300f is a potential therapeutic target in AML. CD300f is a member of the CD300 immunoregulatory family encoded by a gene complex on human chromosome 17q25 (Clark *et al.*, 2009). It is a transmembrane glycoprotein with both inhibitory motifs and PI3K phosphorylation sites in its cytoplasmic region (Alvarez-Errico *et al.*, 2007). Healthy myeloid cells including $CD34^+$ HSPC express CD300f, while proteomic and transcriptomic analyses have shown that it is upregulated in AML samples (Korver *et al.*, 2009; Strassberger *et al.*, 2014). One CD300f monoclonal antibody (mAb) that bound leukemic blasts but not $CD34^+$ HSPCs in the majority of AMLs in a small cohort of human samples had some efficacy in a xenogeneic model of AML using the HL-60 cell line (Korver *et al.*, 2009).

Effective therapeutic antibodies targeting well-defined epitopes mitigate off-target toxicity. CD300f isoforms are expressed in leukemic cell lines; immunoprecipitation of CD300f from the histiocytic lymphoma cell line U937 identified 53 and 59 kDa proteins (Alvarez-Errico *et al.*, 2004). Four RNA transcripts were initially described (Alvarez-Errico *et al.*, 2004), more are listed in databases, and the current NCBI database lists seven protein isoforms resulting from alternative splicing: NP_620587 (IREM-1, Isoform 1), NP_001276011 (Isoform 2), NP_001276012 (Isoform 3), NP_001276013 (Isoform 4), NP_001276014 (Isoform 5), NP_001276015 (Isoform 6), and NP_001276016 (Isoform 7). Studies have examined the binding of CD300f mAbs to the canonical CD300f (Isoform 1) expressed by transfected cells, but their binding to other isoforms is undefined. We have confirmed the validity of CD300f as a target on AML in a cohort of 35 AML patients and demonstrated that different CD300f-specific mAbs recognize independent extracellular epitopes. Further, we showed expression of CD300f isoforms by cell lines and primary AMLs is complex and the different isoform expressed affects mAb binding. This work has defined key differences in the extracellular region of CD300f that will help design novel AML therapeutic antibodies to specific isoforms and minimize hematologic toxicity.

2. Materials and methods

2.1. Antibodies

CD300f mAbs used were UP-D1 (mouse IgG1,κ, eFluor 660 conjugate, Jomar), UP-D2 (mouse IgG1, κ, PE conjugate and purified, BioLegend, San Diego, CA, USA), and 234903 (rat IgG2b, R&D Systems, Minneapolis, MN, USA). An in-house mAb, DCR-2 (IgG1,κ), was generated from a mouse immunized with CD300f Chinese hamster ovary (CHO) transfectants and boosted with recombinant human CD300f-Fc protein (Sino Biologicals, Beijing, China). The polyclonal antibodies used were rabbit antibody to the peptide representing

residues 63–92 of the canonical CD300f sequence (CLM-1, Abcam) and goat anti-human LMIR3 (leukocyte myeloid inhibitory receptor; gLMIR3, R&D Systems). All antibodies detected epitopes on the CD300f Ig-like domain. CMRF-81 (anti-tetanus toxoid mouse IgG_1 (Ju *et al.*, 2008)) was used as an isotype control.

2.2. Cell lines

The myeloid-derived cell lines HL-60, U937, HEL, and THP-1 (all from ATCC) were grown in complete RPMI containing 200 mM glutaMAX, 100U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin, and 10% heat-inactivated fetal bovine serum [all from Thermo Fisher Scientific (Thermo, Melbourne, Victoria, Australia)].

2.3. Human samples

Venous blood and bone marrow (BM) samples were obtained, with informed consent, from healthy volunteers collected through the Department of Hematology, Concord Repatriation General Hospital. Cord blood was obtained through the Sydney Cord Blood Bank with ethical approval to use samples that failed banking volume criteria. Mononuclear cells were prepared using density gradient centrifugation over Ficoll-Paque following the manufacturer's recommendations (GE Healthcare Life Sciences, Uppsala, Sweden). Mononuclear blasts were prepared from excess AML diagnostic samples following patient consent. All consent forms were written and accompanied by participant information sheets. All patient sample protocols conformed to the guidelines set by the Declaration of Helsinki. Table S1 summarizes the characteristics of AML samples. The Concord Repatriation General Hospital Human Ethics Committee approved all protocols. Samples of AML were categorized as having a monocytic differentiation if they met the 2016 WHO criteria of acute myelomonocytic leukemia as well as acute monoblastic and monocytic leukemia by morphology and immunophenotyping, irrespective of genetic abnormalities.

$CD14^+$ monocytes were purified from peripheral blood mononuclear cells (PBMCs) labeled with CD14-FITC (clone M5E2) by cytometric sorting on a BD Influx or with a CD14-positive selection kit from Miltenyi Biotec. AML samples were phenotyped with the following mAbs from BD Biosciences (Sydney, New South Wales, Australia): CD45-V500 (clone HI30), CD34-PE-CY7 (clone 581), CD38-V450 (clone HB7), and CD33-PE (clone WM53). Results were analyzed with FLOWJO software (Treestar, Ashland, OR, USA). The gating strategy for identifying the blast and leukemic stem cell populations is shown in Fig. S1. $SSC^{lo}CD45^{dim}$ blast populations were purified from AML samples labeled with CD45-V500.

2.4. Flow cytometry

Standard protocols were used to stain cells with directly conjugated specific, or isotype control, antibodies as described previously (Clark *et al.*, 2016). Unlabeled mouse mAbs were detected with species-specific Alexa Fluor (AF) 488 or 647F(ab')$_2$ secondary reagents (all from Thermo). Live cells were identified as propidium iodide$^-$ events. Data were collected on either an Accuri C6, Fortessa LSR, or Influx (BD Biosciences).

To determine whether different antibodies bound similar epitopes, we preincubated target cells with saturating amounts of primary antibody in 0.5% BSA/PBS for 30 min on ice. Cells were washed with 0.5%BSA/PBS before incubation with a subsaturating concentration of the test antibody. Experiments were repeated three times. Percent binding of the test antibody was determined from median fluorescence intensity (MFI) by [MFI Test Antibody-MFI Primary isotype]/[MFI Primary Antibody-MFI isotype control] x 100. In cross-blocking experiments using primary samples, $CD34^+$ HSPC, lymphocytes, and monocytes all originated from cord blood (CB) PBMC. AML samples were gated with CD45 and CD34 to exclude nonblasts.

2.5. Immunoprecipitation and western blots

For immunoprecipitation, 2.5×10^7 cells were biotinylated with Sulfo-NHS-Biotin (Thermo) before lysis in M-PER mammalian protein extraction reagent (Thermo) containing protease inhibitors (Roche, Castle Hill, New South Wales, Australia). Proteins were immunoprecipitated with antibodies bound to Protein G Dynabeads according to the manufacturer's recommendations (Thermo). Samples were resolved through a 4–12% Bis-Tris Plus gel (Thermo), with or without antioxidant, and transferred to nitrocellulose using an iBlot system (Thermo). Membranes, blocked with 5% BSA/TTBS, were incubated with primary antibody, followed by HRP-conjugated species-specific antibody, detected with enhanced chemiluminescence (ECL) reagent (Clarity ECL Kit, Bio-Rad), and analyzed using a Bio-Rad ChemiDoc imaging system (Bio-Rad, Galdesville, New South Wales, Australia). Biotinylated protein was detected with streptavidin–HRP and ECL.

2.6. Gene expression analysis

Total RNA was prepared from freshly purified cell populations or cells growing in exponential growth phase using TRIzol reagent as per the manufacturer's

instructions (Thermo). Integrity and quantity of extracted RNA were assessed using an RNA 6000 Nano Bioanalyzer (Agilent Technologies, Mulgrave, Victoria, Australia). All RNA used had a RNA integrity number > 8.8. For cDNA, 100 ng of DNase I (Thermo)-treated RNA was reverse-transcribed into cDNA using SuperScript III (Thermo). Oligonucleotide primers designed to detect splice variants were checked for specificity by BLAST alignment and are listed in the supplementary material. Gene expression was performed by qPCR on the cDNA using optimized primers and Fast SYBR® Green Master Mix (Thermo). Duplicate samples of cDNA were amplified using a 7500 Fast Real-time PCR System (ABI). C_T values for splice variant amplification were normalized to the *HPRT* endogenous gene and presented as fold changes to a $CD14^+$ or U937 cDNA reference sample using the formula: fold change = $2^{-\Delta\Delta CT}$ (Pfaffl, 2001). Primer efficiencies were all greater than 98%.

2.7. Transcriptomic analysis

Healthy bone marrow HSPC (GSE63569 and GSE69239) from seven individuals and The Cancer Genome Atlas (TCGA) acute myeloid leukemia (LAML) data sets from 151 patients were downloaded. Due to differences in treatment, acute promyelocytic leukemia (M3) was removed from the TCGA analysis. Data sets were aligned with STAR RNA-seq aligner version 2.4 to GRCh38.d1.vd1 genome (Dobin *et al.*, 2013). Read quantification was performed with in-house shell scripts. The exon 3 read positions were chr17: 74704478-74704517, and the exon 4 read positions were chr17:74703100-74703141. RKPM was calculated as [(number of target reads)/(total reads/1 000 000)]/(target length in Kb).

2.8. Generation of CD300f transfectants

Full-length CD300f cDNA (Isoform 1) containing an amino-terminal c-myc epitope was expressed under the CMV promoter of the pBud vector in CHO cells. Cells expressing high amounts of surface c-myc were sorted on a BD Influx. Sequences were validated at the Australian Research Genome Facility.

2.9. ELISA

The specificity of antibodies for the CD300f Ig-like domain, and cross-reactivity with CD300b, was tested by ELISA using recombinant proteins obtained from Sino Biological. Antibodies and appropriate species and isotype controls were incubated with the immobilized recombinant protein, and binding was detected with the relevant HRP-labeled secondary antibody and OPD.

2.10. Primer sequences

The primer and probe sequences were Fw_hHPRT1: 5′AATTATGGACAGGACTGAACGTCTTGCT; Rv_hHPRT1: 5′TCCAGCAGGTCAGCAAAGAATTTATAGC; $CD300f^{SI4}$_F (amplifies exon 4 in Isoform 4 or 6): 5′CACGCCTACCTCCACTACGTTT; $CD300f^{C}$_F (amplifies exon 4 in Isoforms 1, 2, 3, 5, 7): 5′ATTGACCCAGCACCAGTCACC; CD300f-Ex4_R (reverse primer to amplify exon 4 in all Isoforms): 5′GGTGGCCGGTCAGAGTTG.

2.11. Statistical analysis

Statistical analysis was performed using PRISM (GraphPad Software, Inc, San Diego, CA, USA). Comparisons between single groups were analyzed with t-tests. Exon 3 and exon 4 expressions of RNA-seq data and UP-D2 binding of AML cell lines and primary samples were analyzed with one-way ANOVA with multiple comparisons between groups.

3. Results

3.1. CD300f antibodies bind to primary AML

We assessed the binding of the CD300f-specific mAb, UP-D1, to 34 newly diagnosed AML samples and healthy bone marrow by flow cytometry using the gating strategy outlined in Fig. S1. UP-D1 bound to $SSC^{lo}CD45^{dim}$ AML blasts in 85% (Fig. 1A) and the $SSC^{lo}CD45^{dim}CD34^+CD38^-$ in 76% of these patient samples (Fig. 1B). There was no significant difference between the ability of UP-D1 and anti-CD33 to bind total AML blasts or the $CD34^+CD38^-$ subset, which is enriched with leukemic stem cells (Fig. 1A,B). UP-D1 also bound to the $Lin^-CD34^+CD38^-CD45RA^-CD90^+$ hematopoietic stem cell (HSC) precursor population within healthy BM (Fig. 1C) similar to CD33. There were no significant differences in the UP-D1 binding to total $CD34^+$ cells, myeloid progenitors, multipotent progenitors (MPPs), or HSC between bone marrow and cord blood (Fig. S1).

3.2. Confirmation that CD300f antibodies bind to the CD300f Ig-like domain

All CD300f protein isoforms listed in NCBI protein database share the CD300f Ig-like domain but differ

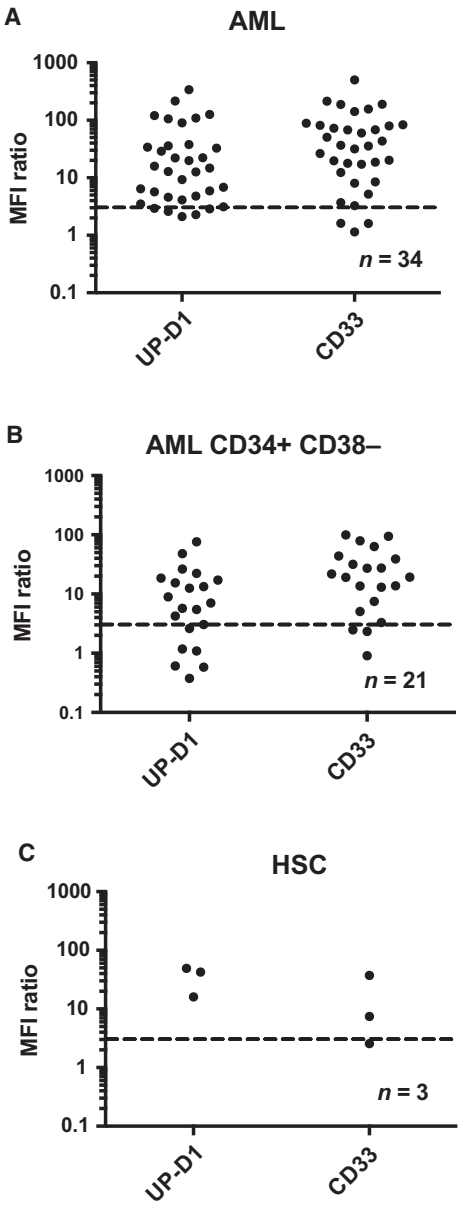

Fig. 1. CD300f is expressed on leukemic cells from AML patients. CD300f (UP-D1) compared to CD33 expression on (A) AML blasts, (B) $CD34^+CD38^-$ subset, and (C) $Lin^-CD34^+CD38^-CD45RA^-CD90^+$ bone marrow HSCs was assessed using multiparameter flow cytometry. The MFI of the population of interest was divided by the MFI of the isotype control to give a MFI ratio. Populations with a MFI ratio ≥ 3, shown above the dotted line, were considered to be positive.

in their leader sequence, exon 4-coded sequence, and their cytoplasmic domain (Fig. S2). We confirmed binding of the CD300f antibodies to CD300f Isoform 1 (Fig. S3A) or Isoform 6 expressed on transfected CHO cells. Because all CD300 molecules share significant amino acid sequence similarity in the Ig domain, we confirmed the specificity of each CD300f mAb to CD300f and not the other family members by either flow cytometry on transfectants or ELISA. Of the CD300 molecules, CD300f shares the highest amino acid sequence identity with CD300b. The CLM-1 peptide antibody and the gLMIR3 polyclonal antibody bound the Ig domain of CD300b (Fig. S3C).

Each antibody bound to the four $CD300f^+CD300b^-$ myeloid-derived cell lines tested, with the exception of the CLM-1 peptide antibody, which only bound to THP-1 (Fig. S3D). Each mAb showed a different MFI ratio pattern. UP-D1 had a low MFI ratio binding to HEL but a high ratio to HL-60, U937, and THP-1. The 234903 clone and DCR-2 mAbs were similar with a high MFI ratio to U937, lower ratio binding to HL-60 and THP-1, and an even lower ratio binding to HEL. The mouse UP-D2 clone and gLMIR3 polyclonal antibody showed similar MFI patterns (Fig. S5). Testing the binding of each antibody to CD300f Isoform 1 expressed on transfectants in the presence of other CD300f demonstrated that no mAb completely cross-blocked binding of another (Fig. S5). These data indicated the presence of at least four distinct CD300f epitopes recognized by the antibody panel. They are the UP-D1 epitope, a DCR-2/243903 epitope, the UP-D2 epitope, and a CLM-1 epitope.

In healthy PBMC, the four mAbs and gLMIR3 antibody bound to both $CD14^+CD16^-$ (conventional) and $CD14^{dim}CD16^{++}$ (inflammatory) monocytes (Fig. 2A). Interestingly, the UP-D2 and DCR-2 clones bound to $CD14^+CD16^-$ monocytes with significantly greater intensity than to the $CD14^{lo}CD16^+$ monocyte population and this pattern was reversed with the 234903 mAb. Having shown that UP-D1 bound to AML blasts and that it recognized a distinct epitope to other CD300f antibodies, we tested the other antibodies on five representative AML samples (Fig. 2B). All antibodies bound AML blasts, including the $CD34^+CD38^-$ subset (Fig. 2B) except for the CLM-1 antibody, which bound the blasts from only one sample. The UP-D1, DCR-2, and 234903 mAb bound to HSC in healthy CB. There was a weak binding of UP-D2 (Fig. 2C) and no binding with the CLM-1 antibody to healthy HSC.

Antigen density of CD300f on the surface of myeloid-derived cell lines and a number of primary AML samples with a high percentage of blasts was tested using a quantitative bead-based kit (data not shown). The myeloid-derived cell lines were expressed in the order of 10^4 CD300f molecules per cell. Primary AML blasts expressed CD300f at levels ranging from 10^1 to 10^4 molecules/cell agreeing with a previous report (Korver *et al.*, 2009).

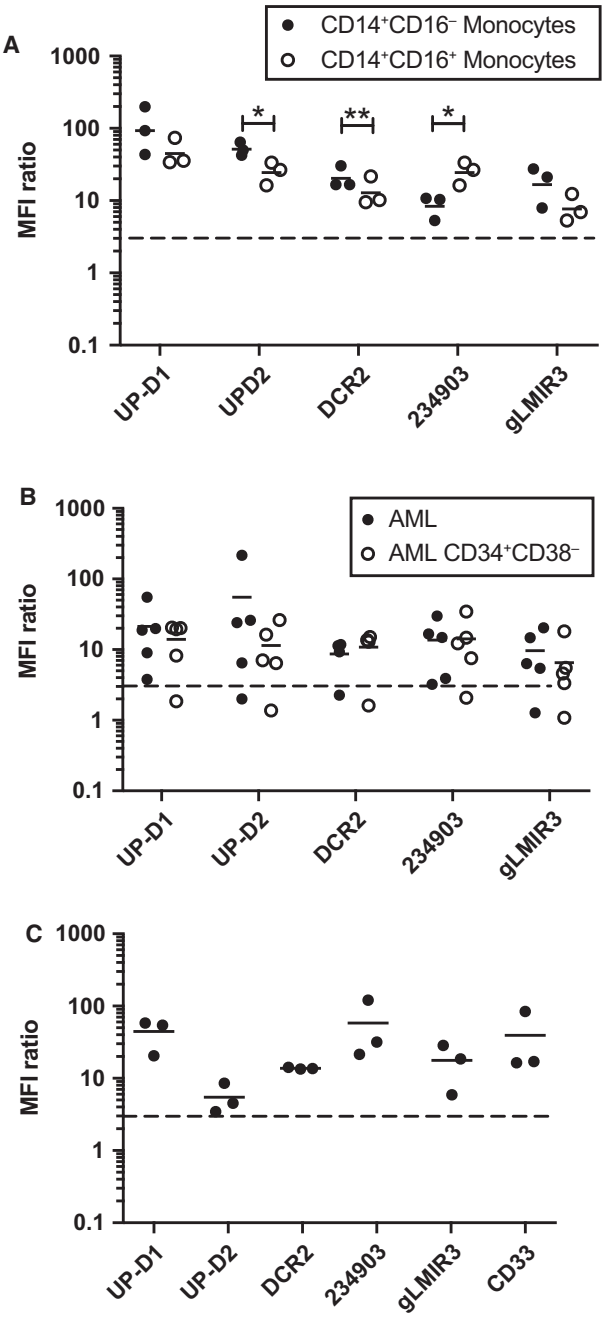

Fig. 2. Binding of CD300f antibodies to healthy and leukemic cells. Comparison of CD300f antibodies binding to (A) healthy PBMC populations assessed by multiparameter flow cytometry, (B) AML blasts and $CD34^+CD38^-$ subset ($n = 5$), and (C) $Lin^-CD34^+CD38^-CD45RA^-CD90^+$ CB hematopoietic stem cells ($n = 3$). Statistical analysis was performed using *t*-tests. $^*P < 0.05$ and $^{**}P < 0.01$.

3.3. Expression of CD300f isoforms

The MW predicted from the CD300f primary sequences ranges from 21.4 kDa to 33.7 kDa. UP-D1 was originally shown to immunoprecipitate molecules of 53 and 59 kDa (Alvarez-Errico *et al.*, 2004). We used the gLMIR3 antibody to immunoprecipitate CD300f from the myeloid-derived cell lines and identified proteins with MW ranging from 40 kDa on HL-60 to 55 kDa on U937 with one predominant band in each cell line (Fig. 3A). Immunoprecipitated CD300f proteins from primary AML lysates that expressed high levels of CD300f had MW of 28 kDa, 40 kDa, and 60 kDa.

Both alternative splicing events and different post-translational modifications are likely to contribute to the variation in isoform expression. To understand the effect the expression of different isoforms has on antibody targeting of $CD300f^+$ cells, we looked in detail at the different extracellular portion of the isoforms. In the databases, the extracellular sequences for CD300f have two forms. The canonical form of CD300f does not express exon 4, and here, it will be identified as $CD300f^C$. CD300f Ig domain, encoded by exon 3, spliced to the transmembrane region is present in protein Isoforms 1, 2, 3, 5, and 7 (Fig. S2). The alternative has a splice insertion of exon 4 after the Ig domain before the transmembrane domain. Exon 4 encodes a Ser-Thr-rich sequence and is found in protein Isoforms 4 and 6 and here will be referred to $CD300f^{SI4}$.

3.4. Differential expression in CD300f Exon 4 between Healthy $CD34^+$ HSPC and AML

The qPCR was used to amplify $CD300f^{SI4}$ and $CD300f^C$ transcripts from cDNA prepared from myeloid-derived cell lines, healthy monocytes, healthy CB $CD34^+$ HSPC, and AML blasts (Fig. S4). We observed that $CD300f^C$ transcripts were more abundant than $CD300f^{SI4}$ present in healthy CB $CD34^+$ HSPC. AML blasts expressed both $CD300f^{SI4}$ and exon $CD300f^C$ transcripts (Fig. 3). There was a significant difference in $CD300f^{SI4}$ expression between the CB $CD34^+$ and AML cells (which were primarily of subtypes with monocytic differentiation) ($P < 0.05$).

To confirm our qPCR findings on a larger sample set, we compared publicly available RNA-seq data from healthy bone marrow $CD34^+$ HSPC with AML RNA-seq data from TCGA project. Exon 4 and exon 3 (expressed on all isoforms) sequences were compared across the bone marrow $CD34^+$ HSPC and both monocytic AML and nonmonocytic AML (Fig. 4). There were significant differences in exon 4 but not exon 3 expression between bone marrow $CD34^+$ HSPC and monocytic AML ($P = 0.033$). There were significant differences between monocytic and nonmonocytic

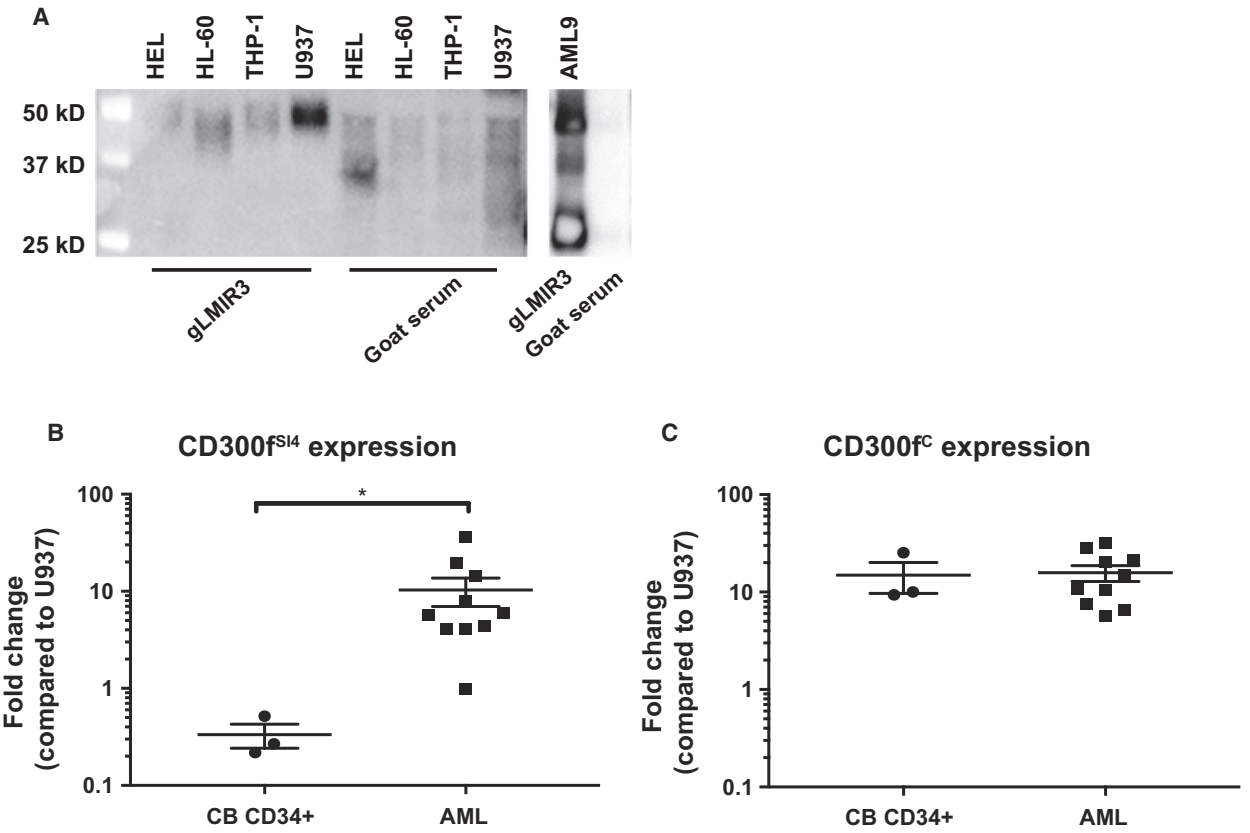

Fig. 3. (A) Immunoprecipitation with a CD300f polyclonal antibody reveals multiple forms of CD300f in AML cell lines and primary AML cell samples. CD300f was immunoprecipitated from biotinylated membrane lysates; HEL, HL-60, U937, THP-1, and a primary AML9 membrane lysate using goat anti-LMIR3 or goat serum. Immunoprecipitated protein was detected with streptavidin–HRP and ECL substrate. (B) CD300f exon 4 splice variants or (C) CD300f^{C} was compared between healthy CB-derived CD34^{+} HSPC and primary AML samples (flow-sorted or blast count > 90%). Statistical analysis was performed using *t*-tests. $^{*}P < 0.05$.

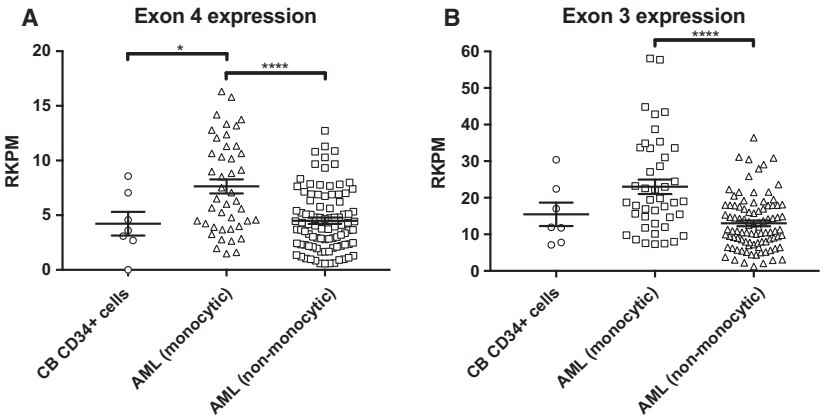

Fig. 4. RNA-seq of CD300f exon 4 between BM CD34^{+} HSPC, monocytic AML, and nonmonocytic AML. Publicly available healthy BM CD34^{+} HSPC (n = 7) and AML (n = 131) data were analyzed for CD300f exon 4 and exon 3 expressions. (A) CD300f exon 4 expression comparison between BM CD34^{+} HSPC, monocytic AML, and nonmonocytic AML. (B) CD300f exon 3 expression comparison between BM CD34^{+} HSPC, monocytic AML, and nonmonocytic AML. Statistical analysis was performed with one-way ANOVA with multiple comparisons between groups. $^{*}P < 0.05$ and $^{****}P < 0.0001$.

AML phenotypes in both exon 4 ($P < 0.001$) and exon 3 ($P < 0.001$) expressions.

3.5. CD300f antibodies showing different binding to CD300f isoforms

All CD300f Abs bound to the CD300f^{SI4} and CD300f^{C} extracellular region when expressed in CHO cells. Comparing the MFI ratios of UP-D1 to each isoform revealed that UP-D1 bound to the CD300f ^{SI4} isoform with a threefold higher ratio compared to the CD300f^{C}. On the other hand, there was less than twofold difference in UP-D2, 234903, and DCR-2 binding to CD300f ^{SI4} isoform compared to CD300f^{C} isoform (Fig. 5). Western blot analysis (Fig. 5) revealed that gLMIR3, DCR-2, and 234903 Abs bound to a 49kD protein from the CD300f ^{SI4} in nonreducing conditions, but there was little binding in reducing conditions, whereas UP-D2 worked poorly on western blot under either condition. There was minimal binding to the CD300f^{C} isoform in either reducing or nonreducing conditions by gLMIR3 (Fig. 5G), even when 10-fold levels of lysate were analyzed. CLM-1 antibody confirmed CD300f^{C} expression in all samples (Fig. 5F). The presence of the exon 4-coded Ser-Thr-rich sequence alters the structure of the CD300f increasing the exposure of the epitope for each mAb.

3.6. DCR-2 mAb binding reveals the UP-D2 epitope

Cross-blocking experiments demonstrated that no CD300f mAbs completely blocked the binding of another CD300f mAb to CD300f (Figs 6 and S3). Interestingly, in these experiments we observed that instead of blocking, DCR-2 significantly enhanced the binding of UP-D2 to CD300f^{C} expressed on CHO cells (Fig. 6). The enhanced binding of UP-D2 by DCR-2 was further augmented on CD300f ^{SI4} expressed on CHO cells. This enhancement of UP-D2 binding was also observed on several AML cell lines. Notably, compared to HL-60 and HEL cells, U937 cells which express more CD300f ^{SI4} by qPCR also showed the most enhanced binding (Fig. S4). Finally, in primary cells, DCR-2 significantly enhanced UP-D2 binding on monocytes but not CD34^{+} HSPC from CB. There was no significant difference in UP-D2 binding with primary staining using PBS or an isotype control (data not shown). This demonstrated that the isoform of CD300f expressed by HSPCs can be distinguished from that expressed on mature monocytes (Fig. 7). The effect of DCR-2 on the binding of UP-D2 was tested on AML samples. Enhancement was evident on AMLs with monocytic differentiation ($P = 0.0314$) but absent on other AMLs compared to CB CD34^{+} HSPC (Fig. 7).

4. Discussion

The development of new antibody-based therapeutics requires the identification of appropriate cell surface protein targets. Our work demonstrates the possibility to develop antibodies to CD300f as therapeutics against AMLs with monocytic differentiation also have the potential for reduced hematologic toxicity compared to currently studied targets. We identified CD300f as a member of the CD300 gene family (Alvarez-Errico *et al.*, 2004; Clark *et al.*, 2009), and data from Korver *et al.* suggested CD300f was a potential AML target for both unconjugated antibodies and ADC (Korver *et al.*, 2009). Our early analysis (Modra *et al.*, 2006) and Korver's data obtained with their CD300f-specific mAb (clone D12) confirmed expression on the surface of AML cells. This present work has further established the validity of CD300f as a target on AML. A high percentage of AML patients express cell surface CD300f on their blast cells, and the percentage of CD300f^{+} AMLs or CD33^{+} AMLs is similar. Our studies distinguished the expression of CD300f extracellular isoforms that included alternatively spliced variants of the extracellular exon 4-encoded sequence.

The great need for new AML therapeutics has brought several potential antibody targets into consideration. In addition to CD33, potential targets identified by proteomic and transcriptomic studies of myeloid cell lines and AML samples include CD123, CD96, CD44, CD47, CD32, CLL-1, IL1RAP, TIM-3, and LILRB4 (Dobrowolska *et al.*, 2013; Gasiorowski *et al.*, 2014; Mardis, 2014; Strassberger *et al.*, 2014). All these molecules are expressed to some degree by normal cells of the myeloid lineage and bone marrow HSPC raising the possibility of therapeutic antibody hematologic toxicity. To date, it has been difficult to determine an AML-specific epitope and careful evaluation of prospective therapeutic mAbs for their therapeutic index of activity against AML versus normal hematopoietic precursors is essential. Potential therapeutic mAb to CD300f will need careful evaluation of their specificity with testing for wider CD300 molecule 'off-target' effects. The success of gemtuzumab ozogamicin suggests there is a 'therapeutic window' whereby ADC targeting myeloid antigens can exert an antileukemic effect without excessive myelosuppression. The excellent efficacy of gemtuzumab ozogamicin in acute promyelocytic leukemia (Breccia and

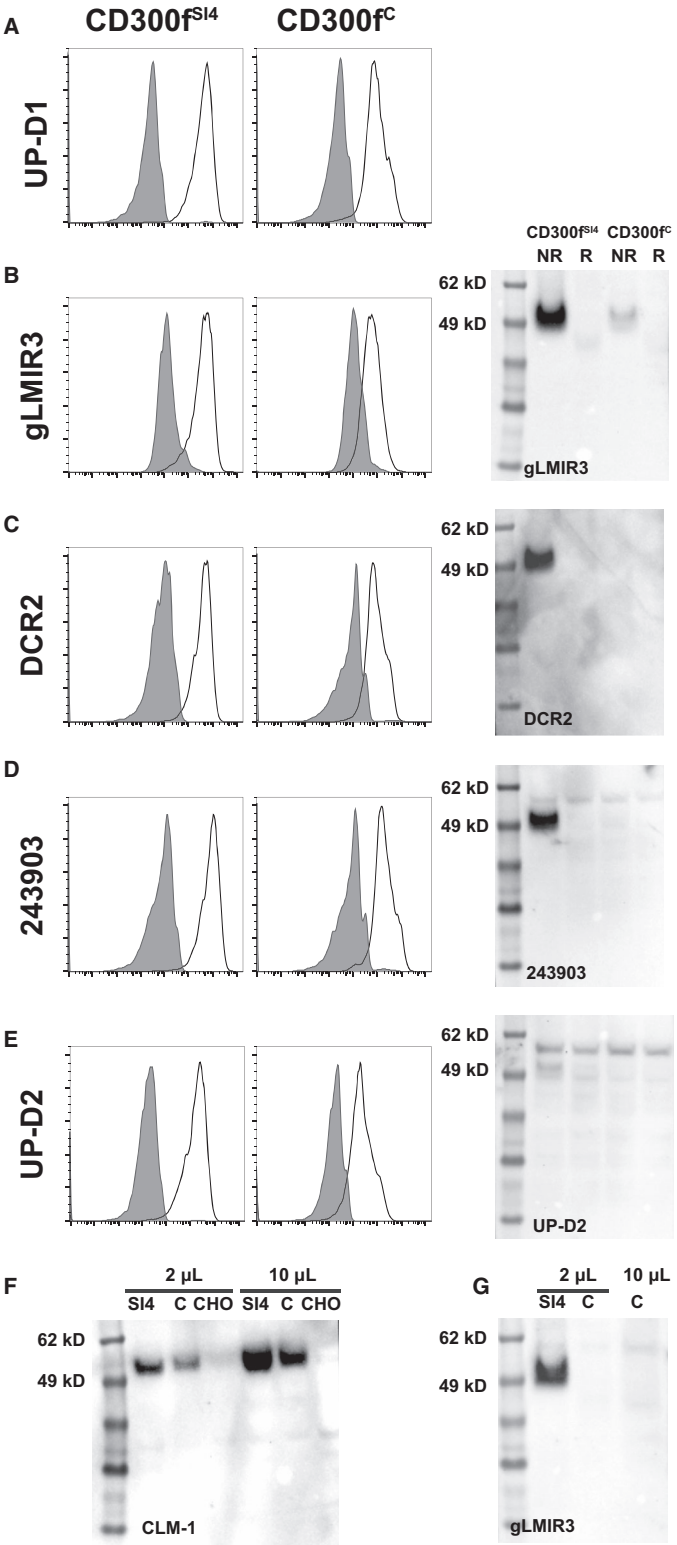

Fig. 5. Influence of CD300f exon 4 on antibody binding. The binding properties of anti-CD300f antibodies (A–E) by flow cytometry and western blot were compared using CHO cells transfected with either the CD300f^{SI4} present or CD300f^{C}. (F) Western blot using CLM-1 demonstrating the presence of CD300f in both CHO transfectants. (G) Western blot using gLMIR3 at different concentrations to confirm the absence of binding to reduced CD300f^{C}-transfected CHO cells.

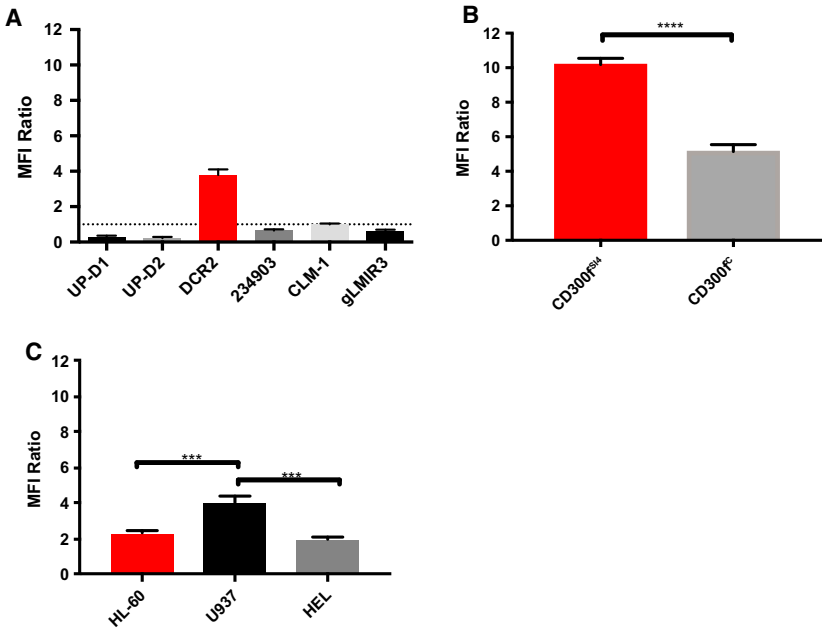

Fig. 6. Cross-blocking studies with DCR-2 and UP-D2. Cells were incubated at the saturation point of a primary antibody or an equal concentration of an isotype control and then stained with UP-D2 PE at 80 ng·mL^{-1}. The MFI ratio is the comparison between primary antibody and isotype control groups. (A) The change in UP-D2 PE binding to CD300f^{C} CHO transfectants with different primary antibody staining. (B) Difference in UP-D2 binding to CD300f^{SI4}- and CD300f^{C}-transfected CHO cells, which were incubated with a saturating amount of DCR-2 or isotype control prior to UP-D2 PE. (C) Difference in UP-D2 binding to AML cell lines, which were incubated with a saturating amount of DCR-2 or isotype control prior to UP-D2 PE. Error bars represent SEM. Panel B was analyzed using a t-test. Panel C was analyzed using a one-way ANOVA with multiple comparisons between groups. ***$P < 0.001$ and ****$P < 0.0001$.

Lo-Coco, 2011) which is CD33-dense suggests that the therapeutic window is likely to be widest when AML subtypes are chosen with high receptor levels.

AML target molecules will inevitably have wider myeloid cell expression. CD300f is expressed primarily within the myeloid cell series and is present on the myeloid cell populations of healthy PBMC (Alvarez-Errico *et al.*, 2004). Three CD300f mAbs bound CD34^{+}CD38^{-} BM-derived HSPC in healthy BM or CB and, importantly, to AML. Korver *et al.* did not detect CD300f on CD34^{+}CD38^{-} BM-derived HSPC using their IREM-1 mAbs (Korver *et al.*, 2009). This difference emphasizes the importance of our observations, which have defined the role multiple isoforms of CD300f will play as potential targets and the fact that different CD300f antibodies clearly target at least four different epitopes.

Our work suggests two possible ways to exploit targeting CD300f in future antibody-based therapies against AML with monocytic differentiation. The first method would be to generate a mAb, ADC, or other antibody-based therapeutic derivative that binds preferentially to an exon 4-related epitope. A second way would be to develop chimeric or humanized versions of DCR-2 and UP-D2 for combination therapy in which UP-D2 could be conjugated with a toxic payload as an ADC or developed into another form of therapeutics. These strategies may result in prolonged monocytopenia. The immunological consequences of a prolonged monocytopenia are unclear, those with germline GATA2 mutations associated with monocytopenia have a higher incidence of opportunistic infections, but these mutations cause additional NK- and B-cell cytopenias (Hsu *et al.*, 2011). The significant enhancement of binding to AML with monocytic differentiation would likely lead to a wider therapeutic window than currently seen. A widened therapeutic window would reduce hematologic toxicity by limiting depletion of HSPC. Either method requires further development to test with an expanded cohort of healthy HSPC and AMLs.

Detailed epitope mapping of the rituximab target found that most CD20 Abs bind one of two overlapping epitopes (Klein *et al.*, 2013). Our studies identified five immunogenic CD300f epitopes. Crystallization studies of the CD300f Ig domain showed an Ig V-like domain with a CDR3 region that was structurally variable, and a protrusion from the Ig structure created by

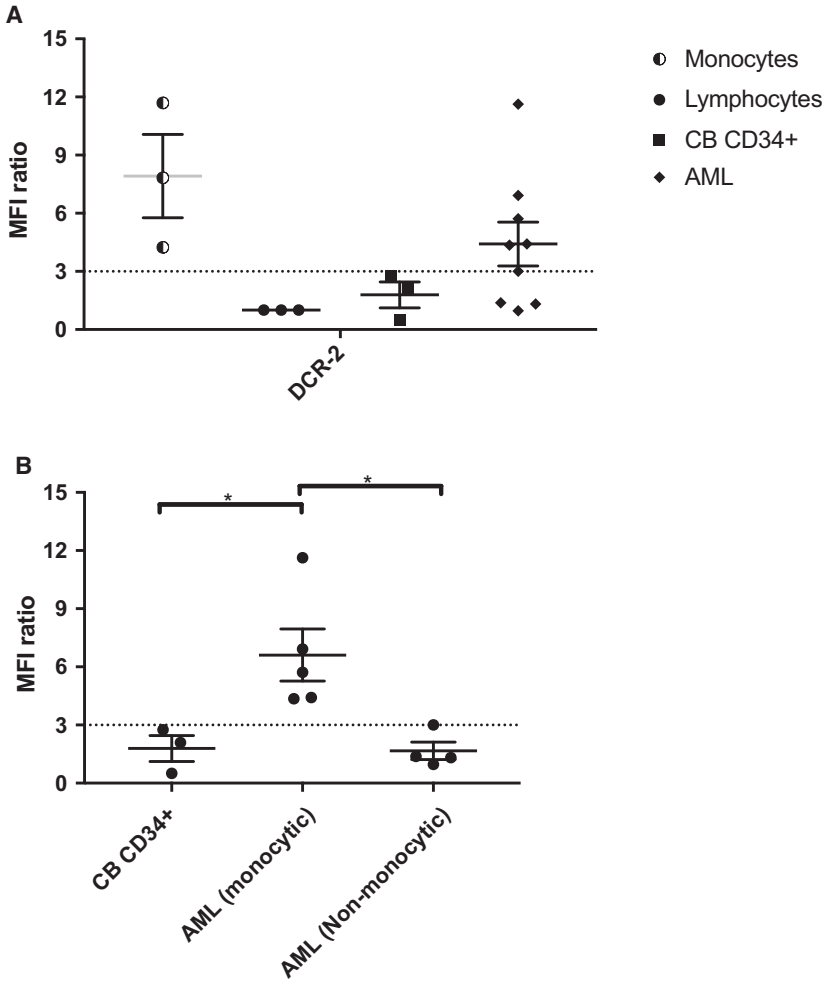

Fig. 7. Binding of DCR-2 to CD300f$^+$ cells enhances the binding of UP-D2 to monocytes, monocytic AML, but not CD34$^+$ HSPC or nonmonocytic AML. CB or primary frozen AML cells were incubated with PBS, the saturation point of DCR-2 (10 $\mu g \cdot mL^{-1}$), or an equal concentration of an isotype control. Following primary incubation, samples were stained with UP-D2 PE at 80 $ng \cdot mL^{-1}$. Data for monocytes, lymphocytes, and CD34$^+$ HSPC were obtained from CB. (A) Difference in UP-D2 PE binding across cell types when saturated with DCR-2 or isotype control, compared to PBS. (B) Differences in UP-D2 PE binding across CD34$^+$ cells between CB, monocytic AML, and nonmonocytic AML. Error bars represent SEM. Statistical analysis was performed with one-way ANOVA with multiple comparisons between groups. $*P < 0.05$.

a second disulfide bond between Cys54 and Cys62 (Marquez *et al.*, 2007). This may explain differences in the ability of polyclonal antibodies to bind more readily to nonreduced compared to reduced forms of CD300f.

The descriptions of CD300f as IREM-1 identified the canonical CD300f and three splice variants, all of which were missing exon 4 (Alvarez-Errico *et al.*, 2004). Our molecular analysis identified CD300f^{SI4} and CD300f^{C} transcript variants that were differentially expressed in both AML and HSPC. The more marked differential in expression of CD300f^{SI4} on AML compared to HSPC using qPCR as compared to the RNA-seq analysis may be due to both techniques and samples that were used. A final functional examination of the difference in CD300f^{SI4} would require a mAb that preferentially binds the exon 4 region of CD300f.

While exon 4 does not contribute to the mAb-binding Ig domain, it contributes to the tertiary structure of the molecule on the cell surface. The fourteen residues coded by the inserted sequence include 2 serine and 7 threonine residues, which have potential to undergo significant post-translational modification by either O-linked glycosylation, phosphorylation, or acetylation. Our studies validated the binding of CD300f antibodies to CD300f ^{SI4} and CD300f^{C}

transfectants but highlighted that each antibody bound differently to the two forms of CD300f.

CD300f was identified as a potential AML target by independent proteomic, transcriptomic, and empirical investigation of its myeloid-restricted expression. Our data describing multiple epitopes on the canonical CD300f isoform advance the process of antibody development to CD300f. Understanding how CD300f variant expression relates to changes in splicing mechanisms common in AML (Adamia *et al.*, 2014) is critical, and future genetic analysis may predict for CD300f expression.

5. Conclusions

The novel finding that CD300f variants resulting from splicing events are more abundant in AMLs with monocytic phenotypes compared to HSPC opens opportunities for a wider treatment window compared to currently tested surface molecules in this subset of AML. The potent effect of DCR-2 binding revealing a conformational epitope has novel targeting prospects for AML with monocytic phenotypes that should be further explored.

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Conflict of interest

GJC is a Director of DendroCyte BioTech Pty Ltd. GJC and REG are listed as inventors on patents protecting DCR-2. Other authors have no conflict of interest to disclose.

Data accessibility

The following publicly available data sets were used for this project: GSE63569, GSE69239, and the TCGA LAML.

Author contributions

EA, REG, FK, PAS, T-HL, PDF, and GJC performed experiments; EA, REG, HJI, PJH, and KB provided samples and intellectual input; EA, REG, PAS, and GJC analyzed results and made figures; PMH and DNJH contributed intellectually to the project design; EA, REG, and GJC contributed to project design, analysis of results, and writing of manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Clinical characteristics of AML samples tested. ND Not determined.

Fig. S1. Gating strategy to identify AML and HSCs. (A) After initially gating on PI negative viable cells, hematopoietic stem cells were identified as lineage-$CD45^{dim}CD34^{+}CD38^{-}CD45RA^{-}CD90^{+}$. Multipotent progenitors (MPP) were identified as lineage-$CD45^{dim}$ $CD34^{+}CD38^{-}CD45RA^{-}CD90^{-}$. Myeloid progenitors are contained in the $CD34^{+}$ $CD38^{+}$ subset. (B) Blasts were identified as $CD45^{dim}SSC^{low}$. The leukemia stem

cell enriched $CD34^+CD38^-$ fraction was identified from this gate. (C) The relative MFI ratios of total CD34 + cells, myeloid progenitors ($CD34^+$ $CD38^+$ subset), MPP and HSC were compared between bone marrow and cord blood cells.

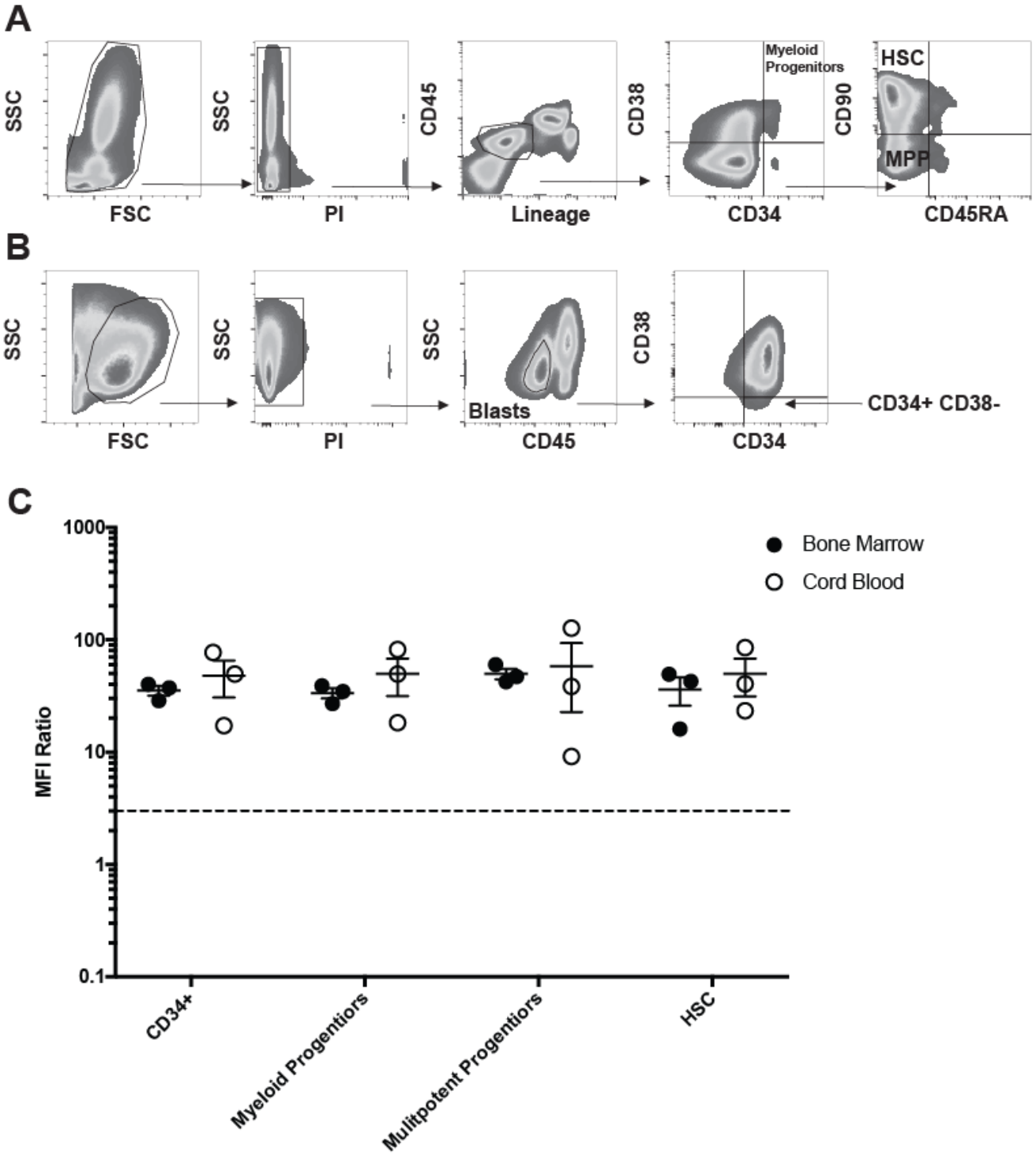
Fig. S2. Sequences of the CD300f isoforms listed in NCBI indicating the alternating exon structure in blue/black type.

Fig. S3. Specificity of CD300f antibodies. (A) Binding of CD300f antibodies to CD300f transfected CHO cells. Antibody (unshaded histogram) compared to isotype for each antibody (shaded histogram). CD300f antibodies were tested by ELISA for binding to (B) CD300f-Ig fusion protein and (C) CD300b-Ig fusion protein. ELISA was performed $n = 2$, error bars represent SEM from duplicate wells of representative result. (D) Graphs showing the geometric MFI mean of CD300f antibodies binding to four myeloid derived cell lines. Error bars represent SEM.

Fig. S4. Expression of multiple CD300f splice variants in primary AML samples. Graph showing fold difference in expression of $CD300f^{C}$ and $CD300f^{SI4}$ in cDNA prepared from AML samples with a high blast count, blasts sorted from AML populations, healthy CD14 monocytes HL-60, HEL and U937. CD300f specific amplicons were normalized to the HPRT endogenous gene transcripts in U937. Error bars represent SEM.

Fig. S5. Cross-blocking studies with CD300f antibodies. CD300f transfected CHO cells were incubated with a saturating amount of each primary antibody, (x axis). Cells were then stained with the test antibodies (A) UP-D1 (B) 234903 (C) CLM-1 (D) LMIR3. The binding of the test antibody in the presence of the primary antibody was calculated as by MFI compared to an isotype control, with 0 binding indicating complete overlap of epitopes, and 1 binding indicating no overlap of epitopes ($n = 3$). Error bars represent SEM.

Supplementary Figure 1

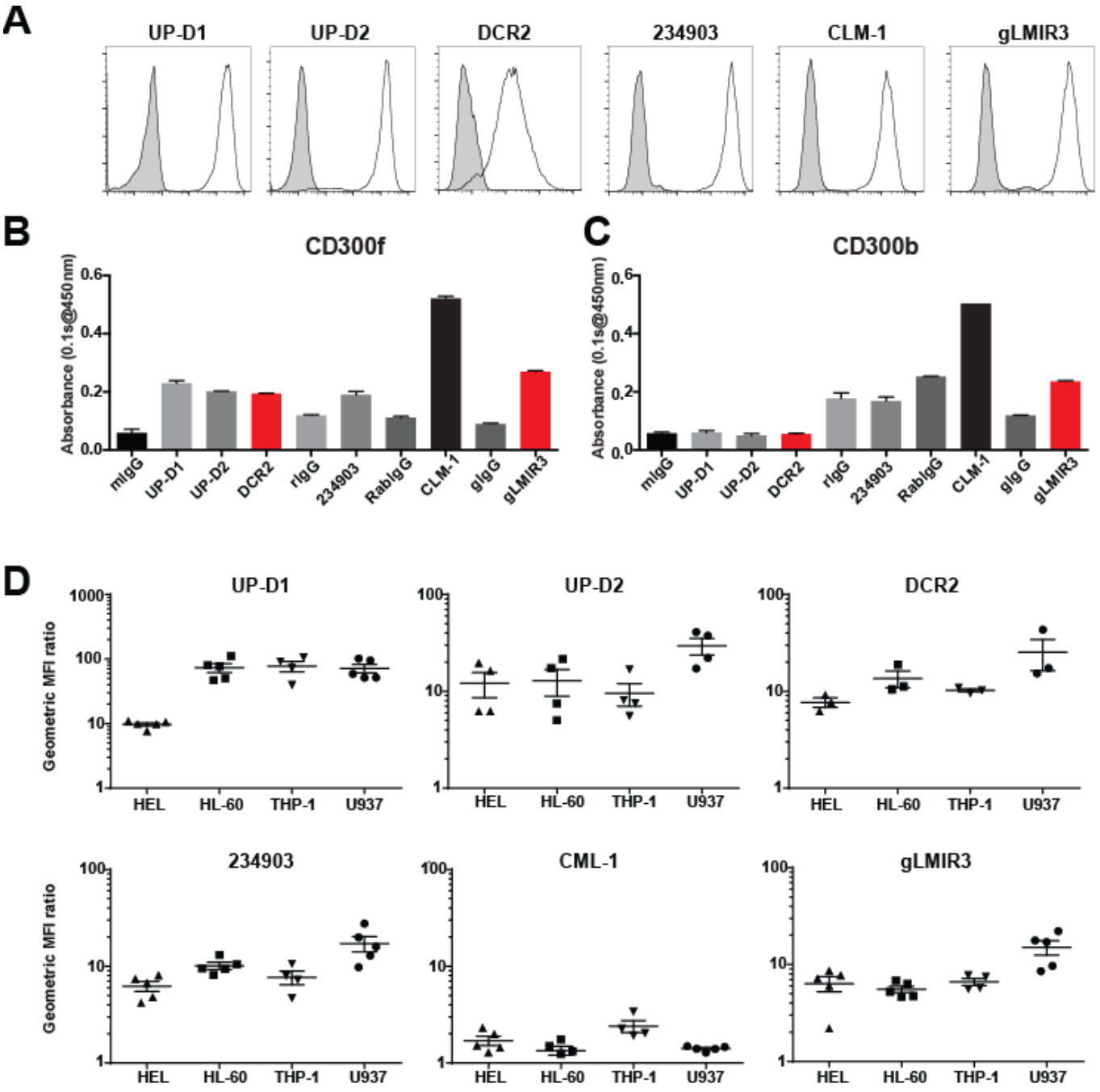


Gating strategy to identify AML and HSCs. (A) After initially gating on PI negative viable cells, hematopoietic stem cells were identified as lineage-CD45dimCD34+CD38−CD45RA-CD90+. Multipotent progenitors (MPP) were identified as lineage-CD45dimCD34+CD38−CD45RA-CD90−. Myeloid progenitors are contained in the CD34+ CD38+ subset. (B) Blasts were identified as CD45dimSSClow. The leukemia stem cell enriched CD34+CD38− fraction was identified from this gate. (C) The relative MFI ratios of total CD34 + cells, myeloid progenitors (CD34+ CD38+ subset), MPP and HSC were compared between bone marrow and cord blood cells.

Supplementary Figure 2

Sequences of the CD300f isoforms listed in NCBI indicating the alternating exon structure in blue/black type.

Supplementary Figure 3



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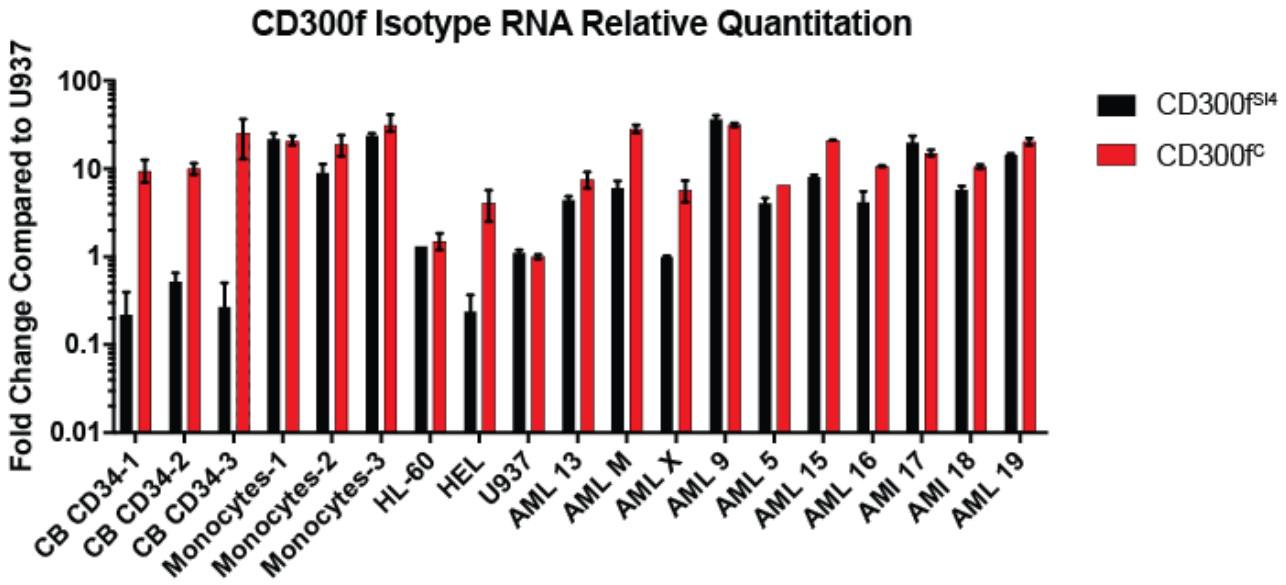
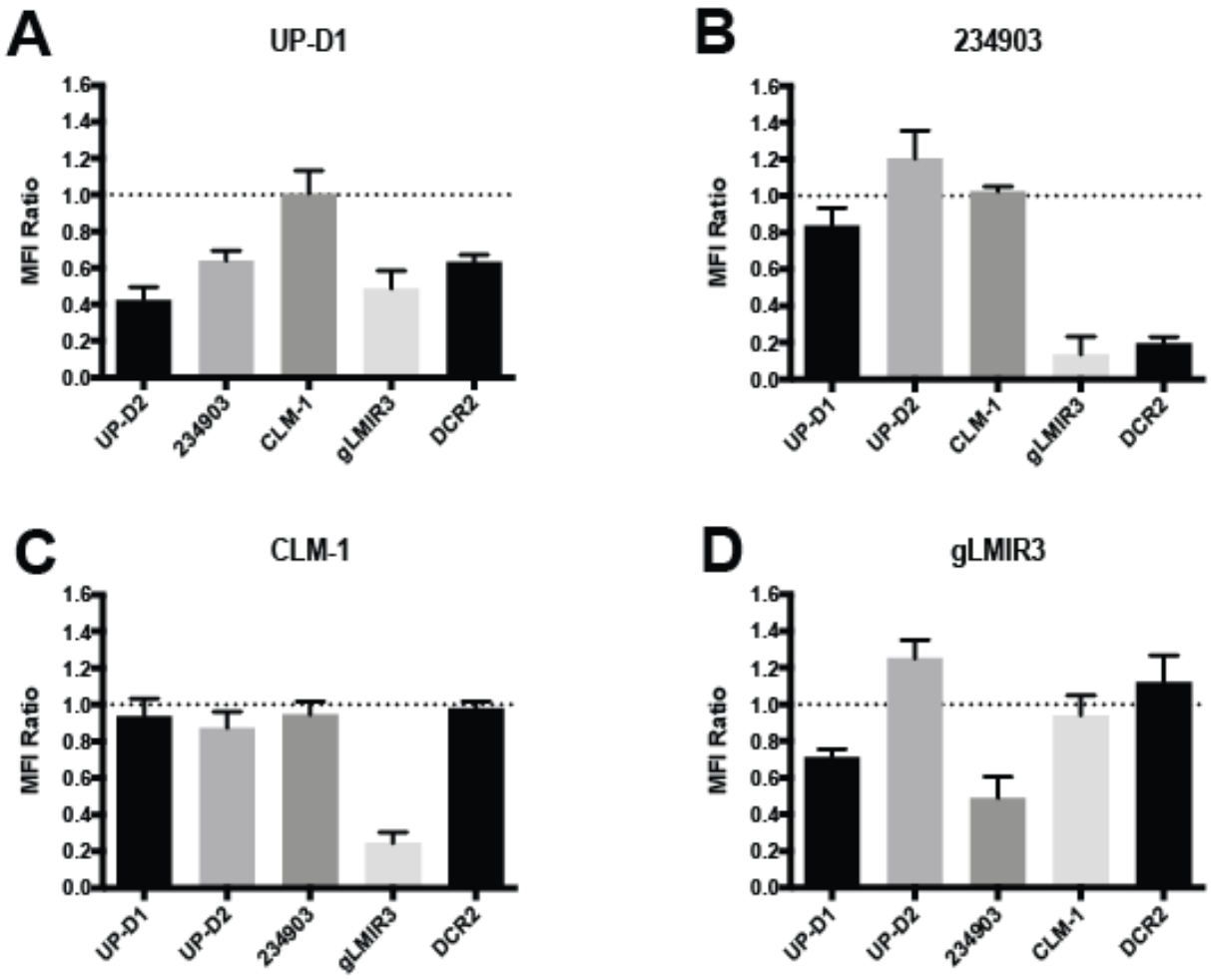
Supplementary Figure 4



Expression of multiple CD300f splice variants in primary AML samples. Graph showing fold difference in expression of CD300fC and CD300fSI4 in cDNA prepared from AML samples with a high blast count, blasts sorted from AML populations, healthy CD14 monocytes HL-60, HEL and U937. CD300f specific amplicons were normalized to the HPRT endogenous gene transcripts in U937. Error bars represent SEM.

Supplementary Figure 5



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Chapter 6: Targeting CD300f to enhance hematopoietic stem cell transplantation in acute myeloid leukaemia

Abadir E, Silveira S, Gasiorowski RE, Ramesh M, Romano A, Mekkawy A, Lo T, Kabani K, Sutherland S, Pietersz G, Ho PJ, Bryant C, Larsen S, Clark G. Targeting CD300f to enhance hematopoietic stem cell transplantation in acute myeloid leukaemia. Blood Advances. 2020;4(7):1206–1216.

REGULAR ARTICLE

blood advances

Targeting CD300f to enhance hematopoietic stem cell transplantation in acute myeloid leukemia

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Key Points

- CD300f is expressed in the majority of AML patients and across HSPCs; an anti-CD300f ADC depletes AML cells and HSPCs.
- In vitro and in vivo data suggest targeted CD300f therapy enhances efficacy and reduces toxicity of HSCT in AML.

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) significantly reduces the rate of relapse in acute myeloid leukemia (AML) but comes at the cost of significant treatment-related mortality. Despite the reduction in relapse overall, it remains common, especially in high-risk groups. The outcomes for patients who relapse after transplant remains very poor. A large proportion of the morbidity that prevents most patients from accessing allo-HSCT is due to toxic nonspecific conditioning agents that are required to remove recipient hematopoietic stem and progenitor cells (HSPCs), allowing for successful donor engraftment. CD300f is expressed evenly across HSPC subtypes. CD300f has transcription and protein expression equivalent to CD33 on AML. We have developed an anti-CD300f antibody that efficiently internalizes into target cells. We have generated a highly potent anti-CD300f antibody-drug conjugate (ADC) with a pyrrolobenzodiazepine warhead that selectively depletes AML cell lines and colony forming units in vitro. The ADC synergizes with fludarabine, making it a natural combination to use in a minimal toxicity conditioning regimen. Our ADC prolongs the survival of mice engrafted with human cell lines and depletes primary human AML engrafted with a single injection. In a humanized mouse model, a single injection of the ADC depletes $CD34^+$ HSPCs and $CD34^+CD38^-CD90^+$ hematopoietic stem cells. This work establishes an anti-CD300f ADC as an attractive potential therapeutic that, if validated in transplant models using a larger cohort of primary AML samples, will reduce relapse rate and toxicity for patients with AML undergoing allo-HSCT.

Introduction

Relapse after allogeneic hematopoietic stem cell transplant (allo-HSCT) for acute myeloid leukemia (AML) occurs in 24% to 36% of patients, and the outcomes for these patients are poor.[1] Disease genetic characteristics can predict for relapse overall and impact post–allo-HSCT relapse rates.[2] The rate of relapse after allo-HSCT is higher in adverse-risk groups, particularly in some subgroups such as monosomal karyotypes.[3,4] Postinduction factors that predict relapse include the presence of residual disease. Minimal residual disease (MRD) positivity prior to allo-HSCT, detected by flow cytometry, quantitative polymerase chain reaction, or next-generation sequencing, correlates with relapse.[5-7] Although allo-HSCT remains the only potential curative option in patients with refractory disease, relapse rates remain high in that setting.[8]

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For data sharing, please e-mail the corresponding author.

The full-text version of this article contains a data supplement.

The role of the immune response and graft-versus-leukemia effect is well established.[9] Evidence demonstrates that the intensity of conditioning plays a clear role in reducing relapse risk. Myeloablative (MA) allo-HSCT conditioning regimens reduce relapse more than reduced-intensity conditioning (RIC) and non-MA regimens.[10] The increased relapse rate seen in patients who are MRD positive or undergo non-MA conditioning suggests that reducing the burden of disease by the time of transplant is critical to improving outcomes.

The advent of RIC and non-MA regimens has transformed transplantation, making it accessible to older patients and those with comorbidities. RIC regimens demonstrate significantly less treatment-related mortality (TRM) than MA regimens.[11] Despite the reduction seen in RIC, TRM remains significant, especially in those >65 years.[12] The development of antibody-based therapies depleting hematopoietic stem and progenitor cells (HSPCs) as part of allo-HSCT conditioning is expanding.[13] Such therapies may reduce or eliminate traditional methods of depleting HSPC such as alkylating agents and irradiation. Preclinical studies demonstrate that antibody-drug conjugate (ADC)–based conditioning limits damage to bone marrow (BM) architecture and accelerates immune recovery compared with traditional conditioning.[14] The advent of targeted condition has the potential to further reduce TRM.

The CD300f protein (encoded by the *CD300LF* gene) is an inhibitory receptor found on healthy myeloid cells, including antigen-presenting cells (APCs).[15,16] CD300f is present on a high proportion of AML cells as well as HSPCs.[17,18] Its distribution makes CD300f an excellent target in both AML therapy and targeted allo-HSCT conditioning. We have completed proof-of-principle work demonstrating how incorporating an anti-CD300f ADC into conditioning for allo-HSCT in AML may decrease relapse and toxicity by reducing/replacing traditional agents.

Methods

Preparation of tissue samples

Blood and BM samples from patients with AML or healthy individuals were collected at Concord Repatriation General Hospital (CRGH) or Royal Prince Alfred Hospital (Sydney, Australia). Patient and sample demographics are provided in supplemental Table 1. Peripheral blood (PB) or BM samples from healthy donors were collected at CRGH. Cord blood (CB) samples were collected by the Sydney CB Bank. Donors provided informed consent under ethical approval obtained from the Sydney Local District Human Research Ethics Committee (HREC/12/CRGH/59, HREC/11/CRGH/61 and 118). Mononuclear cells (MCs) were isolated from samples using density gradient centrifugation through Ficoll-Paque Plus (GE Healthcare) according to the manufacturer's protocols. Samples were passed through a 22G needle to disrupt BM fragments and then filtered prior to MC isolation as above. Human monocytes were purified from MCs by CD14 MicroBeads selection using an AutoMACS Pro (Miltenyi Biotec).

Cell lines

The AML cell lines HL-60 and THP-1 were obtained by Derek Hart at the Christchurch School of Medicine, University of Otago. The U973, Z-138, and Mino cell lines were from the American Type Culture Collection. All cell lines were maintained in complete RPMI 1640 (complete RPMI) containing 10% fetal calf serum, 2 mM Gluta-MAX, 100 U/mL penicillin, and 100 μg/mL streptomycin (ThermoFisher).

Gene expression

AML gene expression data were retrieved from the Gene Expression Omnibus microarray dataset GSE14468.[19] HSPC gene expression was retrieved from GSE42519, GSE17054, and GSE19599.[20-22] The series matrix files were parsed in R, and the probe ID and signal values corresponding to *CD300LF (1553043_a_at)* and *CD33 (206120_at)* extracted. The human GTEx data were analyzed in RStudio. Tissue type and transcription data in transcripts per million *CD300LF* and *CD33* were extracted from all experiments in this data set.

Antibodies

Antibodies used for phenotyping can be found in the supplemental Methods.

Internalization assays

Details of internalization assays can be found in the supplemental Methods.

Generation of DCR-2-PBD and isotype-PBD

DCR-2 is a murine immunoglobulin G_1 antibody that binds all extracellular forms of CD300f. DCR-2 and an isotype control antibody were conjugated to pyrrolobenzodiazepine (PBD) dimers by native cysteine chemistry via a cathepsin B–cleavable linker, MA-PEG4-VA-PBD (SET0212, Levena Biopharma). Briefly, monoclonal antibody (mAb) was reduced with 10 mM dithioerythritol to expose free thiols of interchain disulfides. The reduced antibody was reacted with a 10-fold molar excess of PBD linker (MA-PEG4-PBD) (10 mg/mL) for 3 hours before overnight dialysis into phosphate-buffered saline (PBS). The final drug-to-antibody molar ratios were 3.3 for DCR-2-PBD and 4.6 for isotype-PBD.

Cytotoxicity assays

Details of cytotoxicity assays can be found in the supplemental Methods.

CFU toxicity assay

Human CB or primary AML cells were plated at 2×10^4 cells/well in semisolid methylcellulose medium (MethoCult Classic, Stemcell Technologies) in a 24-well plate (Corning) were cultured at 37°C in 5% CO_2 for 14 days when colony-forming units (CFUs) were scored. DCR-2-PBD or isotype-PBD was added to each well for continuous exposure. Three CB samples were tested with duplicate wells performed in each experiment. Primary AML cells were taken from PB samples with >90% blasts and performed in duplicate.

Activation marker expression and MoDC toxicity assays

Monocyte-derived dendritic cells (MoDCs) were derived from $CD14^+$ monocytes as described previously.[23] MoDC phenotyping used accepted activation markers on dendritic cell populations.[24] Details of the assays can be found in supplemental Methods.

Mouse xenograft assays

NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice (Australian BioResources) were housed under specific-pathogen-free conditions. In the subcutaneous model, 2×10^6 U937 cells were injected under

the skin on the right flank. Tumors were measured daily with electronic calipers. When the mean volume of the tumors was >100 mm^3, DCR-2-PBD, isotype-PBD, or PBS was injected intraperitoneally once. Mice were assigned so each cohort had a similar mean starting tumor volume (range, 130.3-137.2 mm^3). Tumor volume was measured until >1000 mm^3, when mice were euthanized.

For the BM HL-60 model, NSG mice were irradiated with 200 cGy 24 hours prior to IV administration of 5×10^6 HL-60 cells. On day 7, mice were injected with DCR-2-PBD, isotype-PBD, or PBS and monitored for disease progression and survival. Mice were euthanized when their clinical score was ≥4 or on day 70.

The humanized mouse model used $CD34^+$ cells (>95% purity) isolated by positive selection (Miltenyi 130-046-702) according to the manufacturer's instructions from 2 pooled CB samples. Each NSG mouse was injected with 100 000 $CD34^+$ cells 4 hours after receiving a 150-cGy irradiation dose. Engraftment as determined by percentage of human $CD45^+$ cells in venous blood was assessed at 12 weeks. Mice were assigned to treatment groups to have similar means of human $CD45^+$ percentage in the PB (10.16% DCR-2-PBD cohort and 10.74% isotype-PBD cohort). Mice were then injected with 300 μg/kg of DCR-2-PBD or isotype-PBD. Mice were euthanized on day 7, and human $CD45^+$ cells from BM (bilateral femurs and tibias) and 300 μl blood were enumerated and phenotyped by flow cytometry.

For primary AML xenografts, NSG mice were irradiated with 150 cGy 24 hours before 8×10^6 AML cells from CRGH11 (sample 10, supplemental Table 1) were injected IV. Once engraftment was established (>1% human CD45-positive events in PB), mice were given DCR-2-PBD or isotype-PBD. Mice were euthanized 6 days later, and the BM was harvested for enumeration and surface marker analysis by flow cytometry.

Flow cytometry

CD300f expression on primary AML and BM samples was performed on an Influx flow cytometer (BD Biosciences). AML cell line experiments were performed on an Accuri flow cytometer (BD Biosciences). Remaining assays were performed on a Fortessa LSR flow cytometer (BD Biosciences). Analysis, including TiSNE, was performed using FlowJo.

Statistical analysis

Statistical analysis was performed using Prism (GraphPad Software). Error bars correspond to standard error. Differences in means between 2 groups were assessed using Student *t* tests. Multiple-group tests were performed using analysis of variance with posttest comparisons. Differences in survival were assessed using a log-rank (Mantel-Cox) test. The combination index was calculated using Compusyn software.[25]

Results

CD300f is expressed on AML and HSPCs, but not outside of the hematopoietic system

To assess the distribution of CD300f, gene expression data were analyzed. As gentuzumab ozogamicin, which targets CD33, is the only approved AML therapeutic that binds a surface molecule, we compared expression of CD300f to CD33 in AML. We analyzed expression array data from patients with AML (n = 460) to compare *CD33* and *CD300LF* (Figure 1A). The mean *CD300LF* expression level was significantly higher than *CD33* (8.39 $\log_2$ vs 7.89 $\log_2$, $P < .0001$). There was a weak correlation in transcript expression between *CD300LF* and *CD33* ($r = 0.16$, $P = .004$). There were no significant differences in *CD300LF* expression across the European LeukemiaNet risk groups (supplemental Figure 1A).

Protein expression of CD300f and CD33 was compared by flow cytometry (Figure 1B). There was no significant difference in mean fluorescence intensity (MFI) ratios, and there was a moderate to strong correlation in expression ($r = 0.63$, $P = .0001$). CD300f protein expression on the $CD34^+CD38^-$ subset of primary AML showed no significant correlation with CD33 protein expression (supplemental Figure 1B). Single-cell expression was compared on a set of 9 AMLs using TiSNE, with a similar distribution seen for CD300f (supplemental Figure 1C) and CD33 (supplemental Figure 1D). Gene expression array data demonstrated CD300f expression across hematopoietic stem cells and myeloid hematopoietic progenitor cells (Figure 1C), with the lowest expression in megakaryocyte/erythroid progenitors ($P < .0001$). Protein expression by flow cytometry did not reveal any significant differences in MFI ratio across the groups of HSPCs. A standard gating strategy (supplemental Figure 3) was used to determine HSPC subsets. Protein expression on peripheral blood mononuclear cells showed CD300f expression on monocytes and neutrophils, but not lymphocytes (supplemental Figure 2). RNA sequencing (RNA-seq) data from the human GTx database of multiple organs were analyzed for potential off-target effects of anti-CD300f–based therapy. Both *CD33* and *CD300LF* had significantly increased expression in the blood, spleen, and lungs compared with all other organs ($P < .0001$) (Figure 1E-F). Immunohistochemistry analysis by The Human Protein Atlas (https://www.proteinatlas.org/) indicates that CD33 and CD300f are expressed on lung macrophages, but not on pneumocytes.

Antibodies to CD300f are internalized upon binding

The mouse anti-human CD300f antibody, DCR-2 was assessed for its ability to be internalized by flow cytometry. It rapidly internalizes upon binding to HL-60 cells (Figure 2A). Internalization is significant at 30 minutes and is >50% at 180 minutes. Internalization at 30 minutes was confirmed by fluorescence microscopy on HL-60, $CD14^+$ monocytes and primary AML cells (Figure 2B). The internalization data demonstrates that DCR-2 is a suitable mAb for ADC development.

In vitro cytotoxicity of DCR-2-PBD

DCR-2 and an isotype control mAb were conjugated to a PBD toxin to assess potential cytotoxicity. The cytotoxicity of DCR-2-PBD and the isotype-PBD were tested against $CD300f^+$ and $CD300f^-$ cell lines (Figure 3A). DCR-2-PBD killed the $CD300f^+$ AML cell lines HL-60, U937, and THP-1 with 50% inhibitory concentrations (IC_{50}s) in the low picomolar range (5.44, 6.74, and 29.39 pM, respectively). These IC_{50}s are similar to those of other PBD-based ADCs.[26,27] DCR-2-PBD has >200 pM IC_{50} against the $CD300^-$ lymphoma cell line Z-138. The isotype-PBD has an IC_{50} of >200 pM against HL-60 and U937.

AML surface targets, including CD300f, can be heterogeneously expressed within AML cases, and we considered bystander killing advantageous for an effective ADC. We investigated bystander killing by comparing the viability of the $CD300f^-$ lymphoma cell line

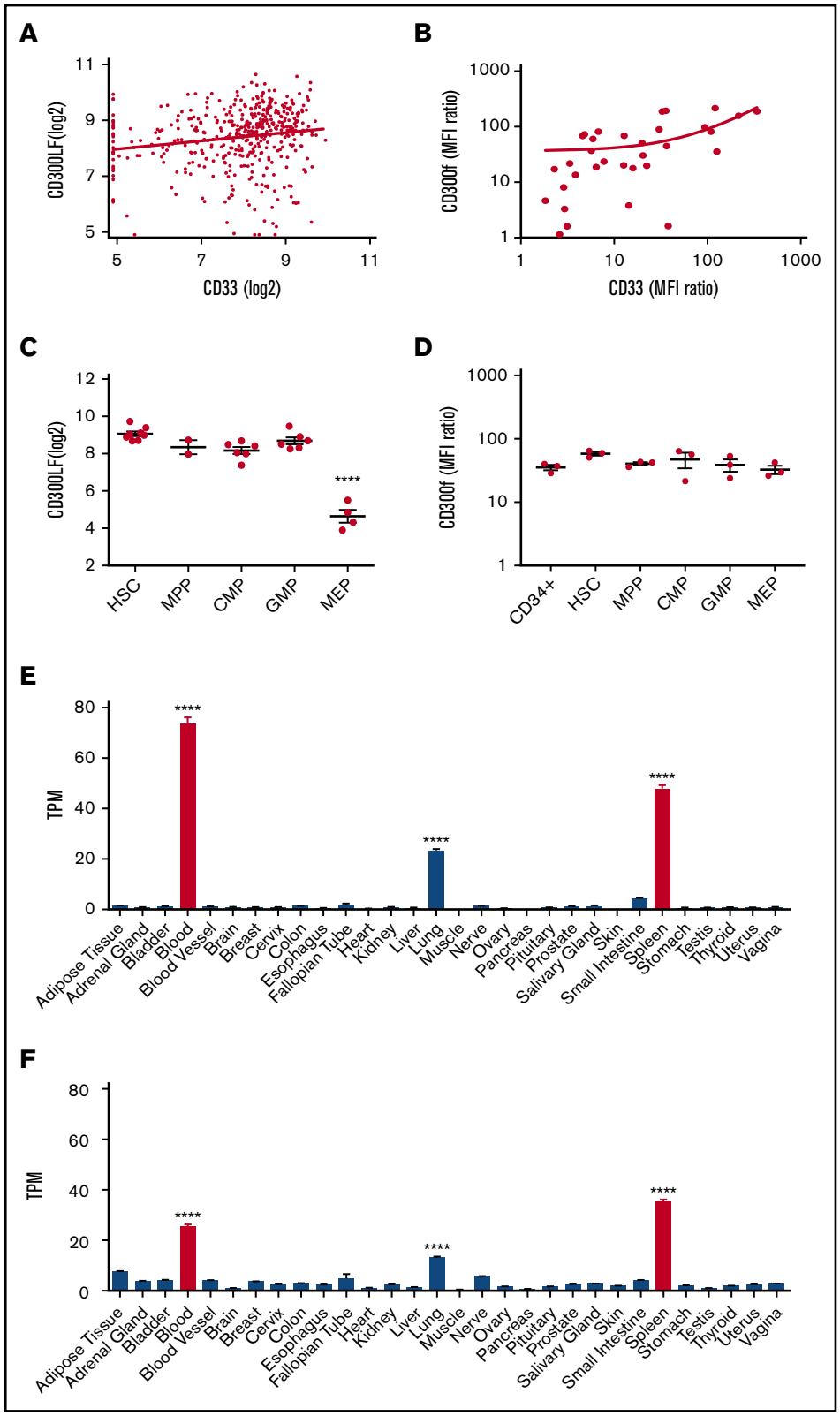

Figure 1. CD300f expression on AML cells, HSPCs, and organ expression. (A) CD33 is compared with CD300f by gene expression using linear regression analysis. (B) MFI ratios of CD33 and CD300f on patient-derived AML cells (n = 33) are compared with linear regression. (C) CD300f gene expression across HSPC subtypes (****$P < .0001$ megakaryocyte/erythroid progenitors vs all other subtypes). (D) MFI ratios of CD300f on HSPC from healthy BM. (E) CD300f gene expression by RNA-seq across multiple organ types from the human GTEx dataset (****$P < .0001$ blood, spleen or lung vs all organs). (F) CD33 gene expression by RNA-seq across multiple organ types (****$P < .0001$ blood, spleen, or lung vs all organs). Red bars indicate hematopoietic organs. TPM, transcripts per million.

Mino cultured on its own with Mino cultured mixed with HL-60 in the presence of DCR-2-PBD (Figure 3B). There was a significant reduction in viability in the 75% Mino condition with DCR-2-PBD at 25 pM ($P = .0002$) compared with the Mino-only control. In the 50% Mino condition, there was a significant reduction in viability in both the 25 pM ($P < .0001$) and 12.5 pM ($P = .0004$) DCR-2-PBD concentrations. The reduction in Mino cells when combined with HL-60 confirms the ability of DCR-2-PBD to generate bystander killing.

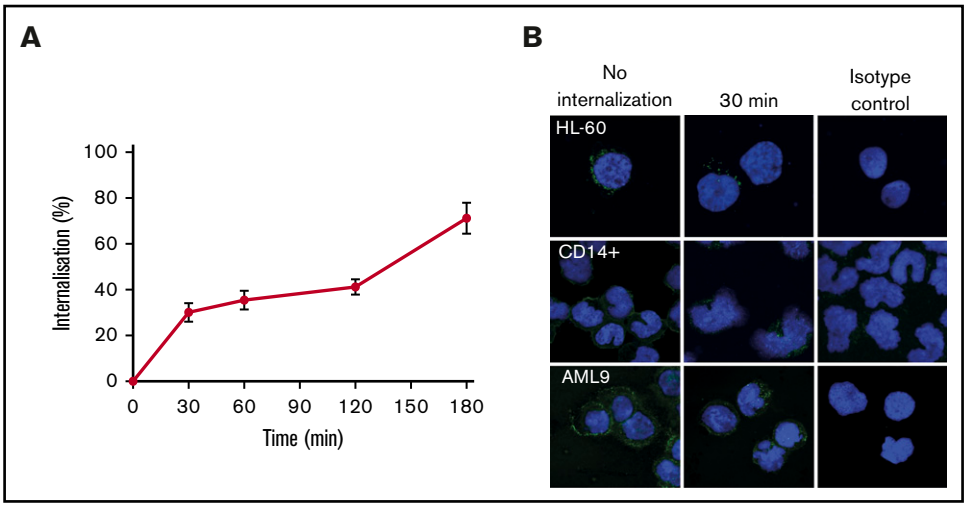

Figure 2. Internalization of DCR-2. (A) Internalization kinetics of DCR-2 on HL-60 (% internalized = relative MFI of total − relative MFI of surface staining). (B) Immunofluorescent microscopy of HL-60, CD14$^+$ monocytes, and primary AML at 0 minutes (no internalization) and 30 minutes showing internalization of CD300f detected by LMIR-3 antibody. Isotype control shown is at 30 minutes. Images were taken at 63× magnification using a 63× HC PL APO CS2 NA 1.40 lens.

Fludarabine is used in many allo-HSCT regimens to facilitate donor cell engraftment by depleting host lymphocytes. Fludarabine combined with PBD demonstrates synergistic cytotoxic properties.[28] An anti-CD300f therapeutic would deplete recipient HSPCs, but not host lymphocytes, as part of a conditioning regimen. Given these complementary roles, we assessed a synergistic relationship between DCR-2-PBD and fludarabine. Cytotoxicity of HL-60 (Figure 3C) and THP-1 (data not shown) was greater in the presence of both DCR-2-PBD and fludarabine than either alone, confirming a synergistic relationship with a combination index of 0.82 and 0.76, respectively. We assessed the exposure time required for DCR-2-PBD to cause cytotoxicity on the HL-60 cell line (Figure 3D). At 12 hours, >98% of HL-60 proliferation was inhibited, while at 24 and 96 hours, inhibition was >99%.

We used a CFU assay to test DCR-2-PBD activity against HSPC and primary AML. Significant reductions in total CFUs were seen at 7.84 ($P = .0033$), 39.21 ($P < .0001$), and 196.1 pM ($P < .0001$) (Figure 3E). All major CFU subtypes were inhibited at the 32.9 and 196.1 pM concentrations by DCR-2-PBD (Figure 3F). Two primary AML samples were tested: CRGH1 with low CD300f expression (MFI ratio of 2.6) and CRGH9 with a high CD300f expression (MFI 109.9) (Figure 3G). Significant toxicity was demonstrated against CRGH1 at 196.1 pM ($P = .036$). Significant toxicity was seen in CRGH9 at all dose levels ($P < .001$).

CD300f is expressed on myeloid APCs; therefore, depletion of CD300f-expressing cells may impair recipient antigen presentation.[29] We examined if DCR-2-PBD would reduce myeloid APC cell numbers, prevent activation, or have a functional inhibitory effect (supplemental Figure 4). MoDCs were used to test the ability of DCR-2-PBD to deplete APCs. DCR-2-PBD did not significantly reduce MoDC numbers. PBD has demonstrated cytotoxicity against quiescent and dividing cells but to our knowledge has not been previously trialed on terminally differentiated myeloid cells.[30] The impact of DCR-2-PBD on myeloid APC activation was assessed by incubating APCs with DCR-2-PBD followed by lipopolysaccharide. DCR-2-PBD did not inhibit the expression of activation markers on Lineage$^-$ HLADR$^+$ CD11c$^+$ myeloid dendritic cells. DCR-2-PBD did not prevent T-cell activation in a 1-way mixed lymphocyte reaction.

DCR-2-PBD prolongs the survival of mouse AML cell line models

DCR-2-PBD in vivo function was tested in subcutaneous and BM cell line models. U937 was injected subcutaneously in NSG mice. Mice were injected with a single dose of PBS, isotype-PBD (150 or 300 μg/kg) or DCR-2-PBD (150 or 300 μg/kg) (Figure 4A), and mice were monitored for tumor growth. Tumor growth was delayed, and survival was significantly increased in both the 150 μg/kg group ($P = .0019$) and 300 μg/kg ($P = .0017$) of DCR-2-PBD compared with the isotype-PBD (300 μg/kg) group. A second model used irradiated mice that were IV injected with HL-60, which led to heavy BM infiltration, met clinical end points at a median of day 23. A single injection of DCR-2-PBD (300 μg/kg) significantly increased survival ($P = .0058$) compared with isotype-PBD (Figure 4B). Six of 8 animals treated with DCR-2-PBD survived to the experiment end point. No human cells could be detected in mice surviving until day 70, and the range of human CD45$^+$ cells in all other mice was 76% to 88% at the time of euthanasia.

DCR-2-PBD depleted both primary HSPCs and AML cells in mouse xenografts

Further proof of principle for an anti-CD300f ADC to contribute to allo-HSCT conditioning was demonstrated using DCR-2-PBD to selectively deplete HSPCs and myeloid cells in a humanized mouse model. Humanized mice injected with a single dose of DCR-2-PBD (300 μg/kg) had a significant depletion in the mean of both total CD34$^+$ (0.29×10^5 vs 9.42×10^5, $P = .001$) and total primitive CD34$^+$CD38$^-$CD90$^+$ cells (0.05×10^4 vs 5.54×10^4, $P = .008$) compared with isotype-PBD–treated groups (Figure 5A). The difference was also significant as a percentage of human CD45$^+$ cells for both the CD34$^+$ (3.09% vs 20.62%, $P < .0001$) and CD34$^+$CD38$^-$CD90$^+$ populations (0.16% vs 2.17%, $P = .008$). The selectivity of DCR-2-PBD compared with isotype-PBD was demonstrated by depleting the myeloid cells (0.31 cells/μL vs 14.89 cells/μL, $P = .0002$), but not lymphocytes (208.5 cells/μL vs 263 cells/μL), which are longer lived and do not express CD300f, from PB samples (Figure 5B).

We demonstrated the efficacy of DCR-2-PBD against primary AML by injecting mice with a single dose of 300 μg/kg DCR-2-PBD or

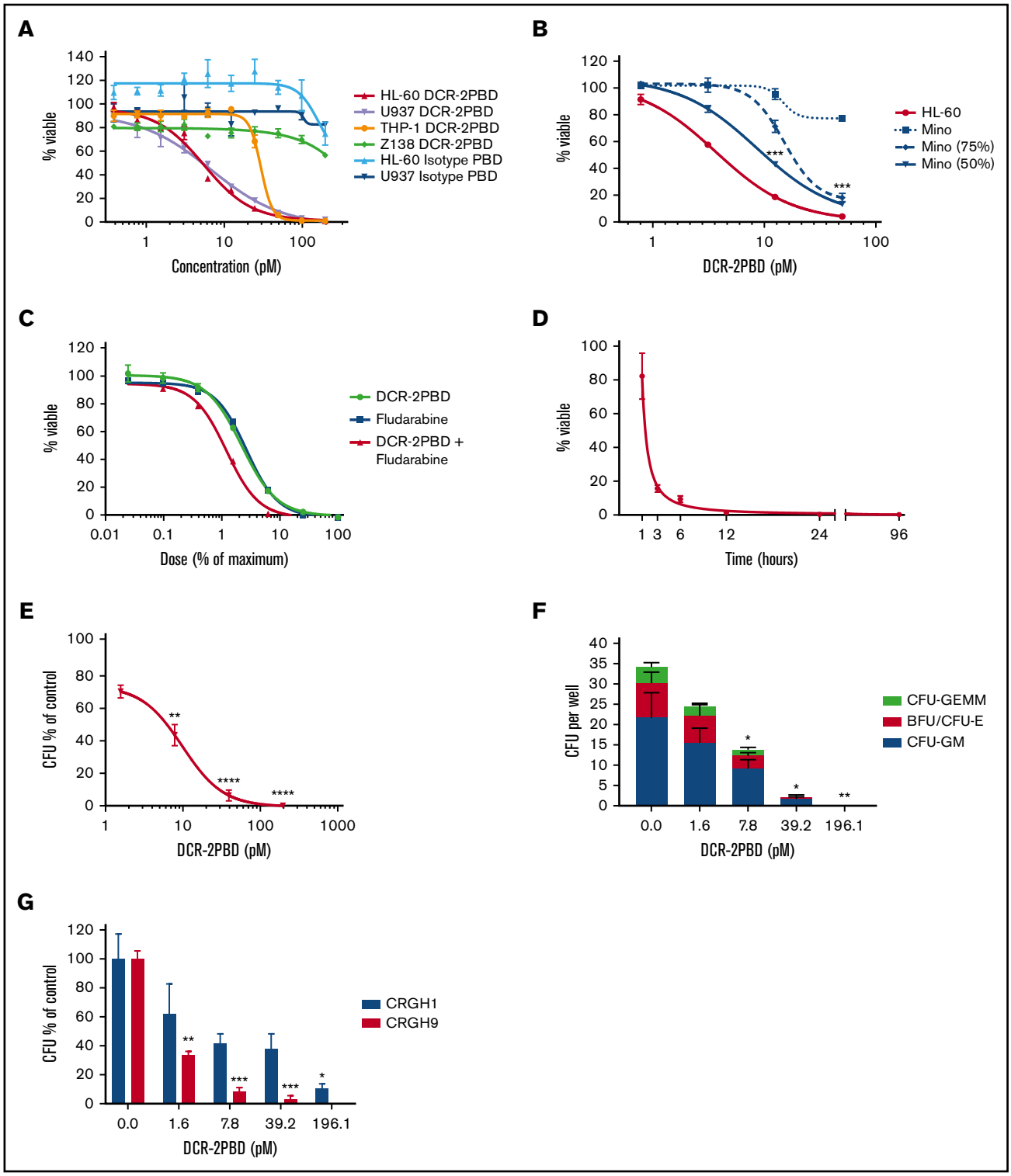

Figure 3. DCR-2-PBD in vitro cytotoxicity. (A) Inhibitory concentration curves on CD300f^{+} AML cell lines (HL-60, U937, and THP-1) and CD300f^{-} lymphoma cell line (Z-138) using DCR-2-PBD or isotype-PBD (performed in triplicate). (B) Bystander killing assay using an CD300f^{-} lymphoma cell line (Mino) and HL-60 either alone or in combination. In combination conditions, only the percentage of viable of Mino cells is shown (performed in triplicate) (***$P < .001$ Mino 50% at 25 and 12.5 pM, Mino 75% at 25 pM). (C) Combination DCR-2-PBD and fludarabine inhibitory concentration curves using HL-60 (performed in triplicate). (D) Time-dependent killing assay of DCR-2-PBD on HL-60, with final viability measured at 96 hours. (E) Total CFU inhibitor concentration curve with DCR-2-PBD (3 CB samples each performed in duplicate) (**$P < .01$ CFU inhibition at 7.8 pM, ****$P < .0001$ CFU inhibition at 39.2 and 196.1 pM). (F) Individual CFU subtype formation inhibition by DCR-2-PBD (*$P < .05$ CFU inhibition at 7.8 and 39.2 pM, **$P < .01$ CFU inhibition at 196.1 pM). (G) Individual AML CFU inhibition with DCR-2 PBD (performed in duplicate) (*$P < .05$ CFU inhibition at 196.2 pM for CRGH1, **$P < .01$ CFU inhibition at 1.6 pM for CRGH9, ***$P < .001$ CFU inhibition at 39.2 pM and 196.2 pM for CRGH9). BFU, burst-forming unit; GEMM, granulocyte, erythroid, macrophage, megakaryocyte; GM, granulocyte macrophage.

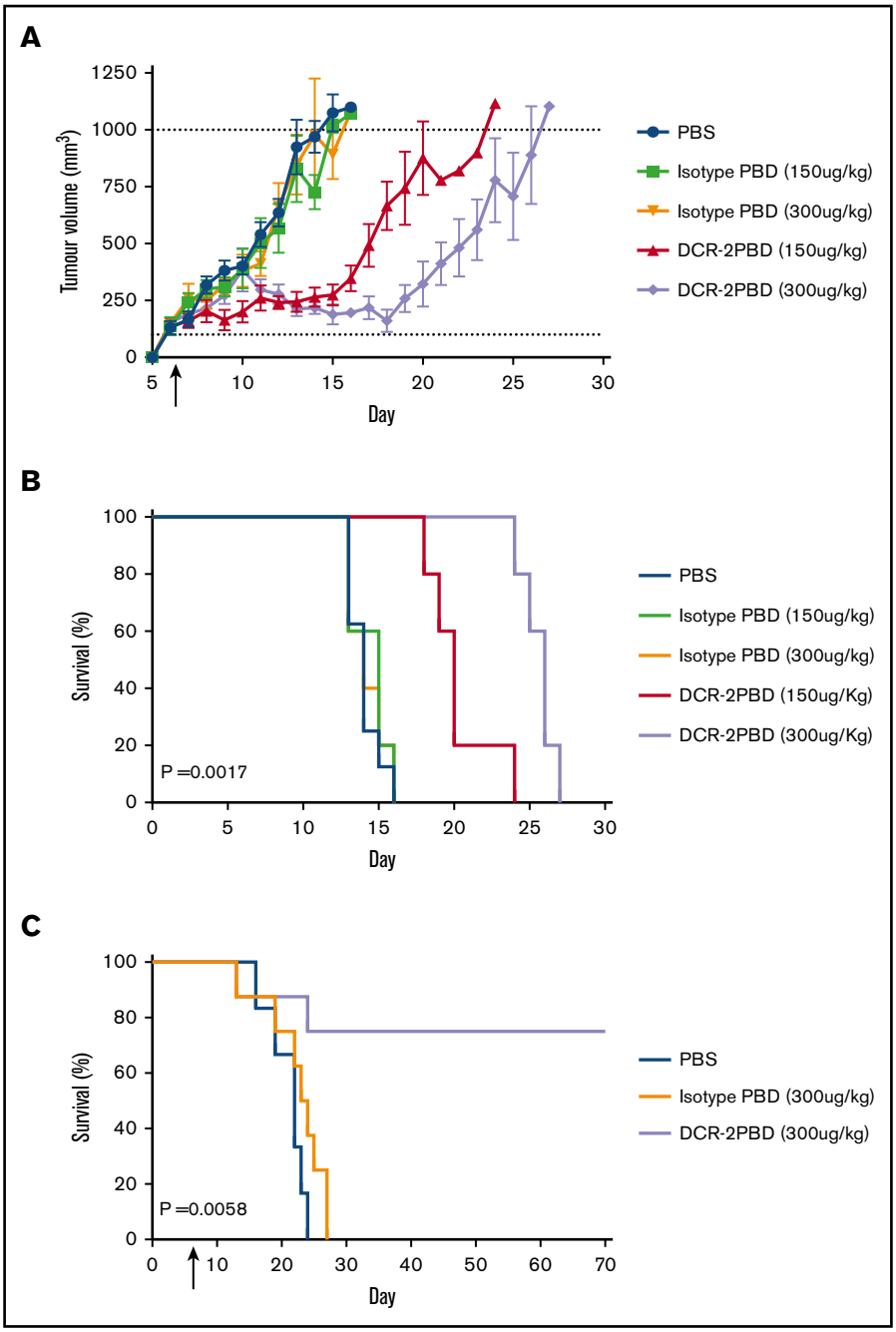

Figure 4. DCR-2-PBD prolongs survival in AML cell line mouse models. (A) U937 subcutaneous tumor volume of mice treated with a single injection on day 6 with either PBS (n = 8) or isotype-PBD (n = 5 both groups) or DCR-2-PBD (n = 5 both groups). (B) Survival data from mice injected with U937 subcutaneously (P = .0017 DCR-2-PBD 300 μg/kg compared with isotype-PBD 300 μg/kg). (C) Mice injected with HL-60 IV then treated day 7 with PBS (n = 6), isotype-PBD (n = 8), or DCR-2-PBD (n = 8) (P = .0058 DCR-2-PBD vs isotype-PBD).

isotype-PBD and enumerating BM engraftment on day 7. DCR-2-PBD significantly depleted the human AML cells residing in the BM compared with isotype control when measured by mean total enumeration (0.37×10^6 vs 1.45×10^6 P = .019) (Figure 5C-E). DCR-2-PBD significantly reduced the AML cells as a mean percentage of total cells (Figure 5F) compared with isotype-PBD (12.32% vs 38.92%, P = .03).

Discussion

The ideal way to use the next generation of surface-molecule–targeted therapy remains unclear. Avoiding an allo-HSCT would be ideal, but unless a true AML-specific antigen without expression on HSPCs is validated, hematological toxicity will remain unacceptably high. CD300f has been explored as an AML target, but a clear method on how to best use an anti-CD300f therapeutic has been lacking.[17,18] Our work has validated CD300f as an ideal target for a novel conditioning agent in AML.

CD300f has an expression level and distribution comparable to CD33 both between and within AML cases. It is also unlikely to have significant expression outside the hematopoietic system. CD300f efficiently internalizes upon antibody binding, making it an excellent candidate for ADC development. DCR-2-PBD demonstrated specific in vitro toxicity of AML cell lines and HSPCs. The PBD component allows for rapid cytotoxicity and bystander killing.

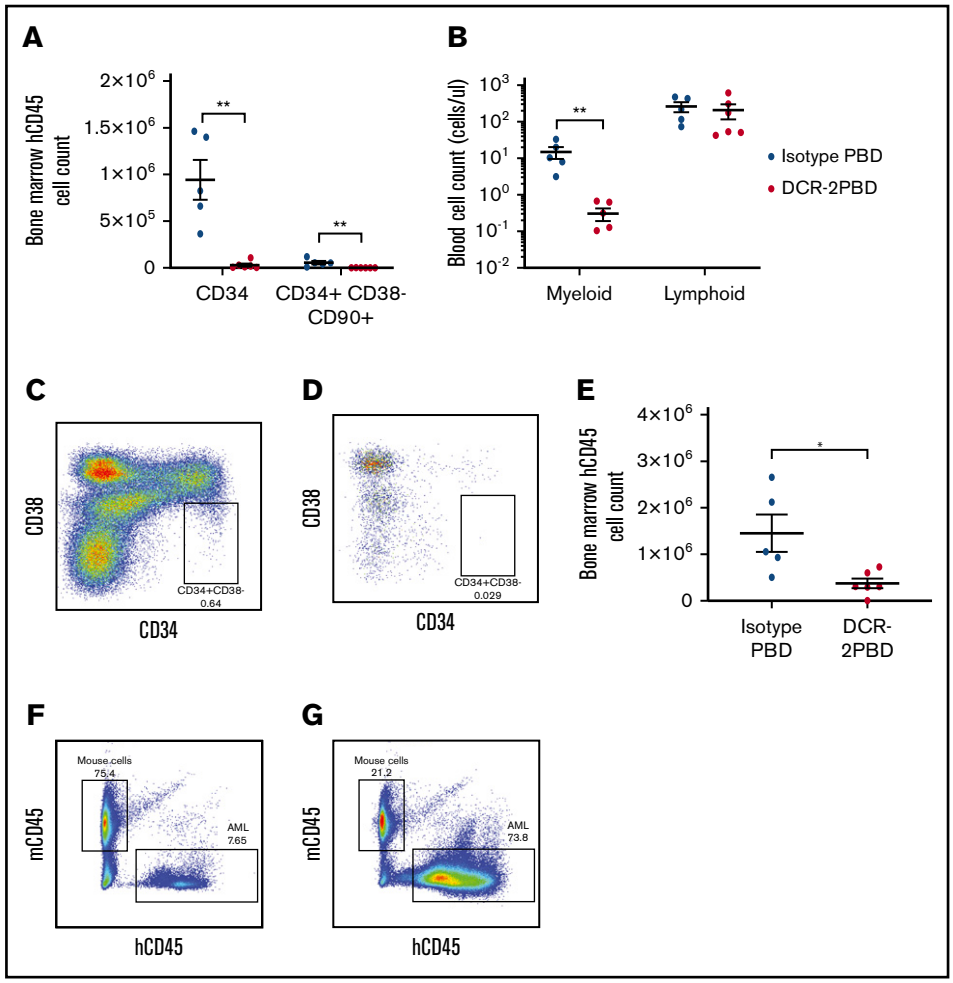

Figure 5. DCR-2-PBD depletes HSPCs in vivo. (A) Total cell count of $CD34^+$ and $CD34^+$ $CD38^-$ $CD90^+$ cells in humanized NSG mouse BM 7 days after injection of DCR-2-PBD (n = 6) or isotype-PBD (n = 5) (**$P < .01$ reduction in $CD34^+$ and $CD34^+$ $CD38^-CD90^+$ cells in the DCR-2-PBD cohort compared with the isotype control cohort). (B) Cell count per microliter of blood from DCR-2-PBD and isotype-PBD treated humanized mice (**$P < .01$ reduction in myeloid cells in PB of DCR-2-PBD cohort compared with isotype-PBD cohort). (C-D) Representative plots of human $CD45^+$ cells in the BM of a control humanized mouse (C) and a DCR-2-PBD treated humanized mouse (D). (E) BM enumeration of primary AML 6 days after injection of mice treated with isotype-PBD and DCR-2-PBD (n = 6 DCR-2-PBD, n = 5 isotype-PBD) (*$P < .05$ reduction of primary AML cells in BM, DCR-2-PBD cohort compared with the isotype-PBD cohort). (F-G) Representative plots of mouse CD45 vs human CD45 cells in mice engrafted with primary AML treated with isotype-PBD (F) and DCR-2-PBD (G).

DCR-2-PBD prolonged survival in AML cell line models as well as depleted primary AML cells and HSPCs in vivo.

Alternatives to traditional chemotherapy-based treatments for AML include ADCs and chimeric antigen receptor T cells. As AML is derived from HSPCs, these cells share many surface membrane molecules, making the choice of a target difficult.[31] Although gentuzumab ozogamicin had setbacks prior to its current listing, it provides evidence that an ADC may alter the course of AML in some patients.[32] Vadastuximab talirine is an ADC that targets CD33 coupled to a PBD, which induced complete remission and MRD negativity in older patients with AML, but the maximum tolerated dose was limited by hematologic toxicity with BM aplasia.[33] Nonhematologic toxicity was minimal. The marked hematologic toxicity demonstrated suggests that vadastuximab talirine targeted both AML and HSPCs. Vadastuximab talirine prior to allo-HSCT demonstrated tolerability and good outcomes for the small number of patients involved, suggesting no permanent effect of the PBD toxin limiting engraftment.[34] The development of vadastuximab talirine ceased after an increased number of deaths due to infections were observed in the phase 3 CASCADE study.[35] Incorporating an ADC into a transplant strategy may limit hematologic toxicity

We have shown that PBD, when internalized on an anti-CD300f antibody, causes significant cytotoxicity even with a brief exposure, which is ideal for a conditioning agent. A limitation with ADC-based conditioning is that the half-lives of most antibodies would endanger donor cells. Alternative strategies to shorten the half-life of an antibody by removing the FcRn recognition site or using an antibody fragment (ie, antigen-binding fragment) would address these concerns.[36,37] A DCR-2–based therapeutic modified in this way will make such a conditioning approach possible.

CD300f has the potential to be variably expressed within a single case of AML, as this is common for many target antigens, including CD33.[38] We have sought to mitigate this by employing a toxin with a bystander effect. The bystander effect of ADCs can be seen with brentuximab vedotin, which can induce a clinical response even in tumors with minimal expression.[39] The ability of a toxin to kill bystander cells would enhance responses in heterogeneous tumors but also increase the potential for off-target effects. Given the inherent heterogeneity and plasticity of AML surface expression, choosing a toxin with a bystander effect, like we have demonstrated with DCR-2-PBD, will significantly increase efficacy. An immunosuppressive agent must combined with $CD34^+$ depletion to facilitate engraftment. Fludarabine was found to be a natural partner to DCR-2-PBD given its widespread use in conditioning and synergistic properties with PBD.[28]

CD300f has some significant advantages to other molecules being explored in targeted conditioning. ADCs targeting CD45 and CD117 have been investigated for AML conditioning.[40] While CD45 may

contribute to both lymphodepletion and the reduction in healthy $CD34^+$ cells and residual AML, it is expressed at low levels in AML and is not efficiently internalized.[41] A higher total dose of ADC may overcome inefficient internalization, but this would increase the prospect of free toxin causing nonspecific toxicity. Anti-CD117 ADCs significantly reduce HSPCs in animal models.[42] A major concern targeting CD117 with a potent ADC is its significant expression outside of the hematopoietic system, which raises the possibility of increased toxicity.[43] The even distribution across the major HSPC subtypes would likely make CD300f a more efficient conditioning agent compared with current AML targets being studied, which often have variable HSPC expression.

We have demonstrated that CD300f is expressed on AML cells and HSPCs, and it is unlikely to be expressed significantly on cells of nonhematopoietic origin. CD300f is expressed on monocyte derived cells such as pulmonary macrophages and microglia in non-hematopoietic tissue.[44-46] While this may suggest the potential for "off-target" toxicities, these same cells express CD33, and no severe nonhematologic toxicities were noted with the development of vadastuximab talirine.[33] Despite this, a major limitation of this work is the examination of off-target ADC effects. The mouse anti-human CD300f ADC does not bind mouse CD300f, and therefore, mouse xenogeneic models would not be appropriate to examine nonhematopoietic toxicity. Any depletion of HSPCs may induce neutropenia given the very short half-life of neutrophils, regardless of the expression of the target on neutrophils themselves. We would expect an anti-CD300f therapeutic to induce neutropenia, but other ADCs used in transplant models have avoided neutropenia altogether while maintaining immunity, even when the target is expressed on neutrophils.[14]

We also investigated the impact of an anti-CD300f ADCs on host APCs. We predict the lack of reduction or effectiveness of myeloid APCs is due to inherent resistance to PBD as they are terminally differentiated. Tissue inflammation caused by nonspecific conditioning agents has a role in the development of graft-versus-host disease, and an ADC conditioning backbone may significantly alter the posttransplant immune landscape.[47] A DCR-2-PBD–based therapeutic would still require a lymphodepleting agent, which may mitigate any potential benefits of limiting tissue damage by removing alkylating agents or radiation. There are now multiple groups developing targeted conditioning, and the impact on graft-versus-host disease and graft-versus-leukemia needs to be studied further.

With the current range of AML targets, an allo-HSCT will be required as part of novel AML treatments to allow the use of highly potent therapies targeting myeloid antigens such as ADCs and chimeric antigen receptor T cells. An advantage of adapting these therapies to become part of transplant conditioning is that it may allow for advancements in transplantation outside of AML. The use of novel less toxic, specific conditioning agents will broaden their application from AML into transplantation for MDS, with the possibility of reducing toxicity in a cohort of patients that traditionally suffer significant morbidity and mortality from allo-HSCT. Recent advances in antibody-based therapeutics have presented the possibility of targeted conditioning agents.[13,48] Clinical trials have begun with unconjugated antibodies as conditioning agents (NCT02963064). The potent effect demonstrated by DCR-2-PBD against healthy HSPCs suggests that therapeutic derivatives targeting CD300f may be incorporated into conditioning for nonmalignant disorders of erythropoiesis or immunodeficiency either as part of an allo-HSCT or a gene-modified autologous HSCT.

This work validates CD300f as a specific target that merits further exploration in targeted conditioning therapy. Testing across a greater number of primary AML samples and implementation of transplant models will be required.

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Authorship

Contribution: E.A. and G.J.C. designed the study and planned the experiments; E.A., P.A.S., R.E.G., M.R., A.R., A.H.M., T.-H.L., K.K., S.S., and G.A.P. performed experiments; E.A., P.A.S., R.E.G., and G.J.C. analyzed data; E.A. and G.J.C. wrote the manuscript; and P.A.S., R.E.G., S.S., C.E.B., P.J.H., and S.R.L. assisted with revision of the manuscript.

Conflict-of-interest disclosure: G.J.C. is a director of DendroCyte BioTech Pty Ltd. G.J.C. and R.E.G. are listed as inventors on patents protecting DCR-2. The remaining authors declare no competing financial interests.

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Supplementary Methods

Antibodies

AML samples were phenotyped with a monoclonal antibody (mAb) panel containing CD300f -efluor660 (clone UP-D1) (ThermoFisher) CD45-V500 (HI30), CD34-PE-CY7 (581), CD38-V450 (HB7), CD33-PE (WM53) and CD11c-APC-AF700 (B-Ly6) from BD Biosciences and HLA-DR-APC (L243) (Biolegend). Healthy BM samples were phenotyped with a lineage (Lin) cocktail containing V450 conjugated CD235a (GA-R2), CD14 (MϕP9), CD20 (2H7), CD19 (HIB19), CD56 (NCAM16.2) and CD3 (SK) (BD Biosciences). A mAb panel containing CD3-V450 (SP34-2), CD19-V450 (HIB19), CD56-V450 (B159), CD14-V450 (M5E2), CD11c-AF700 (B-ly6), CD80-PECy7 (L307.4) all from BD biosciences, HLA-DR-APC and CD83-FITC (HB15a) (Beckman Coulter) was used to assess CD83 and CD80 expression on myeloid cell populations. Healthy PB was phenotyped using CD45-AF488 (HI30), CD3-AF700 (SP34-2), CD56-PECy7 (NCAM16.2) and CD19-V450 all from BD Biosciences. Cell populations from mouse xenografts were phenotyped with anti-human CD45-AF488, CD33-PE (WM53), and anti-mouse CD45-PerCP Cy5.5 (30-F11) (BD Biosciences). Samples from the humanised NSG mice (Hu-NSG) were phenotyped with CD3-V450 (SP34-2), CD19-V450, CD56-V450, CD45-AF488, CD34-PE-CF594 (581), CD33-PE, CD38-APC (HIT2), CD90-AF700 (5E10) and anti-mouse CD45-PerCP Cy5.5 (BD Biosciences). Binding is displayed as a geometric mean fluorescence intensity (geoMFI) ratio which was calculated by: geoMFI test antibody/geoMFI isotype control.

Internalisation Assays

DCR-2 (IgG1), and as an isotype control, the anti-tetanus toxoid mAb, CMRF-81 (IgG1) which were both produced, purified and directly conjugated to phycoerythrin (PE) in house. Cells were incubated with DCR-2-PE or CMRF-81-PE (10µg/ml) on ice for 20 min, washed to remove unbound mAb then incubated at 37°C/5% CO2 to allow internalisation for the indicated times. After incubation, residual mAb remaining on the cell surface was detected with a secondary goat anti-mouse (GAM) IgG-AF488 antibody. Cells were fixed in 1% paraformaldehyde/PBS for flow cytometry analysis. The surface and total relative Mean Fluorescent Index (MFI) was calculated as the (MFI of binding antibody at time point – MFI of isotype control at time point) at 37°C/ (MFI of binding antibody at time point– MFI of isotype control at time point) at 0oC. The percent internalised was assessed by 100- (relative MFI surface staining / relative MFI total staining). In immunofluorescent microscopy experiments cells were stained with DCR-2 then air-dried and fixed with 4% paraformaldehyde or incubated at 37°C for 30 minutes then fixed. Rehydration was performed using 1% BSA/PBS prior to staining with GAM IgG-AF488 antibody and 18µM DAPI (ThermoFisher).

MoDC Toxicity Assay

MoDC were incubated with DCR-2-PBD or isotype-PBD (200pmol) 72 hours prior to enumeration of live cells by flow cytometry.

DC Activation Assay

Peripheral Blood Mononuclear Cells (PBMC) were incubated in complete RPMI media with or without DCR-2-PBD (200pmol). After 12 hours, washed cells were incubated for a further 12 hours with or without LPS then DCR-2-PBD or isotype-PBD (200pmol) were added for 72 hours prior to enumeration of live cells by flow cytometry. The expression of CD83 and CD80 was assessed on Lineage- HLADR+CD11c+ myeloid DC.

Mixed Leucocyte Reaction

T cells were depleted from PBMC by magnetic selection using an AutoMACS Pro with anti-CD3 mAb (HIT3a, Biolegend) with greater than 90% depletion in all samples. The CD3-depleted PBMC were incubated in complete RPMI media with DCR-2-PBD (200pmol), isotype-PBD (200pmol) or PBS for 24 hours. After washing to remove unbound ADC, the CD3-depleted PBMC were used to stimulate allogeneic *Carboxyfluorescein Succinimidyl Ester* (CFSE) labelled naive CD4+ T cells which had been prepared using a RosetteSep Kit (Stem Cell Technologies 17555). On day 5 the proliferation of T cells, identified using anti-CD3 AF700 (SP34-2), was assessed by CFSE reduction using flow cytometry. The results of the DCR-2-PBD and isotype-PBD groups were normalised to the PBS control group. Stimulator populations were prepared from three PBMC donors and experiments were performed in duplicate.

Cytotoxicity Assays

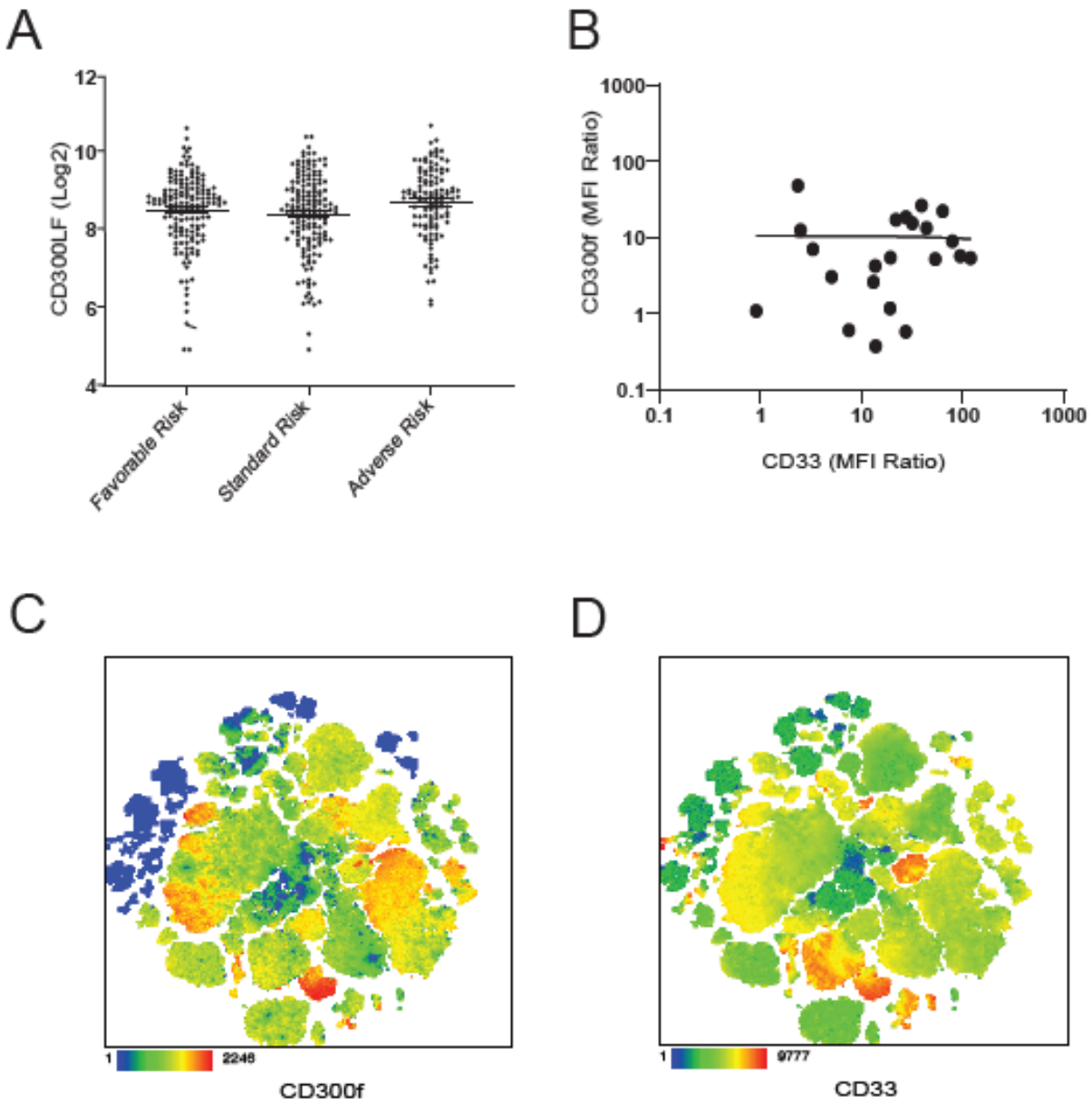
Cytotoxicity was determined by incubating 5000 target cells with DCR-2-PBD, isotype-PBD or PBS in 200µl total volume of complete RPMI for 96 hours at 37°C/5% CO2 after which DAPI- viable cells were enumerated using flow cytometry. Events per condition were compared to the mean of the control group to obtain the % viable. Bystander killing of CD300f-Mino cells was performed as above. After incubation, live bystander cells were identified with CD20-PE (2H7) and DAPI. Kinetic analysis was performed as above. At the indicated time points, washed cells were resuspended in complete RPMI and cultured for 96 hours before analysis. Synergy between ADC and fludarabine was assessed as above by combining DCR-2-PBD and/or fludarabine in samples. All samples were tested in triplicate.

Supplementary Table 1

Sample ID	Age	Sex	Sample	WHO Diagnosis	FAB Classification	Sample Blasts %	Karyotype	NPM1	FLT3-ITD
1	77	F	PB	AML with MDS changes	M2	55	Normal	NT	NT
2	76	F	BM	AML NOS	M4	70	Normal	NT	NT
3	74	M	PB	AML NOS	M2	43	8	NT	NT
4	70	M	BM	Therapy related myeloid neoplasm	M4	82	Normal	Detected	Detected
5	79	M	PB	Therapy related myeloid neoplasm	M4	67	46, XY, add(12) (q24.3)	NT	NT
6	89	M	PB	AML with MDS changes	M2	16	Normal	NT	NT
7	75	M	PB	RAEB-2	N/A	15	8	NT	NT
8	59	M	PB	AML NOS	M5	95	Normal	NT	NT
9	51	M	PB	AML NOS	M0	73	No metaphases	Negative	Negative
10	59	M	PB	AML with MDS changes	M4	56	Normal	Negative	Detected
11	81	M	PB	CMML-2	N/A	10	Normal	NT	NT
12	86	F	PB	AML NOS	M1	NT	unknown	NT	NT
13	95	F	PB	AML with MDS changes	M5		unknown	NT	NT
14	82	M	PB	AML with MDS changes	M5	53	19	NT	NT
15	61	F	BM	AML NOS	M6	26%	complex	NT	NT
16	16	M	BM	AML + inversion 16	M4Eo	45	Inversion 16	NT	NT
17	41	F	BM	AML NOS	M5	78	complex	NT	Negative
18	75	M	PB	AML with MDS changes	M2	60	Normal	NT	NT
19	73	F	PB	AML NOS	M0	85	Normal	Detected	Negative
20	72	F	PB	AML with MDS changes	M5	80	Complex;del(12)(p12),del(13)(q21), add(17)(p11.2)[1]46,XX[1]	NT	NT
21	59	M	BM	AML NOS	M0	60	Normal	Negative	Negative
22	23	M	BM	AML + inversion 16	M4Eo	60	46,XY,inv(16)(p13;q22)	Negative	Negative
23	87	M	PB	Therapy related myeloid neoplasm	M2	62	47,XY,t(8;16)(p11;p13), +8	NT	NT
24	24	M	PB	AML NOS	M5	90	t(11;17), +8	Negative	Negative
25	44	F	PB	AML + inversion 16	M4Eo	50	Inversion 16	Negative	Negative
26	79	M	BM	AML NOS	M2	60	6	Negative	Negative
27	55	M	BM+PB	Blastic pDC Neoplasm	N/A	85	Normal	Negative	Negative
28	61	M	BM	AML NOS	M2	25	No metaphases	NT	NT
29	44	UD	BM	AML NOS	M1	UD	t(9:11)	NT	NT
30	51	UD	BM	AML + inversion 16	M4Eo	UD	inv 16	NT	NT
31	86	UD	BM	AML NOS	M4	UD	8	NT	NT
32	19	UD	BM	AML NOS	M2	UD	monosomy 7	NT	NT
33	61	UD	BM	AML NOS	M4	UD	Normal	NT	NT

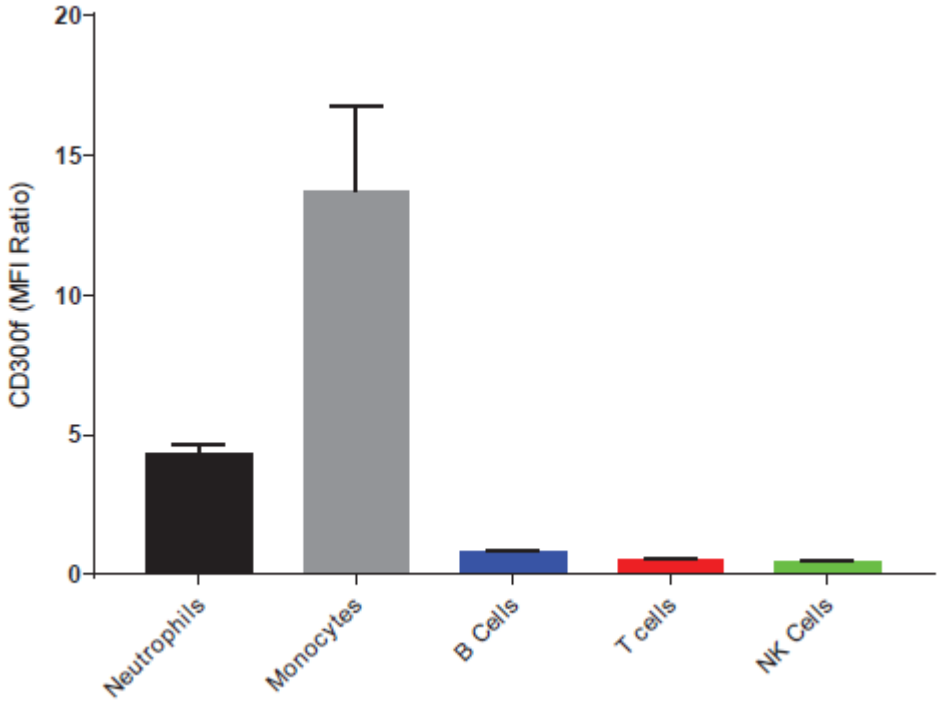
Clinical and pathological characteristics of AML patient samples used in the current study.

Supplementary Figure 1



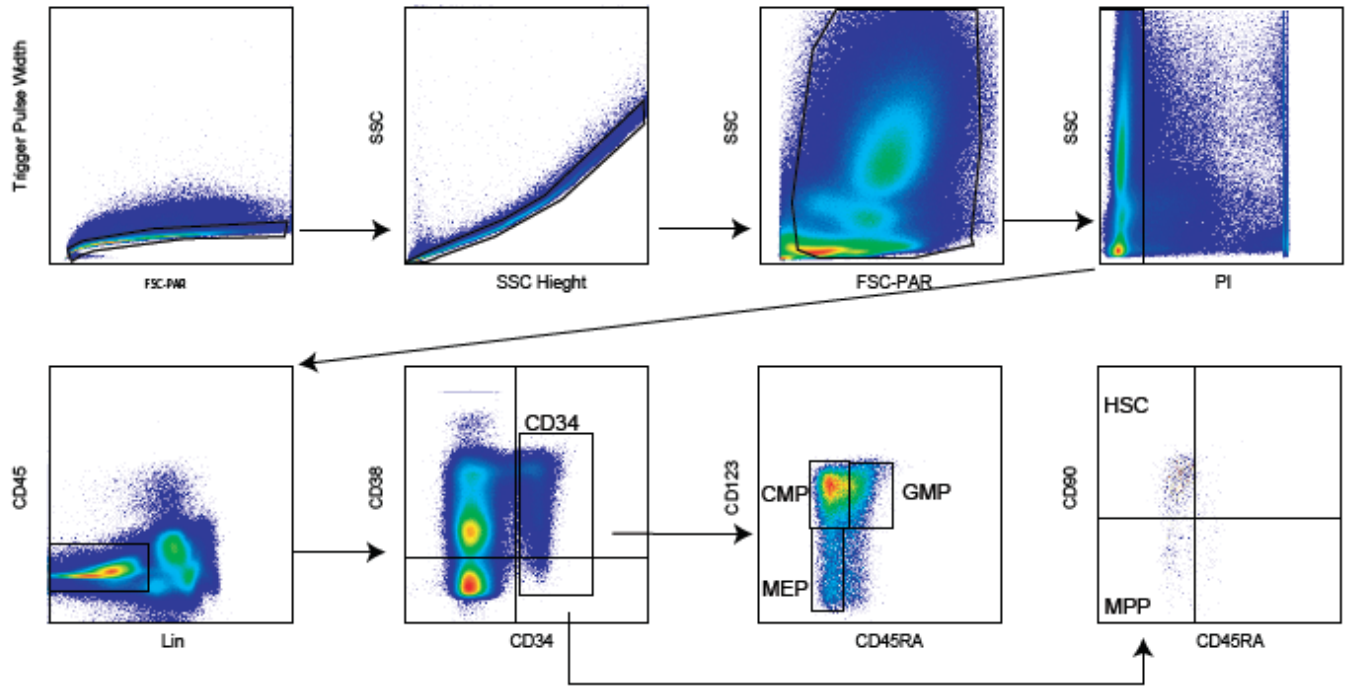
(A) AML CD300f gene expression by ELN risk status. Mean with SEM. (B) MFI ratios of CD33 and CD300f on the CD34+ CD38- subset of patient derived AML (n = 22) are compared with linear regression. (C) TiSNE of CD300f expression on AML samples from 9 patients. (D) TiSNE of CD33 expression on AML samples from 9 patients.

Supplementary Figure 2



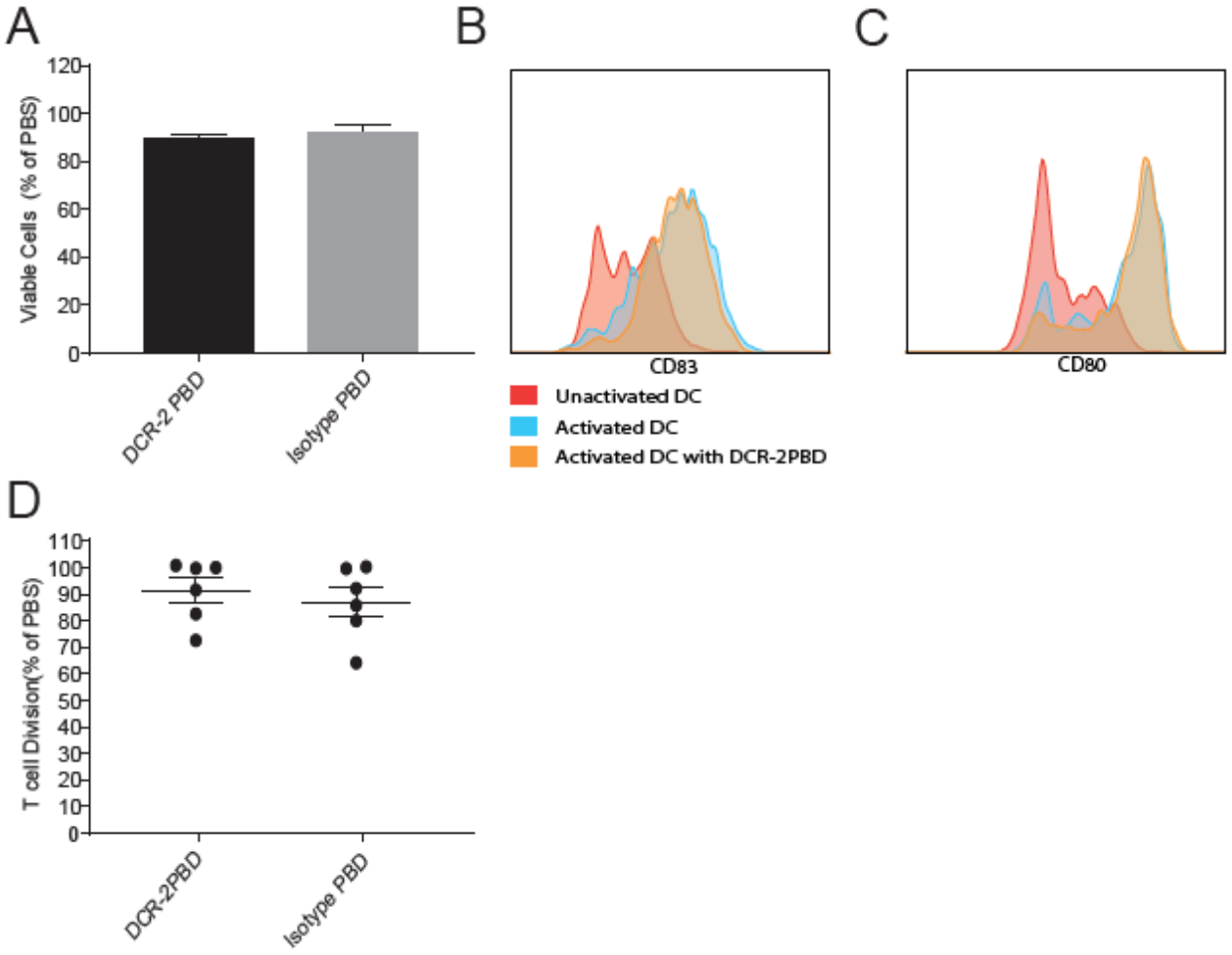
MFI ratios of CD300f on the peripheral blood subsets from healthy donors (n = 3). Mean with SEM.

Supplementary Figure 3



Gating strategy to identify HSPC groups via flow cytometry.

Supplementary Figure 4



(A) Viability of MoDC treated with either DCR-2-PBD or isotype-PBD after 96 hours compared to PBS control. Representative figures of activation markers CD83 (B) or CD80 (C) on non-activated or activated myeloid DC with or without DCR-2-PBD. (D) T cell division using a one way MLR with DCR-2-PBD or isotype-PBD, readout after 96 hours, both groups compared to PBS control.

Chapter 7: Targeting the niche: depleting haemopoietic stem cells with targeted therapy.

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REVIEW ARTICLE

Targeting the niche: depleting haemopoietic stem cells with targeted therapy

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Abstract
Haemopoietic stem cell transplantation is an expanding procedure worldwide but is associated with significant morbidity and mortality. Depletion of resident haemopoietic stem and progenitor cells (HSPC) is required for both autologous and allogeneic haemopoietic stem cell transplantation. Current conditioning protocols utilise chemotherapy or radiation to effectively reduce HSPC but are toxic in both the short and long term. The initial trials to use monoclonal antibodies to target HSPC were limited with marginal efficacy but platforms including antibody drug conjugates and chimeric antigen receptor T cells have made targeted conditioning strategies achievable. In this review we summarise the work developing targeted conditioning that may replace or reduce alkylating agents and total body irradiation. The prospect of conditioning with significantly reduced toxicity will improve outcomes and open transplantation to patients unable to tolerate current conditioning protocols.

Targeting the niche: depleting haemopoietic stem cells with targeted therapy

Haemopoietic stem cell transplantation (HSCT) is a rapidly expanding procedure with over 20,000 and 40,000 transplants performed annually in the United States and Europe, respectively [1, 2]. The majority of HSCT are performed for haematological malignancies but there is increasing use in non-malignant conditions. Substantial donor engraftment is only possible when the haemopoietic stem cell (HSC) bone marrow niche has been cleared of recipient cells to allow space for donor cells to reside [3, 4]. While allogeneic grafts can use graft-versus-host effects to generate their own bone marrow niches without prior depletion of haemopoietic stem and progenitor cells (HSPC), this is characterised by incomplete engraftment and very slow kinetics [5]. To achieve complete engraftment and rapid kinetics, conditioning regimens traditionally incorporate radiation and/or alkylating agents [6–8]. Conditioning regimens have three functions: depletion of HSPC from the niche, suppression of the anti-graft immune response in allogeneic transplantation and, if performed to treat malignancy, to reduce the burden of residual disease. Replacing radiation/alkylating agents with targeted therapy to clear the HSC niche may reduce both short and long-term toxicity, especially in high-risk patients which include those at the extremes of age or with primary immunodeficiencies. The rapidly expanding procedure of genetically modified autologous HSCT would become available to more patients with the development of less toxic conditioning regimens.

Chemotherapy and radiotherapy meet all three functions of conditioning but, as nonspecific agents, are associated with significant toxicity. The initial regimens for allogeneic (allo)-HSCT used myeloablative conditioning (MAC) but the toxicity limited use in the older patients and those with significant comorbidities. Reduced intensity conditioning (RIC) and non-MAC regimens have been developed opening allo-HSCT to those patients for whom MAC is too toxic [9]. RIC leads to both partial and full donor chimerism and is associated with reduced non-relapse mortality (NRM) compared to MAC [10]. Targeted conditioning has the potential to be incorporated into RIC protocols, reducing or replacing alkylating agents to further diminish their toxicity.

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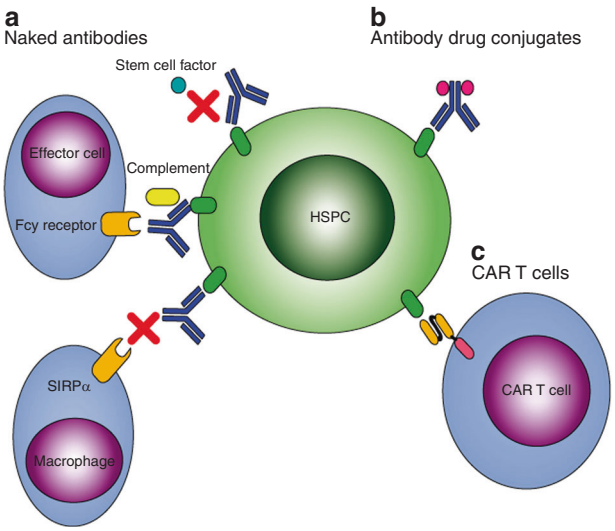

Fig. 1 Methods studied for targeted conditioning modalities. **a** Naked mAbs to CD45 and CD117 deplete HSPC through complement and cellular mediated mechanisms of cytotoxicity. Antibodies to CD117 block the binding of SCF which inhibits proliferation and differentiation of HSPC. Blockade of CD47 prevents the binding of the inhibitory molecule SIRPα increasing the ADCC potential of other co-administered mAb. **b** ADC against CD45 and CD117 are internalised upon binding to their target and administer their cytotoxic payload. **c** CAR T cells bind to CD117 or CD123 where they are activated leading to HSPC depletion

Despite improvements in HSCT and the adoption of RIC, the 100 day mortality remains high in allo-HSCT [11]. Short term toxicity associated with conditioning regimens include myelosuppression, mucositis, organ toxicity and sinusoidal obstruction syndrome [12]. Many patients are ineligible to undergo HSCT due to the toxicity, overcoming the short term toxicity would increase access of allo-HSCT for patients who otherwise have poor long term outcomes. Long-term toxicities resulting from HSCT conditioning include secondary malignancies, cardio-pulmonary toxicity, endocrinopathies and musculoskeletal disorders [13]. Long term survivors have high rates of infertility and increased rates of spontaneous abortion during pregnancy [14, 15]. Conditioning agents are associated with therapy related leukaemia, myelodysplasia and solid organ malignancies [16, 17]. The mortality in allo-HSCT patients who have survived for more than 5 years post-transplant remains 5–9 times higher than the general population, with a 30% reduction in life expectancy in the absence of relapse [18].

The advent of gene therapy for inherited disorders, including haemoglobinopathies and primary immunodeficiencies, has resulted in a new indication for autologous stem cell transplantation. Prior to the development of gene therapy, the only curative option for those with inherited disorders of haematopoiesis was allo-HSCT. Trials using genetically modified autologous products in HSCT have been conducted in patients with thalassaemia, sickle cell disease and primary immunodeficiency disorders [19–23]. Transplantation of genetically modified autologus HSPCs requires myeloablative doses of conditioning to enable significant engraftment, a conditioning agent that provides potent but targeted ablation of HSPCs would be ideal in this scenario [24].

The ideal target molecule would be expressed on HSPC and not outside of the haemopoietic system. Attempts to develop mAbs to deplete HSPC have been limited by the lack of sustained depletion using the cytotoxic and biological properties of naked antibodies, but new approaches with CD47 blockade, antibody-drug conjugates (ADC) and chimeric antibody receptor (CAR) T cells have opened the possibility of achieving targeted conditioning regimens (Fig. 1). To date, only four targets have been studied as outlined in Table 1.

Naked antibodies

CD45

CD45 (leucocyte common antigen) is expressed on all leucocytes and not outside of the haemopoietic system [25, 26]. CD45 is expressed as multiple isoforms, all of which are found on $CD34^+$ cells but the earliest progenitors are $CD45RA^-$ [27]. Despite the isoforms, mAbs directed at pan CD45 epitopes target all leucocytes. The effect of an anti-CD45 mAb on reduction of engraftment was demonstrated using an anti-CD45 ($RT7^a$ allotype) mAb in a rat heart transplant model where passenger donor leucocytes were reduced to <0.1% in peripheral blood compared to 4–5% for those not treated with the mAb [28]. The anti-CD45 $RT7^a$ antibody prevented engraftment of $RT7^a$ allotype HSPC in a competitive engraftment experiment, as well as causing lethal bone marrow failure in $RT7^a$ rats. The mAb depleted HSPC, mature myeloid and T cells while allowing for stable engraftment by donor cells from CD45 $RT7^b$ rats [29]. B cell progenitors were depleted but mature B cells were resistant despite being coated with the antibody. The anti-CD45 $RT7^a$ mAb facilitated high haploidentical donor chimerism when used at myeloablative doses [30]. The rat anti-mouse CD45 mAb 30-F11 transiently depleted mature myeloid and lymphoid cells but not HSPC in immunocompetent mice [31]. 30-F11 conditioning alone did not allow for donor engraftment in syngeneic or allogeneic models but did increase donor chimerism when combined with radiation.

Unconjugated rat anti-human CD45 mAb depleted human lymphoid and myeloid populations prior to HSCT. The rat anti-human CD45 mAbs YTH25.4 and YTH54.12 were administered in equal combination two to ten days prior to traditional chemo-radiotherapy conditioning for

Table 1 Targeted conditioning studies to date

Target	Construct	Findings	References
CD45	Anti-rat mAb (RT7[a] allotype)	Depletes mature lymphocytes and HSPC in rats, conditioning allows for haploidentical HSCT	[29, 30]
	Anti-mouse mAb (30-F11)	Depletes mature lymphocytes and myeloid cells but not HSPC in mice, increases donor chimerism when used with radiation	[31]
	Anti-human mAbs YTH25.4 and YTH54.12	Depletes mature lymphocytes and myeloid cells but not HSPC, reduction in peripheral AML blasts in 2/3 cases. Incorporated into minimal intensity conditioning regimen with 11/16 patients achieving full or high level chimerism	[32–34]
	Anti-mouse ADC (clone 104-Saporin)	Depletes mature lymphoid cells and HSPC, conditioning allows for high level sustained multilineage engraftment of congenic mice	[49]
CD117	Anti-mouse mAb (clone ACK-2)	Causes a transient neutropenia, anaemia and thrombocytopenia with a reduction of progenitor subsets in immunocompetent mice. Can deplete HSPC when combined with radiation. Only leads to significant donor engraftment in immunocompromised mice and not immunocompetent mice	[4, 40, 41]
	Anti-human mAb (clone SR-1)	Conditioning allows for long term engraftment in 1 of 5 macaques with non-myeloablative irradiation or busulfan in an autologous gene modified model. Depletes normal and MDS human HSPC in an mouse xenograft model	[42, 43]
	Anti-human mAb (clone AMG 191)	Depletes normal and MDS human HSPC in an mouse xenograft model, clinical trial (NCT02963064) for patients with primary immunodeficiencies to receive AMG191 conditioning prior to allo-HSCT is currently recruiting	[43]
	Anti-mouse ADC (CD117-saporin)	Combined with T cell depleting agents allowed for significant and durable engraftment in an immunocompetent mouse allo-HSCT model	[50]
	Anti-human CD117- ADC (conjugate unspecified)	Depletes human HSPC in vitro and in mouse xenografts	[51]
	Anti-mouse CD117 CAR T cells	Depletes mouse HSPC in vitro and in vivo, allow durable limited chimerism in congenic and CGD models	[57]
CD47	Anti-mouse CD47 FAB	Depletes immunocompetent mouse HSPC when combined with anti-CD117 mAb and allows for durable chimerism in a congenic HSCT and allo-HSCT (with added T cell depletion)	[47]
CD123	Anti-human CD123 CAR T cells	Causes ablation of healthy human haematopoiesis in a mouse xenograft, after depleation of the CAR T cells a second sex mismatch healthy human graft can be performed. Depletes co-engrafted healthy human HSPC and AML	[58, 59]

HSPC haemopoietic stem and progenitor cells, *HSCT* haemopoietic stem cell transplant, *allo-HSCT* allogeneic haemopoietic stem cell transplant, *AML* acute myeloid leukaemia, *MDS* myelodysplastic syndrome, *CGD* chronic granulomatous disease

allo-HSCT in 14 patients with myelodysplastic syndrome (MDS) or acute leukaemia [32, 33]. Doses of 400 µg/kg (maximum tolerated dose) over four days depleted mature lymphoid and myeloid cells [32]. Even at this maximum dose myeloid progenitor cells were spared. The half-life of the mAb was 12 h and 12 of 14 patients engrafted. Two of the three patients with measurable leukaemic blasts at the time of mAb administration had reductions in blast percentage. In a subsequent trial in children less than one year of age receiving allo-HCT for primary immunodeficiency, the combination of YTH25.4 and YTH54.12 was incorporated into a reduced intensity conditioning regimen including alemtuzumab, fludarabine and low dose cyclophosphamide [34]. Fifteen of 16 patients engrafted and 11 achieved full or high percentage donor chimerism. Three patients achieved T lineage engraftment only and one patient required a second transplant. 81% of the patients survived with immune restoration observed at a median of 40 months.

CD117

CD117 (c-Kit) is highly expressed on HSPC and with its ligand, stem cell factor (SCF), is essential for haemopoiesis [35–37]. A concern about targeting CD117 is its expression outside of the haemopoietic system; CD117 is expressed on melanocytes and epidermal cells, as well as in the CNS and GI tract [38, 39]. The rat anti-mouse CD117 antibody, ACK-2, transiently reduced HSC in the RAG2$^{-/-}$ γc$^{-/-}$ immunodeficient mouse, and when used as the only conditioning modality, facilitated stable engraftment [4]. ACK-2 also inhibited HSPC proliferation when exposed to SCF in vitro confirming a non Fc dependant mechanism. ACK-2 could only induce significant long term reduction of HSPC in immunocompetent mice when combined with low dose radiation [40]. This combination facilitated the transplant of a lentivirus vector transduced autologous HSC in mice with X-linked chronic granulomatous disease (CGD) as a model for mAb conditioning with gene therapy. ACK-2 conditioning allowed for long term donor engraftment in immunocompetent mice when administered in utero with HSCT performed on day one after birth [41].

The mouse anti-human CD117 mAB, SR-1, produced long term engraftment in one of five subjects when combined with non-myeloablative irradiation or busulfan in an autologous gene modified macaque model [42]. Autologous CD34 cells were transfected with a lentiviral vector to allow for long term tracking and reinfused after administration of reduced dose total body irradiation (TBI) and SR-1 (3 subjects) or reduced dose busulfan and SR-1 (2 subjects). The only subject that demonstrated long term engraftment (<1% at 300 days) was in the TBI cohort . SR-1 reduced both human and macaque colony forming units in the absence of effector cells.

SR-1 and a humanised CD117 mAb, AMG 191, deplete HSPC derived from patients with MDS in an NOD/SCID-γ (NSG) model [43]. The anti-CD117 mAbs cleared MDS derived cells from low and intermediate risk MDS for greater than 8 weeks, whilst high risk and very high risk MDS cells would rebound to near baseline levels within 8 weeks. The rebound of MDS derived cells was prevented with a second transplant of healthy HSPC performed one week after the mAb administration. A clinical trial is currently recruiting (NCT02963064) patients with severe combined immunodeficency to receive AMG 191 conditioning prior to allo-HSCT. The trial design does not incorporate other conditioning agents. To avoid the loss of donor HSPC, pharmacokinetic levels of the AMG191 will be monitored and patients will receive the graft only after antibody has cleared from the circulation. The long half-life of mAbs in circulation may limit their utility unless modifications that increase their clearance (such as removing the FcRn region) are made to ensure timely and safe delivery of the donor cells.

CD47

CD47 is an immunoglobulin like protein that interacts with its ligand SIRPα to inhibit phagocytosis of CD47 expressing cells in what is known as a “don’t eat me” signal [44, 45]. Blocking CD47 with mAb has been shown to potentiate the antibody dependent cell mediated cytotoxicity (ADCC) effect of different mAb directed at other surface antigens [46]. Combining the anti-CD117 antibody ACK-2 with an anti-CD47 Fab increased ADCC in vitro using immunocompetent mouse HSC [47]. This combination facilitated engraftment in an immunocompetent CD45 congenic mouse model. ACK-2 was then combined with an anti-CD47 Fab and recipient T cell depletion in an allo-HSCT minor MHC mismatch model. Durable but variable engraftment of myeloid, T, B, NK and HSPC populations was achieved. Naked antibodies have demonstrated the potential to target HSPC but lack the potency to play a key role in conditioning for most types of HSCT. Blocking CD47 overcomes the limited efficacy of naked antibodies. The limitations of naked antibodies can be further overcome using the ability of mAb targeting in more potent therapeutics.

Antibody drug conjugates

ADC are a rapidly emerging class of therapeutic in which a mAb is bound to a drug or toxin. The appeal of this design rests with the targeted potent toxins leading to a wide therapeutic window. The key elements of ADC are the choice of target for the mAb, the design and function of the

antibody, the linker and toxic payload chosen. Most current payloads are small molecules with sub-nanomolar inhibitory concentration 50 (IC_{50}) values, which led to improved efficacy compared to early generation ADC that often used conventional chemotherapy payloads [48].

CD45 ADC in HSCT conditioning was demonstrated with a rat anti-mouse mAb conjugated to a ribosomal inhibitory saporin (SAP) payload [49]. Anti CD45.2, CD49d, CD84, CD90, CD133, CD135 and CD184 mAbs were evaluated using a SAP payload. CD45.2-SAP caused the greatest depletion of HSPC in immunocompetent C57BL/6 mice (CD45.2 allotype). The cytotoxicity of the ADC was dependant on the anti-CD45 mAb clone. Clone 104-SAP depleted 98% of HSPC at the highest dose level but colony forming progenitors were less effected. A 3mg/kg dose of CD45.2-SAP produced chimerism levels of 75–90% using a CD45.1 donor. At 8 months, normal numbers of donor myeloid, B and T cells were recorded suggesting unbiased stem cell engraftment. CD45.2-SAP preserved normal bone marrow architecture compared to TBI, which reduced vascular integrity and bone marrow cellularity. Mice conditioned with CD45-SAP had a quicker recovery of their peripheral myeloid cells and had a survival advantage when exposed to *C. albicans* compared to mice conditioned with TBI. Conditioning with CD45.2-SAP resulted in significant chimerism when recipient mice with a knock-in human sickle cell gene were transplanted with wild type donors, leading to normalisation in haemoglobin, reduction of splenomegaly and an absence of sickle cells on blood films.

An anti-CD117-saporin immunotoxin combined with T cell depleting agents allowed for significant and durable engraftment in an immunocompetent mouse allo-HSCT model [50]. Multi-lineage chimerism developed with B cell chimerism being most pronounced. The transplanted mice tolerated same donor skin transplants without rejection. A human anti-human CD117 mAb ADC has been developed that kills >95% of human HSPC in vitro [51]. In vivo studies using humanised NSG mice demonstrated that the CD117-ADC depleted >98% of HSPC. B and T cells were spared in the in vivo testing. The mAb has been engineered to have a half-life of less than 12 h to allow for donor cell infusion without real time pharmacokinetic monitoring. The increased potency of ADC allows for highly efficient stem cell depletion, but also increases the risk of off target effects.

CAR T cells

Chimeric antigen receptor (CAR) T cells are genetically modified T cells that use the variable regions of mAb to direct the T cells to the chosen antigen. The extracellular CAR region activates a signal independently of the T cell receptor [52]. Success in the treatment of B cell acute lymphoblastic leukaemia, non-Hodgkin lymphoma and chronic lymphocytic leukaemia with CAR T cells demonstrate their powerful cytotoxic ability [53–56]. Murine CAR T cells directed against CD117 deplete mouse HSPC in vitro and in vivo and facilitate long term, but limited engraftment when used as conditioning in a CGD mouse model [57]. The murine derived CAR T cells initially had limited expansion and migration into the bone marrow in vivo but this was overcome by co-transducing mouse CXCR4 DNA (with the anti-CD117 CAR) and pre-treating with cyclophosphamide. CAR T cells were derived from $Thy1.1^+$ mice while recipients were $Thy1.2^+$, this divergence allowed the CAR T cells to be depleted with an anti-Thy1.1 antibody prior to donor cell infusion. In a mouse model using CD45.1 recipients CD45.2 donors, donor chimerism ranged from 20–30% at 36 weeks. In a CGD mouse model, the activation of reactive oxygen species (ROS) in neutrophils was assessed, the neutrophils of CGD mice do not produce ROS, but a partial ROS response was obtained at 12 weeks after engraftment with wild type donor cells using CAR T cell conditioning.

CAR T cells directed against the CD123 (IL-3 Receptor α subunit) to target acute myeloid leukaemia (AML) have been trialled in xenograft models, but their utility is limited by significant myelotoxicity [58]. A potential way to circumvent myelotoxicity is to use CD123 directed CAR T cells as part of allogeneic conditioning. In a humanised mouse model, subsequently engrafted with an AML cell line, anti-CD123 CAR T cells eliminated both the AML cell line and human graft [59]. The CAR T cells were depleted with alemtuzumab or were co-transfected at the time of production with the CD20 gene and could be eliminated with rituximab. Elimination was evaluated with flow cytometry and further confirmed with AML rechallenge. Mice engrafted with an AML cell line cleared the disease with anti-CD123 CAR T cell administration and exhibited a large T cell expansion as well preventing engraftment on AML rechallenge. Mice who had CAR T cell depleting therapy after initial AML clearance did not undergo T cell expansion and died with AML progression after the second presentation. Administration of CD20 expressing CAR T cells in NSG mice depleted healthy human xenografts, subsequently rituximab eradicated the CAR T cells and allowed for a second sex mismatch healthy human graft, which was confirmed using FISH on myeloid cells. Total CAR T cell depletion is required for graft safety. Targeting surface molecules as a method to deplete CAR T cells carries the risk of increased immunosuppression with T cell depletion using native targets or potential failure of CAR T cells to express a transfected target.

Conclusions

Early attempts to target HSPC for conditioning with mAb were met by limited efficacy using naked antibodies. New methods with antibody combinations, ADC and CAR T cells have now put targeted conditioning within reach but several questions remain. With highly potent cytotoxic strategies available, the concern for off target effects of new conditioning agents increases. Stringent evaluation of therapeutics with targets expressed outside of the haemopoietic system will be required prior to human trials. Another major safety concern is ensuring that the depleting agent is no longer active at the time of donor infusion. Many of the animal models used, circumvent this concern by targeting recipient specific isoforms. Real time drug monitoring may prevent primary graft failure but if the agent has an extended half-life this would prolong neutropenia. A robust and predictable elimination of ADC or CAR T cells prior to donor cell infusion is required.

The choice of target and modality is critical, with different antibody clones towards a single target having a range of cytotoxicity even when matched with identical toxins [49]. Implementation of targeted conditioning therapy will most likely be initially in the genetically modified autologous transplant setting or those with primary immunodeficiency, as only a single conditioning agent would be required. Targeted conditioning in allo-HSCT would be more complex as T cell depletion and possibly NK depletion is required to avoid graft loss [60, 61]. If the targeted conditioning regimen did not reduce these populations, conventional chemotherapies (e.g., purine analogues such as fludarabine) or other targeted therapies would need to be incorporated. Traditional conditioning regimens lead to tissue inflammation and the activation of host antigen presenting cells which influence graft-versus-host and graft-versus-tumour effects [62, 63]. How targeted conditioning may alter these factors has not been explored and requires further study. Targeted conditioning agents may have a role in reducing tumour burden (including minimal residual disease) at the time of transplant by binding to surface molecules shared in both HSPC and malignant cells, a number of CD45 radioisotopes have been trailed in conditioning regimens in high risk AML to reduce relapse risk [64].

Targeting the populations of the stem cell niche may be able to reduce the toxic effects of conditioning thereby expanding the therapeutic window of HSCT. Significant challenges remain but renewed interest with the accessibility of new modalities has increased the potential that targeted conditioning may become a reality.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Chapter 8: Conclusions and future directions

The aim of this thesis was to assess novel antigen targets in AML. This work made several substantial contributions to this field. I reviewed the literature and provided original insights into the major factor limiting immunotherapy for AML, which is the inability to differentiate between leukaemic cells and HSPC using surface molecules. Another addition to the literature made by this work is the first comprehensive review of targeted conditioning agents. We examined the potential of CD302 as a target in AML and provided the first publication demonstrating its expression on this disease as well as HSPC. The understanding of CD300f in AML has benefited from this work, both in exploring the potential to target individual isoforms specific for AML with monocytic differentiation as well as targeting all isoforms of CD300f to facilitate allo-HSCT. This work has achieved the aims of the thesis which was to assess novel targets for AML immunotherapies.

CD302 will be a challenging choice for cytotoxic immunotherapy in AML. The expression on the liver, even if intracellular, will always pose theoretical risks not shared by other targets. The role of CD302 in migration and our demonstration of reduced engraftment when AML is pre-coated with an anti-CD302 antibody suggests that it may play a role in the bone marrow niche micro-environment.[1] Any further work on CD302 in AML would best be placed to determine if disruption of the molecule can alter the bone marrow niche, in which AML evades traditional therapies.[2]

CD300f is a more promising target in AML. Using its unique epitopes and isoforms it may be possible to generate a true leukaemia specific immunotherapy. We demonstrated increased expression of exon four and enhanced binding of UP-D2, in the presence of DCR-2, on AML with monocytic differentiation. AML with monocytic differentiation is made up of acute myelomonocytic leukaemia (M4) and monoblastic/monocytic (M5) AML. The latter is relatively homogeneous, and the former is always heterogeneous by surface marker expression. It is possible that a greater response may be achieved with a M5 classification over M4 given the more consistent surface marker expression. Potential therapeutics using this approach may be antibody based therapy that target the exon four region or a therapeutic based on the UP-D2 mAb/ADC that is activated by the co-administration of DCR-2 (Figure 8.1).

Figure 8.1

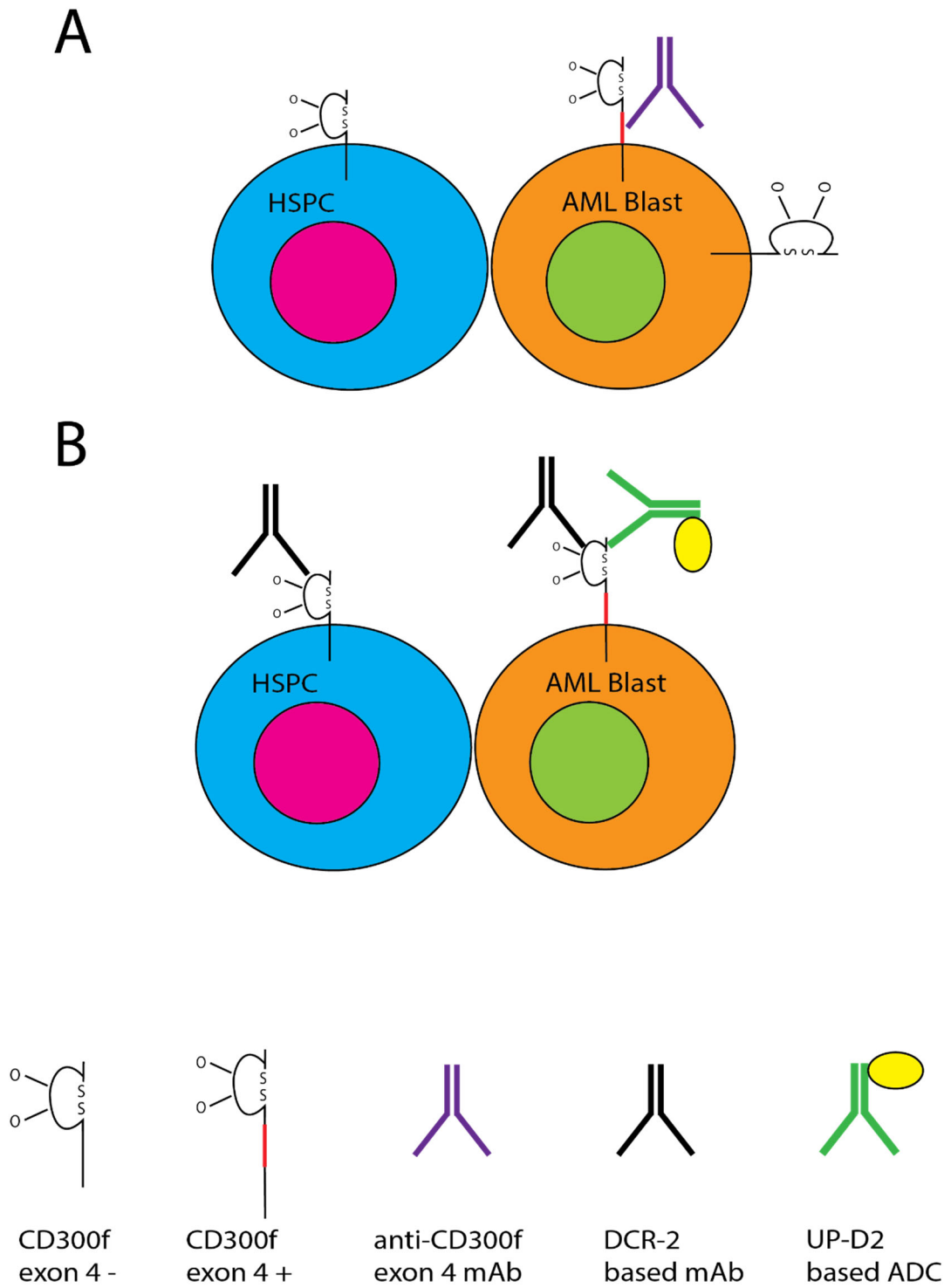

Figure 8.1 Strategies targeting CD300f on AML with monocytic differentiation sparing HSPC. Developing a mAb based therapy against exon 4 of CD300f (A). Inducing enhanced binding of a ADC with the UP-D2 binding site by a DCR-2 based mAb (B).

The most developed idea presented in this work is the use of an ADC against CD300f as part of a targeted conditioning regimen. This approach acknowledges the limitations of current AML targets and seeks to enhance a proven therapy, allo-HSCT. Incorporating allo-HSCT does restrict the number of potential patients that may benefit as it is inherently risky and resource intensive. The risks of HSCT may be reduced by limiting exposure to radiation and alkylating agents. We do demonstrate that CD300f has several positive features to develop into an ADC for targeted conditioning in AML. CD300f appears to have little expression on non-hematopoietic cells, is expressed across most AML, is expressed evenly across all major HSPC, and DCR-2 was efficiently internalised. This work demonstrated that CD300f had a similar expression profile as CD33 overall. The only significant difference in CD33 and CD300f as a target may be the expression on HSPC. CD300f has even expression across all HSC and myeloid HSPC. CD33 has enough expression on HSPC to limit potent immunotherapies (outside of allo-HSCT) but has variable expression across HSC and myeloid progenitors which may make it less efficient as a conditioning agent.[3-5] The next step is to remove the murine components of DCR2 and reduce its half-life by converting it into an antibody fragment (Fab) or producing a mAb without the FcRn region. A targeted anti-CD300f agent may be able to reduce relapses rates and replace alkylating agents/ radiation limiting non relapse mortality (Figure 8.2). This could confer the relapse reduction rate of MAC conditioning with a risk profile more similar to NMA/RIC regimens, which would be a substantial benefit, especially in older patients.

Figure 8.2

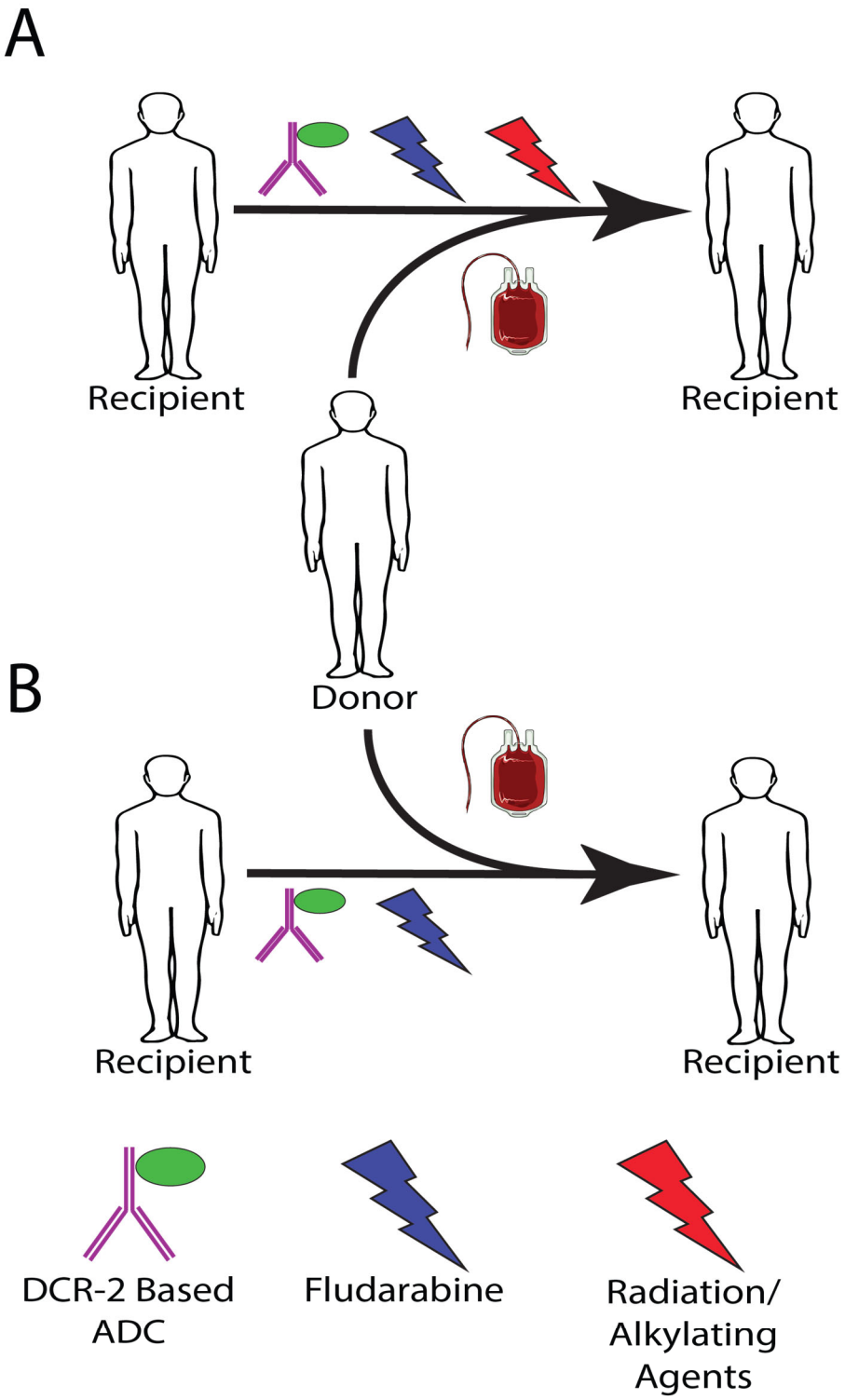

Figure 8.2 Potential future roles for a DCR-2 based ADC conditioning. The ADC could either added to existing conditioning regimens to reduce relapse rates (A) or replacing radiation/alkylating agents to reduce toxicity (B).

The anti-CD300f antibody, DCR-2, was further validated by this work. Anti-CD300f antibodies may have roles outside the realm of AML. CD300f reduces neutrophil chemotaxis and function upon binding to ceramide and it may be possible that blocking this interaction with an antibody could prove useful in sepsis or neutropenic states.[6, 7] The CD300f expression profile validated by this work will open up the possibility of further exploring this molecule in roles outside of AML.

An anti-CD300f ADC based conditioning will have benefits outside of AML. There is great appeal of less toxic conditioning agents for MDS and exploring a DCR-2 based conditioning agent in patients with this disease should be performed. A DCR-2 based conditioning agent would also likely have use in the emerging field gene modified autologous HSCT, where the toxicities of traditional conditioning agents are a major barrier to development. A key challenge in developing CD300f based conditioning further is the expression on HSPC differs between mice and humans. While human HSPC express CD300f, mouse HSPC do not.[7, 8] Therefore further validation of an anti-CD300f ADC as a transplant conditioning agent would require the ongoing use of xenografts.

Other groups are exploring targeted conditioning with other agents, but these may not directly complete.[9, 10] It is possible that different targeted conditioning agents, such as mAb or ADCs with against CD300f and CD117, could be combined and be possibly synergistic. Subsequent preclinical studies should be performed using a short half-life DCR-2 mab based therapeutic in conditioning regimens in xenografts of AML, MDS and inherited disorders of haematopoiesis. The knowledge derived from this thesis will continue to advance the field of allo-HSCT as part of the ongoing goal to establish less toxic more efficacious therapy for patients with AML.

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Appendix A: CD83: activation marker for antigen presenting cells and its therapeutic potential

Li Z, Ju X, Silveira PA, Abadir E, Hsu WH, Hart DN, Clark G. CD83: activation marker for antigen presenting cells and its therapeutic potential. Frontiers in Immunology. 2019;10:1312.

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CD83: Activation Marker for Antigen Presenting Cells and Its Therapeutic Potential

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CD83 is a member of the immunoglobulin (Ig) superfamily and is expressed in membrane bound or soluble forms. Membrane CD83 (mCD83) can be detected on a variety of activated immune cells, although it is most highly and stably expressed by mature dendritic cells (DC). mCD83 regulates maturation, activation and homeostasis. Soluble CD83 (sCD83), which is elevated in the serum of patients with autoimmune disease and some hematological malignancies is reported to have an immune suppressive function. While CD83 is emerging as a promising immune modulator with therapeutic potential, some important aspects such as its ligand/s, intracellular signaling pathways and modulators of its expression are unclear. In this review we discuss the recent biological findings and the potential clinical value of CD83 based therapeutics in various conditions including autoimmune disease, graft-vs.-host disease, transplantation and hematological malignancies.

Keywords: CD83, antigen presenting cells, immune suppression, therapeutic antibody, transplantation

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INTRODUCTION

The immune system's primary function is to protect the host from foreign pathogens, but its dysregulation can lead to serious illness or even death. For instance, failure of immunological tolerance has the potential to cause autoimmune diseases (1). In the transplantation setting, uncontrolled allogeneic immune responses leads to donor organ rejection or graft-vs.-host disease (GVHD) in which grafted T-cells respond to host tissue antigens presented by activated donor or host antigen-presenting cells (APC) (2). Current approaches to prevent or treat these diseases conventionally include non-specific immune suppression agents such as steroids or cyclosporin. However, these agents compromise the patient's immune function against pathogens and malignancy (3). There is a need for selective immune suppressive agents targeting specific inflammatory cells that prevent undesirable immune responses but preserve beneficial responses against infection and cancer.

Expression of membrane-bound (m)CD83 on the surface of activated dendritic cells (DC) and other APC make it an attractive therapeutic target, for achieving selective immune suppression. Anti-CD83 specific antibodies with the ability to deplete $CD83^+$ cells have shown efficacy in the treatment of pre-clinical models of GVHD without significantly affecting viral or tumor specific memory T-cell responses (**Table 1**). Alternatively, recombinant soluble extracellular CD83 constructs (rsCD83) mimicking the natural soluble (s)CD83 variant have demonstrated potent immunosuppressive properties in animal models of autoimmune disease and transplantation

(**Table 1**). In this article, we review the most recent literature updating our understanding of CD83 biology and discuss the value of applications using anti-CD83 antibodies or sCD83 in mediating immune suppression or targeting $CD83^+$ malignancies.

PHYSIOLOGICAL CHARACTERISTICS OF CD83

CD83 Structure and Expression

CD83 is a member of the immunoglobulin (Ig) superfamily. The human gene maps to Chromosome 6p23 and consists of 5 exons: exon 1 encoding the leader sequence, exons 2–3 the extracellular domain, exon 4 the transmembrane domain and exon 5 the intracellular domain (26). Similar gene organization is found in other mammals (27). The human CD83 protein comprises of 205 amino acids with extensive glycosylation resulting in a molecular weight of 45 kD. The mouse protein shows 63% similarity but is smaller (196 amino acids) due to the absence of an eleven amino acid sequence within the extracellular region (28, 29).

In mammals, fish and birds, mCD83 is recognized as an activation marker on the surface of immune cells (30). In humans and mice, the highest and most stable expression is found on activated DC from various tissues, including plasmacytoid and myeloid subsets (31–33). Nevertheless, mCD83 is found on the surface of other activated hematopoietic cells, including B-cells (34–36), macrophages, monocytes (37), neutrophils (38) and NK cells (39). In germinal centers, CD83 is expressed on B-cell centrocytes within the light zone undergoing selection and Ig class switching (40). mCD83 is only found on minor proportions of non-regulatory human T-cells that have engaged with APC, with surface expression primarily due to trogocytosis (35). mCD83 was not detected on the surface of human natural regulatory $CD4^+$ T-cells (Treg) (35) but could be detected on induced or expanded Treg (41, 42). In mice, natural $CD4^+$ Treg show high levels of CD83 promoter activity, and upon activation rapidly express mCD83 on their surface (35, 43, 44). mCD83 is also present on non-hematopoietic cortical thymic epithelial cells (TEC) in mice (45, 46), which is yet to be examined in humans.

Intracellular preformed CD83 protein is detected in a wide range of immune cells, including immature DC, monocytes, macrophages, natural-killer (NK) cells and lymphocytes (35). CD83 is rapidly transported to the surface from golgi and recycling endosome pools in DC, macrophages, monocytes and B-cells upon TLR or TNF engagement (37, 47). CD40/CD40L and BCR-ligation induces mCD83 in B-cells (48). Detailed analysis of the human CD83 gene promoter found SP-1 and NF-κB sites were critical for induction of the gene (49), with interferon regulatory factor-1, -2, -5 and NF-κB-p50, -p65, and -cRel involved in regulating CD83 expression in DC (50). The post-translation modulation of CD83 comprises of a golgi transport related protein, GRASP55, which binds to the CD83 C-terminal TELV-motif and plays a role in CD83 glycosylation (51).

sCD83 can be detected at low levels in healthy human sera but is elevated in the sera of patients with hematopoietic malignancies or autoimmune diseases (12–14, 52–55). Similarly, low to undetectable levels are found in the sera of healthy mice, which is elevated during pregnancy (56) or induction of autoimmunity (57). Culturing experiments determined that most sCD83 is produced by activated B-cells and DC (52, 56), as well as Treg in mice (43). The sequence of natural sCD83 in both species remains unconfirmed and as a result, it is not clear whether the product derives from cleavage of the extracellular portion of mCD83, CD83 splice variants (26), or both. The abundant amount of sCD83 produced by the Hodgkin derived cell line KM-H2, which only express the full-length CD83 transcript, supports cleavage (35); whereas detection of sCD83 in supernatants of cell lines transfected with certain CD83 splice variants supports the alternative mechanism (26).

CD83 Ligands

CD83 forms homodimers in prokaryotic expression systems (29) confirmed in protein crystal structure analysis (58). Strong structural similarities between CD83 and B7 family members were revealed, suggesting that, like B7 family members, CD83 could exert its immunological activity by either homotypic or heterotypic interactions with a ligand (58). rsCD83 constructs bind to the surface of DC (59), B-cells (60), and monocytes (61), cells reported to express CD83 themselves. Similar constructs bound a CD83 transfected but not the wild-type Chinese hamster ovary cell line and failed to bind DC with a CD83 knock-down (62). This indicated the potential for homotypic binding of mCD83 in trans to mCD83 on other cells, but to date, investigators have failed to demonstrate a clear biophysical interaction. These studies do not preclude the possibility that CD83 binds to other ligands as predicted by structural analysis. Indeed, human rsCD83 has been shown to bind myeloid differentiation factor-2 (MD-2), a co-receptor associated with the TLR4 signaling complex, on monocytes (63).

mCD83 Function

The important role of mCD83 in T-cell development became evident in CD83 knockout mice, which exhibit a severe reduction of $CD4^+$ T-cells (45). This phenotype was intrinsic to the non-hematopoietic compartment and could be reversed by intra-thymic injection of wild-type TEC. The underlying mechanism by which mCD83 controls T-cell selection by TEC was attributed to its transmembrane region (64), which binds, and functionally inhibits, the membrane-associated RING-CH8 (MARCH-8) ubiquitin ligase (46, 65). Since MARCH-8 is responsible for the internalization and degradation of surface MHC class II (MHC-II) through ubiquitination, CD83 expression by cortical TEC stabilizes MHC-II on their surface permitting positive $CD4^+$ T-cell selection. mCD83 similarly

Abbreviations: ADCC, antibody-dependent cell cytotoxicity; APC, antigen presenting cell/s; DC, dendritic cell/s; DLBCL, diffuse large B-cell lymphomas; EAE, experimental autoimmune encephalomyelitis; EAU, experimental autoimmune uveitis; GCA, giant cell arteritis; GVHD, graft-vs. -host disease; HL, Hodgkin lymphoma; HRS, Hodgkin Reed-Sternberg; HSV-1, Herpes-Simplex virus-1; IDO, indolamine 2,3-dioxygenase; Ig, immunoglobulin; mAb, monoclonal antibody; mCD83, membrane CD83; MHC-II, MHC class II; MS, multiple sclerosis; NK, natural-killer; rsCD83, recombinant soluble CD83; sCD83, soluble CD83; TEC, thymic epithelial cell/s; TLR, toll-like receptor; Treg, regulatory $CD4^+$ T-cell.

TABLE 1 | Therapeutic applications of CD83.

Therapeutic	Indication	Potential mechanism
Anti-CD83 antibody	Diagnostic mature DC marker	CD83 upregulation when DC are activated (4–8)
	Therapeutic target for GVHD	Depletion of mature $CD83^+$ DC (9, 10)
	Therapeutic target for GCA	Depletion of mature $CD83^+$ DC in GCA arteries (11)
Anti-CD83 antibody/antibody drug conjugates	Biomarker and therapeutic HL target	Depletion of $CD83^+$ malignant cells; neutralizing peripheral sCD83 (12)
Serum sCD83	Biomarker in HL, chronic lymphocytic leukemia, and mantle cell lymphoma	Serum sCD83 shed from $CD83^+$ malignant cells (12–15)
rsCD83	**Immunosuppressive agent in solid organ transplant rejection**	
	Skin transplant	Inhibition of recipient T-cell proliferation and IL-2, IFN-γ production (16)
	Kidney transplant	Tolerogenic DC generation; induction of IDO (17)
		Reduced number of infiltrating T-cells and monocytes and lower levels of inflammatory cytokines in graft (18)
	Corneal transplant	Regulatory T-cell induction mediated by IDO and TGF-β (19)
	Cardiac transplant	Attenuating DC maturation and function, such as down modulating MHC-II expression and reducing the DC allogeneic stimulatory capacity (20)
	Immunosuppressive agent in autoimmune diseases	
	MS (EAE)	Reduced T-cell proliferation and production of IFN-γ (21)
	Inflammatory Bowel Disease	Induced tolerogenic IDO^+ DC (22)
	Autoimmune Uveitis (EAU)	NK cells reduced and the expression of CD11b and CD83 in NK cells decreased (23)
		Induced tolerogenic IDO^+ DC by decreased expression of co-stimulatory molecules and hampered DC calcium response (17)
	Systemic Lupus Erythematosus	Reduction of splenic and peripheral IgG-secreting cells and peripheral T-cells (24)
	Rheumatoid arthritis	Reduced arthritis by increasing Treg numbers in an IDO and TGF-β dependent manner (25)

promotes upregulation of surface MHC-II and CD86 on activated APC including DC and B-cells through transmembrane regulation of the haematopoietically-restricted ubiquitin ligase MARCH-1 (64).

Despite being present on the surface of activated APC and causing upregulation of MHC-II and CD86 required for T-cell activation, the outcomes of signaling through surface mCD83 appears to lead to suppressed or regulatory functions in various immune cell populations. For DC, engagement of mCD83 with antibody or homotypic binding with CD83 expressing cell lines *in vitro* or transgenic CD83 expression by non-hematopoietic cells *in vivo* reduced their capacity to mature and secrete pro-inflammatory cytokines, a feature dependent on the MAPK signaling pathway (62). On the other hand, mice with a conditional knockout of CD83 in DC exhibited increased susceptibility to severe colitis, further indicative of a role for CD83 in DC regulation.

CD83 expression by mouse B or T-cells was shown to increase their longevity *in vivo* (66). However, transgenic overexpression of CD83 in mouse B-cells resulted in inhibitory function, as demonstrated by a decreased capacity to proliferate, class-switch and secrete Ig upon immunization (despite increased surface MHC-II and CD86 levels) as well as augmented secretion of the immunoregulatory cytokine IL-10 by marginal zone B-cells (67). Treating mice with anti-CD83 antibodies significantly augmented their IgG1 responses to T-cell independent antigens, which was underpinned by increased marginal zone B-cell isotype switching (68). Ablating CD83 expression conditionally in B-cells did not result in major changes to their response to antigen, though some changes were noted in germinal center composition and IgE class-switching (69). So far, little is known about CD83 function in human B-cells. However, targeting them *in vivo* with an anti-CD83 monoclonal antibody (mAb) in a human PBMC xenograft model inhibited B-cell responses to specific antigens without causing pan B-cell depletion (70).

In mice, CD83 expression is associated with regulatory function in T-cells. Using reporter mice, CD83 expression was associated with T-cells which mediate Treg-like functions *in vitro* and *in vivo* (43). Transduction of CD83 into mouse $CD4^+$ $CD25^-$ naïve T-cells imparted them with suppressive capabilities comparable to naturally occurring Treg including prevention of experimental autoimmune encephalomyelitis (EAE) in a mouse model (71). While expression of mCD83 on Treg could act in trans to downregulate the function of DC expressing mCD83, the molecule was shown to have essential intrinsic function in Treg differentiation and

retention of their regulatory phenotype (42). In humans, continuous expression of CD83 on activated human $CD4^+$ T-cells is indicative of their differentiation into induced Treg (41).

sCD83 Function

To evaluate the potential function of sCD83, several studies have used rsCD83 constructs consisting of the human or mouse CD83 extracellular domain fused to an Ig Fc chain or a polyhistidine tag (4, 21, 26, 59, 62, 72–76). These all showed similar immune suppressive properties compared to control constructs, inhibiting human monocyte differentiation into DC (72, 76), changing the DC cytoskeleton (75), preventing DC maturation (59, 62), and reducing DC-mediated T-cell proliferation (4).

The ligand of sCD83 and how it exerts its immune inhibitory function is under investigation. Homotypic interaction of rsCD83 with mCD83 on DC blocks the production of inflammatory cytokines monocyte chemoattractant protein-1 and IL-12p40 through MAPK signaling (62). Another study showed that rsCD83 binding to DC suppressed f-actin mediated calcium signaling, preventing co-localization of ORAI1 and mitochondria at the DC-T-cell synapse (57). Binding of rsCD83 to the TLR4/MD-2 complex on monocytes induced anti-inflammatory mediators, such as indoleamine 2,3-dioxygenase (IDO), IL-10, and PGE2 in a COX-2-dependent manner, leading to inhibition of T-cell proliferation and IL-2 secretion (63, 72). The increased generation of IDO and TGF-β by rsCD83 leads to the induction of Treg and allograft tolerance, which was confirmed in mouse kidney or corneal transplant models (17, 19).

TRANSLATION OF CD83 INTO THE CLINIC

CD83 as a DC Activation Marker and Viral Infection Target

mCD83 is an informative DC maturation marker (77, 78) and has been used in clinical trials of solid organ transplant rejection (clinicaltrials.gov, NCT01678937), DC vaccination for the treatment of melanoma (clinicaltrials.gov, NCT01425749) and acute myeloid leukemia (5) or as an inflammatory indicator for novel psoriasis therapy (clinicaltrials.gov, NCT01736696). $CD83^+$ DC are reported to have prognostic value as an inverse correlate of gastric cancer outcomes (6).

Viruses have evolved a number of strategies to subvert host immunity including the targeting of CD83 on APC. Immature DC infected with Herpes-Simplex virus-1 (HSV-1) failed to express mCD83 during maturation (79) and the virus contributed to rapid downregulation of mCD83 on mature DC (7). Interestingly, HSV-1 secretes soluble factors, such as L particles, that interfere with CD83 expression on HSV-1-negative (uninfected) bystander DC (80). Similar observations of virus induced DC downregulation of mCD83 were found with varicella-zoster (81) and cytomegalovirus infection (4, 82). The latter downregulates mCD83 on mature DC via major immediate early viral effector protein IE2 and induces sCD83 secretion (82). In contrast, Epstein-Barr virus produces latent membrane protein-1, which induces rapid upregulation of mCD83 and a strong immune response against infected B-cells, establishing viral latency (8).

sCD83 in Autoimmune Disorders and Solid Organ Transplant Rejection

rsCD83 proteins that exploit the suppressive function of CD83 are demonstrated to be effective in the treatment of various mouse models of autoimmune and inflammatory diseases (**Figure 1**). Intra-peritoneal injection of rsCD83 was effective in preventing EAE, a model of multiple sclerosis (MS), with a reduction in T-cell cytokines including IFN-γ, IL-2, IL-4, and IL-10 (21). In an inflammatory bowel disease model, rsCD83 prevented the symptoms of colitis by decreasing inflammatory cell infiltration and destruction of colonic architecture through inducing long-term expression of IDO by DC (22). IDO^+ DC actively diverted T-cell responses toward tolerance (83). Similarly, in an experimental autoimmune uveitis (EAU) model, topical application of rsCD83 showed a protective effect resulting from the induction of tolerogenic IDO^+ DC, suppressing $CD4^+$ T-cell activation in eyes and spleen. rsCD83 application also reduced mCD83 expression by $CD83^+CD3^-NK1.1^+$ cells that normally infiltrate inflamed eyes. When rsCD83 treated-NK cells were transferred into EAU mice, retinal tissue damage was also relieved (23). In a systemic lupus erythematosus model, rsCD83 significantly delayed onset of pathogenic anti-dsDNA autoantibodies and reduced the concentration of anti-histone IgG autoantibodies compared to the control group (24).

In addition to autoimmunity, sCD83 has potential therapeutic value for preventing solid organ transplant rejection. Treatment with rsCD83 delayed acute cellular rejection of MHC-mismatched skin allografts in mice, significantly reducing the recipient's T-cells capacity to respond (16). In other mouse models, rsCD83 prevented renal allograft rejection and corneal transplantation rejection by inducing IDO and TGF-β (17–19). In a cardiac transplant model, the prolonged allogeneic heart graft and donor specific graft tolerance induced by rsCD83 correlated with a reduction in DC activation markers and allogeneic stimulatory capacity (20). Interestingly, while rsCD83 is immunosuppressive in animal models, elevated levels of natural sCD83 were noticed in various human autoimmune and inflammatory diseases, e.g., in synovial fluid (55) and sera of rheumatoid arthritis patients (54) and MS patient sera (53). The significance of elevated sCD83 in these diseases is not yet understood and may be due to self-regulation of immune system.

Antibody Targeting of $CD83^+$ Cells for Treatment of GVHD

Antibody targeting of CD83 offers the possibility of specifically depleting activated APC capable of stimulating allogeneic T-cells while retaining non-activated APC that impart tolerance and memory T-cells crucial for protective immunity against infection and tumors (**Figure 1**). This therapeutic strategy was initially trialed in pre-clinical models using a polyclonal rabbit anti-human CD83 antibody (9) and later repeated with the high-affinity human anti-human CD83 IgG1 mAb, 3C12C (10). Both antibodies were adept at mediating antibody-dependent cell

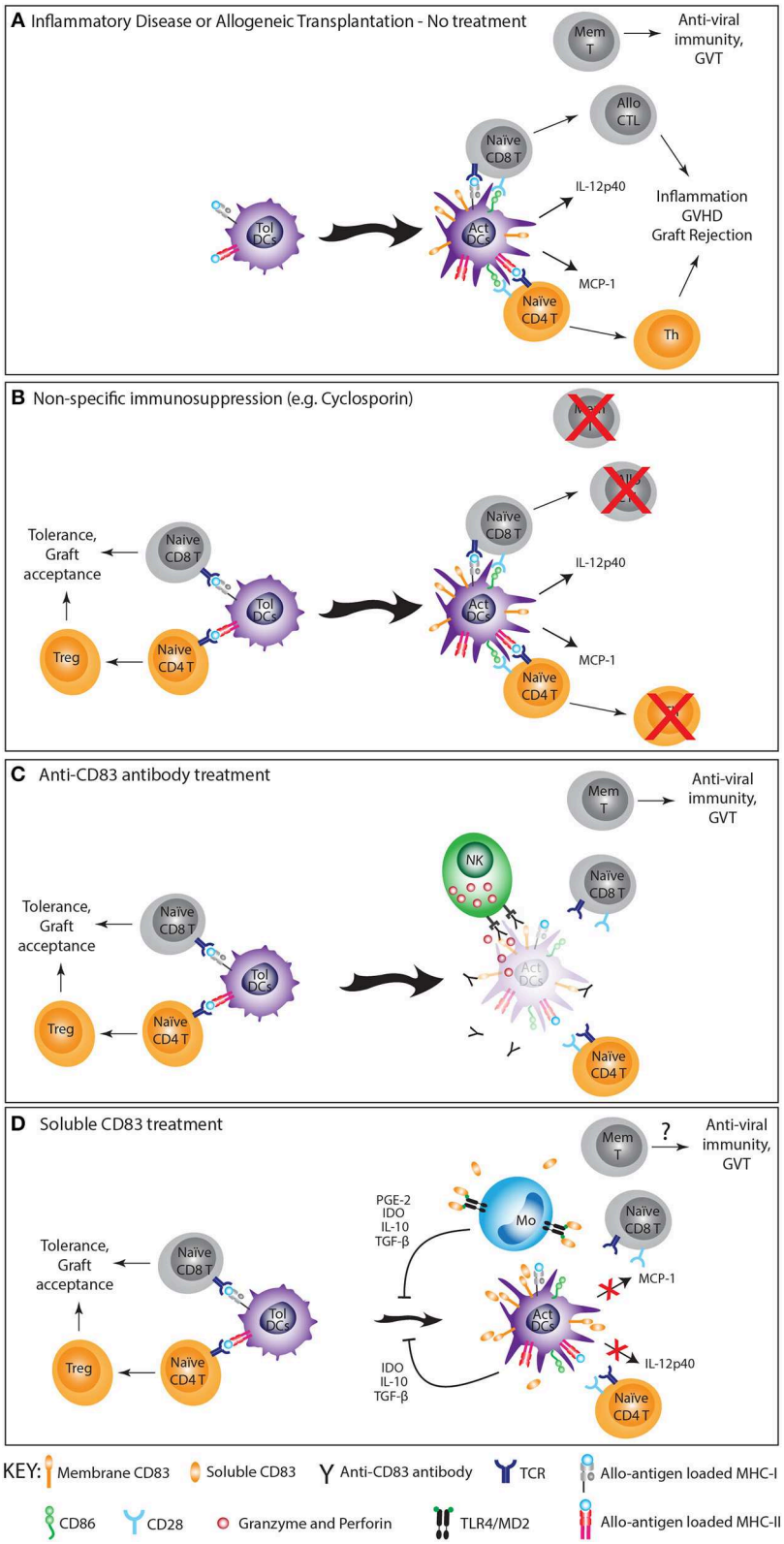

FIGURE 1 | The immunosuppressive effect of anti-CD83 antibodies and rsCD83 in allogeneic transplantation and inflammation. **(A)** Activated (Act) DC stimulate T-cells to initiate a damaging alloimmune/inflammatory response caused by allogeneic transplantation or autoimmunity. **(B)** Conventional treatment with non-specific

(Continued)

FIGURE 1 | immune suppressive agents (e.g., cyclosporin) controls inflammation, GVHD and graft rejection at the expense of protective infectious and tumor memory T-cell responses. **(C)** Anti-CD83 mAb deplete activated DC but not tolerogenic DC (Tol-DC) at the initiation of the immune response, preventing T-cell activation leading to graft rejection/inflammation but promoting T-cell tolerance. It also preserves memory (mem) T-cells capable of protective anti-viral and graft-vs.-tumor (GVT) activities. **(D)** rsCD83 induces IL-10, IDO, PGE-2, and TGF-β from monocytes and DC through homotypic and heterotypic ligand binding, promoting Treg generation, inhibiting DC activation and reducing CD83 surface expression on DC, leading to reduced T-cell activation, graft acceptance and tolerance. The effect of rsCD83 on memory T-cell responses has not been investigated.

cytotoxicity (ADCC) against $CD83^+$ expressing cells, particularly activated DC, and by doing so prevented allogeneic T-cell proliferation in mixed leukocyte reactions without affecting memory T-cell reactivity to cytomegalovirus and influenza antigens. A single 125 μg dose of either antibody prevented acute GVHD in a preclinical xenogeneic model where human PBMC were transplanted into SCID mice. This without significantly compromising the donor's overall T-cell and Treg numbers nor memory T-cell responses against viral or tumor antigens. Treatment of non-human primates with up to 10 mg/kg 3C12C was found to have no adverse clinical effects or significantly affect total blood cell counts (12). However, specific reductions were noted in the $CD83^+$ populations including $CD1c^+$ DC and B-cells. Anti-CD83 antibodies can be effective in other inflammatory settings, as demonstrated in a xenogeneic mouse model of giant cell arteritis (GCA). Treatment of SCID mice with a mouse anti-human CD83 mAb depleted activated DC in GCA-affected human artery grafts, preventing graft infiltration and activation of co-transferred human T-cells (11).

CD83 as a Therapeutic Target and Biomarker in Cancer

The first malignancy to be reported exhibiting surface CD83 expression was Hodgkin lymphoma (HL) (84). Since then, other tumors have been shown to express CD83, including diffuse large B-cell lymphomas (DLBCL), small cell lung cancer and other lung adenocarcinomas and gastric mucosa-associated lymphoid tissue lymphomas (85–88). In addition, CD83 polymorphisms and mutations have been reported in some cancers. For example, somatic mutations of CD83 with unknown significance have been reported in DLBCL (89, 90) and polymorphisms of CD83 were correlated with prognosis of cervical cancer (91).

HL is a B-cell neoplasm defined by the presence of Hodgkin Reed-Sternberg (HRS) cells. Approximately 30% of patients with advanced disease either relapse or become refractory to chemotherapy and their survival is substantially reduced (92). Recently, we determined that CD83 is expressed on HRS cells (12). In the HL setting, antibody-drug conjugates have advantages over naked antibodies in being able to bypass the suppressive tumor microenvironment that can prevent ADCC (15). We showed CD83 was internalized after antibody engagement and developed 3C12C-monomethyl auristatin E toxin conjugates that were effective in killing $CD83^+$ HRS cells (12). This provides impetus for the further investigation of anti-CD83 therapeutics for HL.

HL cells were found to secrete sCD83 that suppressed T-cell proliferation, suggestive of a potential mechanism of immune evasion (12). Blocking sCD83 with anti-CD83 antibodies could mitigate sCD83's effect. Interestingly, serum sCD83 protein is increased in patients with HL as well as other hematological malignancies, including chronic lymphocytic leukemia and mantle cell lymphoma, which correlated with decreased survival and clinical therapeutic response (12–14). These studies raise the possibility that sCD83 could be developed as biomarker for HL and other hematological malignancies and be targeted to enhance immune therapies.

CONCLUDING REMARKS

While recognized as a biomarker for activated APC, greater knowledge of the expression and function of CD83 has given rise to therapeutic strategies that target this molecule or its ligands to suppress inflammatory immune responses. rsCD83 constructs accomplish this by exploiting the regulatory signals induced by CD83 whereas anti-CD83 antibodies act by depleting activated antigen presenting cells that promote inflammatory T-cell responses (**Figure 1**). Both products have shown great promise for treating inflammatory disease in preclinical models, but some key questions regarding their mechanism of action remain. Determining the significance of differences between mouse and human CD83 (particularly for T-cells) would be important for translating research from animal models into humans. For sCD83, the contribution of homotypic and heterotypic ligand binding and the specific signals induced by these interactions require further elucidation. Also pertinent would be examining whether differences exist between natural sCD83 and the recombinant extracellular sCD83 used in the treatment of disease models. Regarding anti-CD83 antibodies, continued work is required to determine whether depletion of activated DC is the main mode of immune suppression given that targeting CD83 with antibodies has also been shown to suppress DC maturation (62). In addition, it is unclear whether targeting other $CD83^+$ cells (e.g., T or B-cells) promotes the efficacy of anti-CD83 treatment. It is of great interest to determine if anti-CD83 antibodies could be therapeutic in other inflammatory settings involving activated DC such as solid organ transplant rejection or autoimmunity. The application of anti-CD83 antibodies or derivatives (e.g., antibody-drug conjugates) are likely to extend to the management of cancer, especially hematological malignancies. The translation of therapeutics targeting CD83 hold great promise as more selective strategies for achieving immunosuppression without significantly compromising protective immunity and have the potential to supersede the broad immunosuppressive drugs currently used to treat inflammatory diseases in the clinic.

AUTHOR CONTRIBUTIONS

Paper writing by ZL, W-HH, and PS. Editing by XJ, EA, and GC. Figure drawing by PS and W-HH. DH provided the concept and supervised our CD83 work.

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Conflict of Interest Statement: GC is a Director of DendroCyte which has intellectual property associated with CD83.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix B: CD83 is a new potential biomarker and therapeutic target for Hodgkin lymphoma

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ARTICLE

CD83 is a new potential biomarker and therapeutic target for Hodgkin lymphoma

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ABSTRACT

Chemotherapy and hematopoietic stem cell transplantation are effective treatments for most Hodgkin lymphoma patients, however there remains a need for better tumor-specific target therapy in Hodgkin lymphoma patients with refractory or relapsed disease. Herein, we demonstrate that membrane CD83 is a diagnostic and therapeutic target, highly expressed in Hodgkin lymphoma cell lines and Hodgkin and Reed-Sternberg cells in 29/35 (82.9%) Hodgkin lymphoma patient lymph node biopsies. CD83 from Hodgkin lymphoma tumor cells was able to trogocytose to surrounding T cells and, interestingly, the trogocytosing $CD83^{+}$T cells expressed significantly more programmed death-1 compared to $CD83^{-}$ T cells. Hodgkin lymphoma tumor cells secreted soluble CD83 that inhibited T-cell proliferation, and anti-CD83 antibody partially reversed the inhibitory effect. High levels of soluble CD83 were detected in Hodgkin lymphoma patient sera, which returned to normal in patients who had good clinical responses to chemotherapy confirmed by positron emission tomography scans. We generated a human anti-human CD83 antibody, 3C12C, and its toxin monomethyl auristatin E conjugate, that killed CD83 positive Hodgkin lymphoma cells but not CD83 negative cells. The 3C12C antibody was tested in dose escalation studies in non-human primates. No toxicity was observed, but there was evidence of CD83 positive target cell depletion. These data establish CD83 as a potential biomarker and therapeutic target in Hodgkin lymphoma.

Introduction

Hodgkin lymphoma (HL) is a B-cell neoplasm that is defined by the presence of Hodgkin Reed-Sternberg cells (HRS). During recent decades, the long-term survival of HL patients has increased, and most patients can be cured through multi-agent chemotherapy, radiotherapy and/or hematopoietic stem cell transplantation.[1] Despite this, 25-30% of patients experience either disease relapse or are refractory to chemotherapy and their survival is substantially reduced, especially for elderly patients who do not tolerate intensive therapy.[2,3] New targeted therapies for HL are warranted, especially for refractory/relapsed patients and elderly patients where limiting treatment toxicity is essential. Recent studies have focused on the development of therapeutic agents that target HL-specific antigens or regulate the natural immune response in patients. Antibodies targeting HL surface antigens such as CD25 (daclizumab),[4] CD20 (rituximab, tositumomab)[5,6] or CD30 (brentuximab)[7-10] have shown promising results. The programmed death-1(PD-1)/PD-ligand 1 (PD-L1) checkpoint inhibitors (nivolumab, pembrolizumab), that reverse the suppres-

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sive communication between the tumor and immune system in tumor microenvironments have also been effective in HL patients.[11-13]

To date, the main utility of identifying membrane-bound CD83 has been to define activated dendritic cells (DC), but CD83 is also expressed on the surface of some activated B cells, T cells, macrophages and neutrophils.[14-18] In addition to a membrane-bound form, there is a membrane cleaved soluble (s) form of CD83. We reported that lymphoma tumor cells (HL and non-Hodgkin lymphoma [NHL]) expressed CD83 and released sCD83 into serum.[19,20] Recombinant sCD83 protein has immune inhibitory function in mice and humans.[21,22] Recently, CD83 was identified as one of the four classifiers to distinguish HL with anaplastic lymphoma kinase (ALK)-anaplastic large cell lymphoma.[23] Despite its potential as a relatively specific target, CD83 has not been investigated as a therapeutic target on either HL or NHL. We generated a human anti-human CD83 antibody, 3C12C, which prevents graft-*versus*-host disease (GvHD) but preserves anti-tumor T-cell function in mice after transplantation with human peripheral blood mononuclear cell (PBMC).[24,25] The availability of this potential therapeutic anti-CD83 antibody prompted us to investigate CD83 biology in HL. We show herein that an antibody that detects CD83 in paraffin sections stains Hodgkin and Reed-Sternberg (HRS) cells in most HL lymph node biopsy samples, that HL tumor cells secrete sCD83, and the serum sCD83 level in HL patients correlates with the clinical response. The 3C12C antibody, and its toxin conjugate, killed HL lines and 3C12C depleted CD83 target cells in non-human primate studies without any evidence of toxicity.

Methods

HL tissue section and serum samples

Lymph node biopsies and serum of HL patients were collected after approval by the Sydney Local Health District (SLHD) Human Research Ethics Committee, consistent with the Declaration of Helsinki. Archival paraffin embedded lymph node biopsies were obtained from 35 HL patients at initial diagnosis (Table 1), while serum samples were collected from six HL patients at diagnosis and during chemotherapy.

Immunohistochemistry

Immunohistochemical staining was performed on 3μm sections of formalin fixed paraffin embedded lymph node biopsies of HL patients or non-human primates. The primary antibodies used were mouse anti-human CD20 (L26, Dako), CD83 monoclonal antibodies (mAb; F5, Santa Cruz Biotechnology), CD30 (Ber-H2, Dako), and staining was performed on a Leica Bond III Autostainer (Leica Biosystems) using a Bond Polymer Refine Detection kit for visualization with 3, 3'-diaminobenzidine (DAB). Images were taken with an Olympus BX51 microscopy with an Olympus PP71 camera using Olympus labSens software.

sCD83 analysis

For the analysis of sCD83 levels, KM-H2, L428 and HDLM2 cells were cultured at concentrations of 106 cells/ml in complete roswell park memorial institute (RPMI) medium containing 10% fetal calf serum, 2mM glutaMAX™, 100U/ml penicillin, 100μg/ml streptomycin (Thermo Fisher Scientific) at 37°C, in 5% CO2. Cell culture supernatant were collected 24 hours after fresh medium change. Human sCD83 was analyzed by a sCD83 ELISA kit (Sino Biological Inc.).

Table 1. Characteristics of 35 Hodgkin lymphoma patients.

Age at enrolment (mean, range)	35 (17-71)
Sex	
Male; n (%)	18 (51.4%)
Female; n (%)	17 (48.6%)
Histologic subtype	
cHL-Nodular sclerosis (NS)	21 (60.0%)
cHL-Mixed cellularity (MC)	7 (20.0%)
cHL-Lymphocyte rich (LR)	1 (2.9%)
cHL-unspecified (CHL-U)	2 (5.7%)
Nodular lymphocyte predominant (NLP)	4 (11.4%)
Stage at onset	
I	3 (8.6%)
II	19 (54.3%)
III	5 (14.3%)
IV	8 (22.9%)

cHL: classic Hodgkin lymphoma.

Antibody Dependent Cell Cytotoxicity (ADCC) Assays

Target HL cells labeled with 25μM Calcein-AM (Life Technologies) were co-cultured at a ratio of 1:25 with human PBMC of a healthy donor used as effector cells. Supernatants were collected after three hours to measure released calcein using an enzyme-linked immunosorbent assay (ELISA) Reader (Perkin Elmer). The percentage of specific cytolysis was calculated as described.[25]

3C12C conjugation with monomethyl auristatin E (3C12C-MMAE) and cytotoxicity on CD83$^+$ cell lines

3C12C is a human immunoglobulin G1 (IgG1) anti-human CD83 mAb selected from a phage display library[26] and further engineered to improve affinity.[25,27] To produce 3C12C-MMAE, a lysosomal cathepsin B-cleavable, self-emolative dipeptide (ValCit) maleimide linker was prepared from MMAE for conjugation to partially reduced 3C12C using a similar method to brentuximab vedotin.[28] The cytotoxic activity was assessed by 7-amino-actinomycin D (7AAD, Thermo Fisher Scientific) staining using flow cytometry.

3C12C trials in non-human primates

The SLHD Animal Research Ethics Committee approved the study of five non-human primates (*Papio Hamadryas baboon*), which received intravenous human-IgG (Intragam, CSL) (10 mg/kg) or 3C12C mAb (1, 5, 10, 10 mg/kg) at days 0, 7, 14 and 21. PBMC were analyzed for immune cell populations including Dendritic cells (DC), T cells and B cells on a Fortessa X20 flow cytometer (BD Biosciences). Liver and kidney function were assessed by measuring alkaline phosphatase (ALP), aspartate transaminase (AST) and creatinine in serum samples using the Cobas 8000 (Roche). Lymph nodes were taken from 3C12C (10mg/kg) or human IgG (10mg/kg) treated animals at day 28 for immunohistological staining.

Statistical Analysis

Mean values with standard error of mean (SEM) bars are shown in graphs. Statistical analyses were performed using Prism 6.0 (GraphPad Software). A Mann-Whitney or one-way analysis of variance (ANOVA) test with Greenhouse-Geisser correction for multiple comparisons were used. Differences with $P<0.05$ were considered significant.

Results

CD83 is expressed on HL cell lines and HRS cells in lymph node biopsies of HL patients

Expression of CD83 was analyzed using the mouse anti-human antibodies HB15a, HB15e and potential therapeutic human anti-human CD83 antibody 3C12C.[25] KM-H2 cells expressed the most expressive CD38 cell surface, stained as it was with all three anti-CD83 antibodies, whilst the L428 and HDLM2 lines expressed less CD83. All three lines expressed CD30 (Figure 1A). This data was confirmed by confocal CD83 staining on KM-H2 cells (Figure 1B), detection of *CD83* messenger ribonucleic acid (mRNA) transcripts by reverse transcription polymerase chain reaction (RT-PCR) and intracellular CD83 expression in the three HL lines (*Online Supplementary Figure S1*).

Next, CD83 expression was analyzed on the paraffin-embedded lymph node biopsies of 35 HL patients (Table 1). The HRS cells were identified as $CD30^+$ (Figure 2A). Of note, 8/35 (22.9%) biopsies expressed high levels of CD83 on the HRS cells (>90% positive), 21/35 (60%) expressed middle levels (10-90% positive), and 6/35 (17.1%) expressed low levels of CD83 (<10% positive) (Figure 2B,C). Of the 29 biopsies with high or middle expression, 21 (72.4%) had strong or moderate intensity, while 8/29 (27.6%) showed weak intensity of CD83 on HRS cells (*Online Supplementary Table S1*). The subtype analysis showed that 16/21 (79.2%) of HRS cells in nodular sclerosis (NS) HL were CD83 high or middle, and 85.7% were CD83 high or middle in mixed cellularity (MC) HL. Most (20/22, 90.9%) of stage I-II HL were CD83 high or middle, and 9/13 (69.2%) HL in stage III-IV were CD83 high or

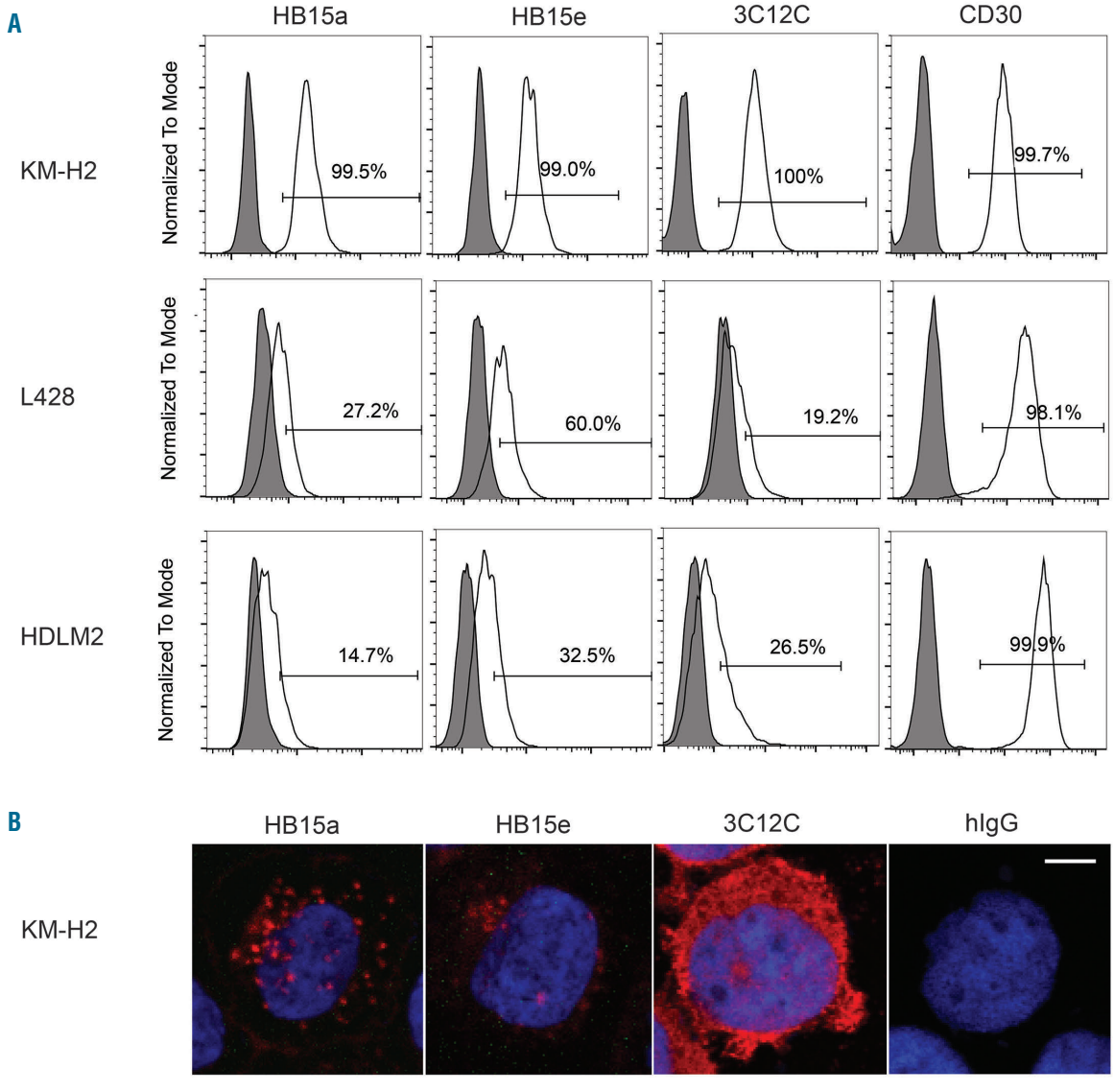

Figure 1. CD83 is expressed on Hodgkin lymphoma cell lines. (A) Expression of CD83 was analyzed by flow cytometry on KM-H2, L428 and HDLM2 cell lines, which were stained with HB15a-fluorescein isothiocyanate (FITC), HB15e-FITC or 3C12C-FITC anti-CD83 mAbs, respectively. Gray histograms represent isotype control, while open histograms represent anti-CD83 antibodies. CD30 staining was used as a positive control. These data are representative of three independent experiments with comparable results. (B) CD83 expression (red) on KM-H2 cells with HB15a, HB15e or 3C12C mAb were imaged by confocal microscopy. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Human IgG1 was used as control for 3C12C mAb. Scale bar: 5μm.

middle. Interestingly, strong CD83 expression on HRS cells was found in two out of three relapsed HL (*Online Supplementary Table S1*). Epstein-Barr virus (EBV) infection is associated with an increasing risk of developing EBV-positive HL. A number of viral products, including EBV nuclear antigens (EBNA), EBV latent membrane proteins (LMP1 and LMP2) and EBV encoding small ribonucleic acids (RNA; *EBER*) have been implicated. LMP1 induced CD83 in EBV-infected human B cells by activation of NF-kB.[29] CD83/LMP1 has been reported to be correlated in MC HL, but not for NS HL.[23] By *in situ* staining of *EBER* of 35 HL samples, we found that seven HL were *EBER* positive, including 2/7(28.6%) MC and 3/22 (13.6%) NS HL (Figure 2D). On six out of seven *EBER* positive HL samples, CD83 staining of HRS were strong or moderate (*Online Supplementary Table S1*).

CD83 is trogocytosed from HL cells to T cells

We found previously that CD83 was able to transfer from the membrane of DC to T cells *via* trogocytosis.[15] Similar trogocytosis was observed to occur between HL cell lines and T cells. When these two cell types were co-cultured for four hours, CD83 surface expression was detected on 5-15% of T cells (Figure 3A,B), whereas no

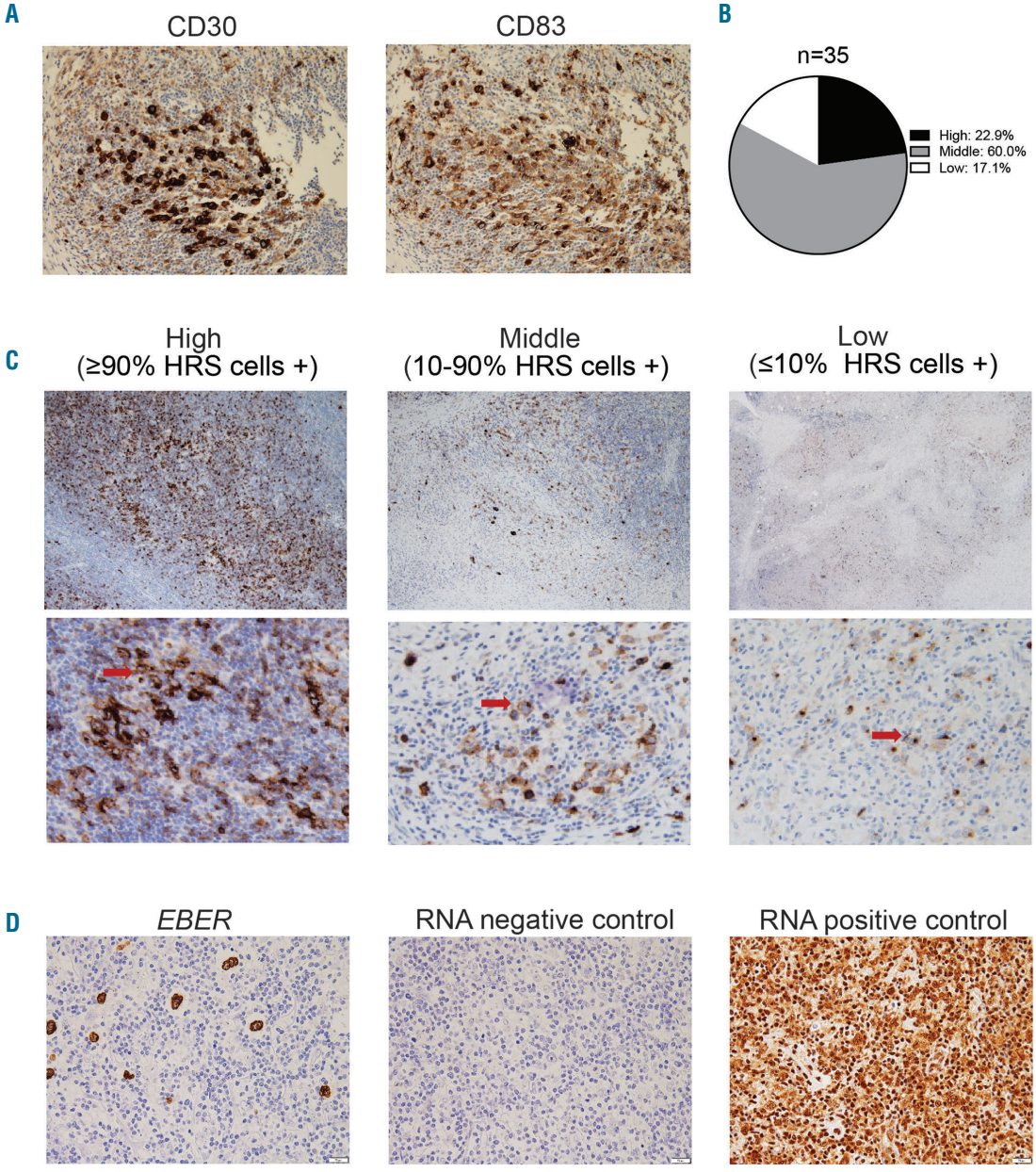

Figure 2. CD83 is expressed on Hodgkin and Reed-Sternberg (HRS) cells in Hodgkin lymphoma patients. (A) CD83 and CD30 expression (brown) on paraffin-embedded lymph node biopsy samples of HL was imaged by microscopy with ×200 magnification. One representative sample of 35 biopsies shown. (B) Pie chart analysis of CD83 expression level in HRS cells of HL patients (n=35). High: CD83 positive in >90% HRS cells; middle: 10-90% CD83^{+}in HRS cells; low: 10% CD83^{+} in HRS cells. One representative sample of each group is shown in (C), upper panel: original magnification ×40, lower panel shown with high amplification (×200). Arrows indicate HRS cells expressing CD83. (D) Epstein-Barr virus encoding small ribonucleic acids (RNA; *EBER*) in 35 HL biopsies were detected by *in situ* hybridization; one of the seven *EBER* positive samples is shown.

CD83 was detected on T cells in the absence of KM-H2 cells. Furthermore, separating the T and KM-H2 cells during culture by a 0.4μm transwell filter prevented trogocytosis (*Online Supplementary Figure S2*). To confirm the trogocytosis involved membrane transfer, KM-H2 cells were labeled with fluorescent dye (CellVue Claret) and co-cultured with CD3$^+$ T cells. Cell membrane transfer from KM-H2 cells to T cells was confirmed by flow cytometry and confocal microscopy (Figure 3C,D, and *Online Supplementary Figure S2*). No differences were observed in the CD4$^+$ and CD8$^+$ T-cell ratio during the co-culture of KM-H2 and T cells within four hours (*data not shown*). However, the CD83$^+$ T cells expressed significantly higher levels of PD-1 than CD83$^-$ T cells (P=0.048) and T cells cultured without KM-H2 (P=0.005) (Figure 3E). The increase in PD-1 was significantly higher on the trogocytosed CD83$^+$CD4$^+$ T cells than non-trogocytosed CD83$^-$ T cells (P=0.049). In contrast, no difference in PD-1 expression was seen between the CD83$^+$ and CD83$^-$ CD8$^+$ T cells, (P=0.185) although both KM-H2 co-cultured CD4$^+$ and CD8$^+$ T cells had higher PD-1 expression than

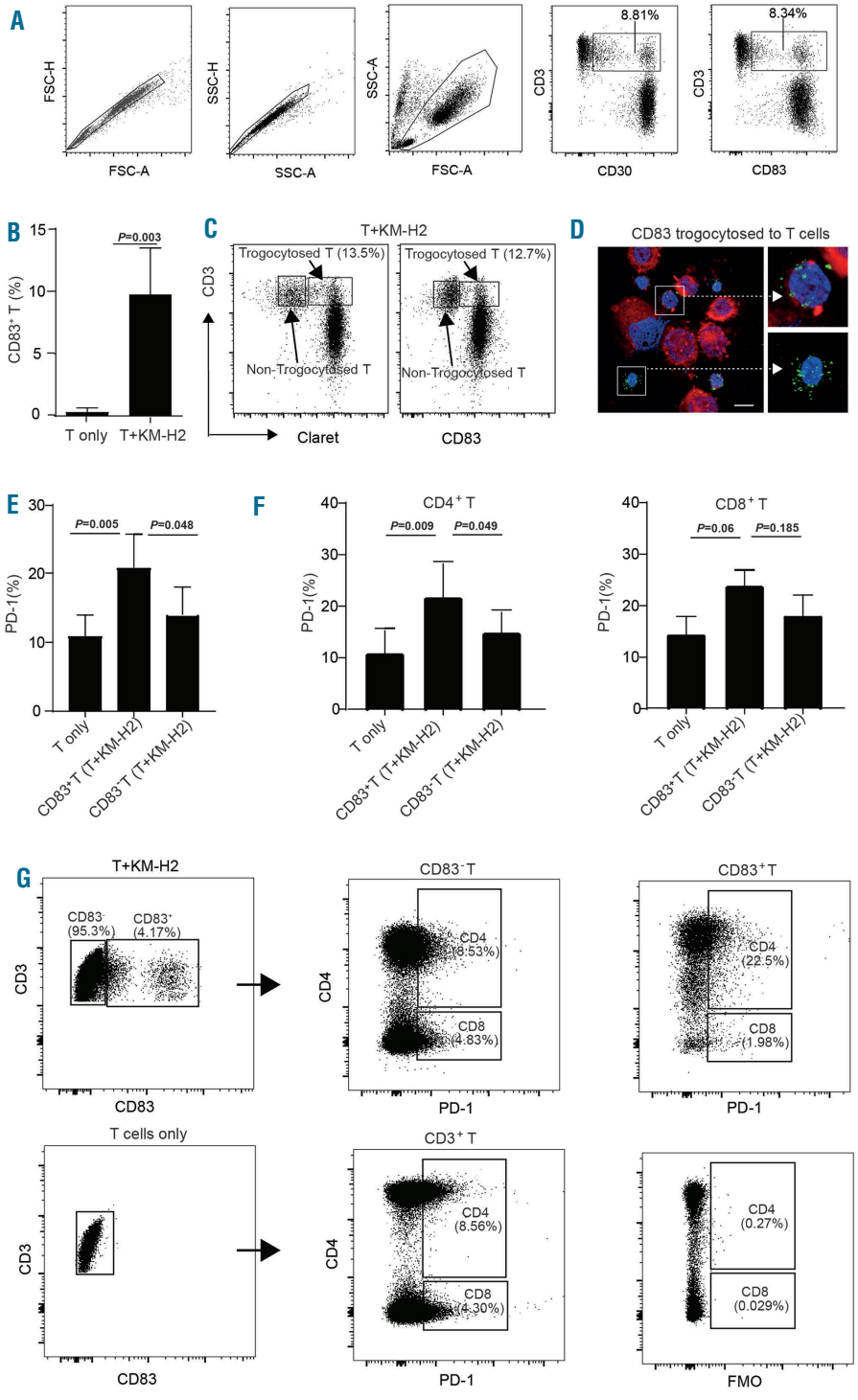

Figure 3. Trogocytosis of CD83 molecule from Hodgkin and Reed-Sternberg cells to T cells. (A) T cells from healthy donor PBMCs were co-cultured with KM-H2 cells for four hours at a ratio of 1:5. CD83 and CD30 expression on CD3$^+$T cells was analyzed by flow cytometry, data were from one of seven experiments and summarized data (mean± SEM and P-value) are shown in (B). (C) KM-H2 cells were labeled with CellVue Claret (red) and co-cultured with purified T cells (green) at a ratio of 5:1 for four hours. CellVue Claret and CD83 expression on T cells was analyzed by flow cytometry. (D) Confocal microscopy image of Claret labeled KMH2 cells co-cultured with T cells that stained with biotinylated mouse anti-human CD3 mAb and Stepdavidin-AF488. Nuclei were stained with DAPI. Scale bar: 5μm. Upper insert: trogocytosed T cells, lower insert: non-trogocytosed T cells. Data representative of three experiments. (E) PD-1 expression on CD83$^+$ trogocytosed T cells co-cultured with KM-H2 cells for four hours was determined by flow cytometry (n=4). P-value of one-way ANOVA analysis shown. (F) PD-1 expression on trogocytosed CD4$^+$T or CD8$^+$ T cells after co-culture with KM-H2 cells for four hours was analyzed (n=4). P-value of one-way ANOVA analysis shown. A representative experiment shown in (G). FSC: forward scatter; SSC: side scatter; PD-1: programmed death-1; FMO: fluorescence minus one.

T cells cultured alone (Figure 3F,G). The CD83+CD4+ T cells had the same proportion of regulatory T cells (Treg) as non-trogocytosed CD4+ T cells (*Online Supplementary Figure S2*).

Supernatant from HL cell lines inhibits T-cell proliferation

Surface CD83 can be cleaved into sCD83.[15,20] We detected it in the supernatants of activated DC and B lymphocytes,[20] as well as the serum of NHL and chronic lymphocytic leukemia patients.[30] High levels of sCD83 were found in the supernatant of KM-H2 (460.6±11.8 pg/ml) and L428 (200.8±53.2 pg/ml), but low in HDLM2 (21.67±1.45 pg/ml) (Figure 4A). HL patients had significantly higher serum sCD83 (360.5±54.82 pg/ml, n=10) at diagnosis than healthy donors (52.6±9.5 pg/ml. Figure 4A).

We then tested the effect of KM-H2 cell supernatant on T-cell function. KM-H2 supernatant containing sCD83

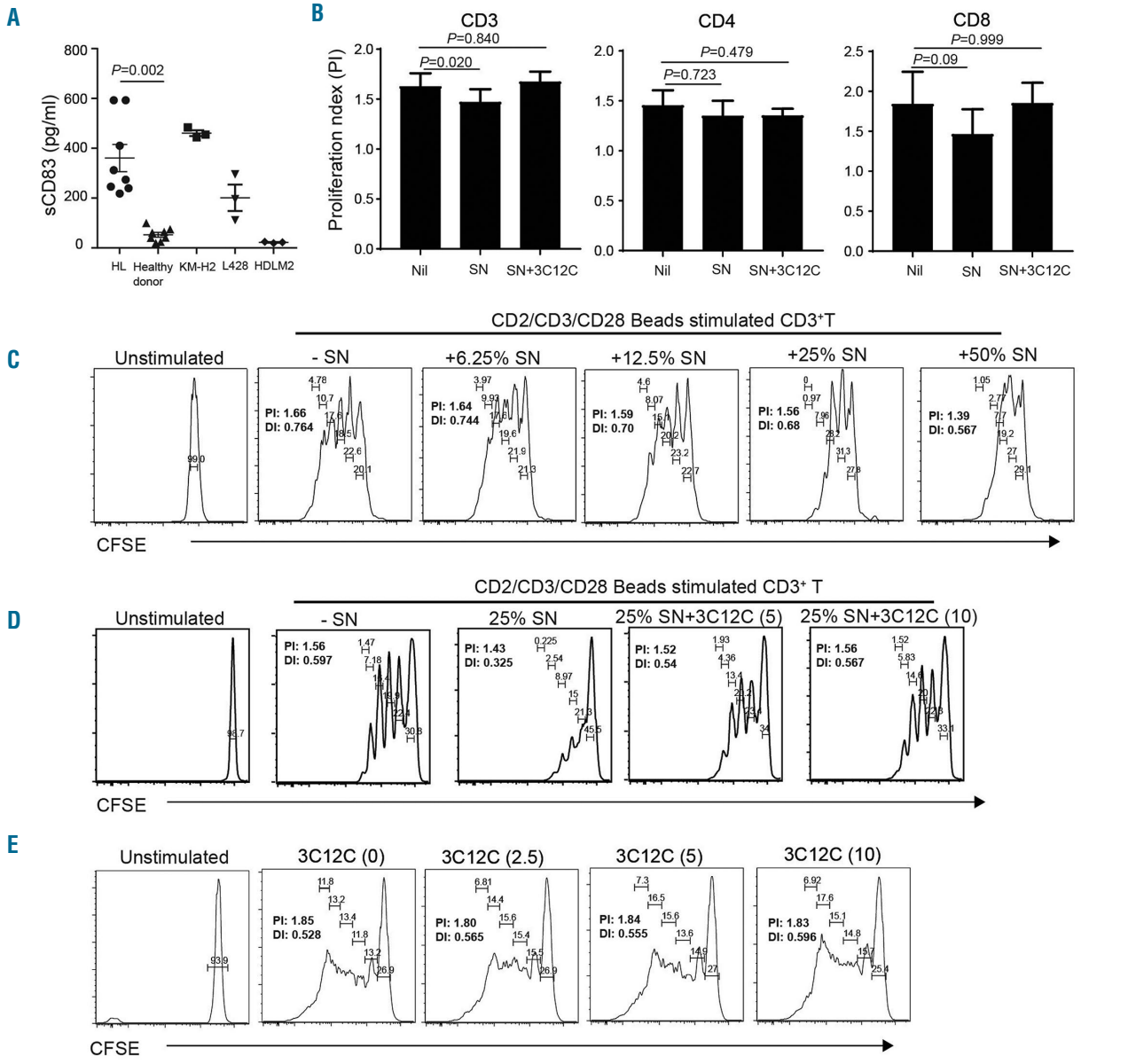

Figure 4. Soluble CD83 (sCD83) from Hodgkin lymphoma (HL) cell lines inhibits T-cell proliferation which is abolished by binding to 3C12C. (A) sCD83 was detected in the supernatant of KM-H2, L428, and HDLM2 lines that were cultured for 24 hours at 1x10^6/ml after changing fresh complete Roswell Park Memorial Institute (RPMI) medium and diagnostic sera of HL patients by ELISA. The *P*-value of A Mann-Whitney test is shown. (B) Carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE) labeled purified T cells were stimulated with CD2/CD3/CD28 beads (3:1) in the presence of 25% supernatant (SN) of KM-H2 or plus 3C12C (5 μg/ml) for five days. Cells were analyzed by flow cytometry and the proliferation index (PI), that is defined as the total number of divisions divided by the number of cells that went into division, were calculated for total CD3+, CD4+ and CD8+ T cells using Flow Jo (n=6). The *P*-value of one-way ANOVA analysis is shown. (C) Different volumes (v/v) of KM-H2 supernatant were added to CD2/CD3/CD28 microbead-stimulated CFSE-labeled human T cells. T cells were collected and CFSE was analyzed by flow cytometry at day five. The PI and division index (DI), that is the average number of cell divisions that a cell in the original population has undergone, were calculated as indicators for proliferation. Representative data from one of three similar experiments shown. (D) CFSE-labeled T cells were stimulated with CD2/CD3/CD28 microbeads. T cells were then cultured in 25% (v/v) KM-H2 SN with 3C12C (5 and 10 μg/ml). T-cell proliferation was analyzed on day five. (E) The effect of different concentrations of 3C12C on proliferation of CFSE-labeled T cells was determined after CD2/CD3/CD28 microbead stimulation.

inhibited CD2/CD3/CD28 bead stimulated T-cell proliferation (Figure 4B) in a dose-dependent manner (Figure 4C). Only proliferation of $CD8^+$ T cells seemed inhibited by KM-H2 supernatant (P=0.09), and not $CD4^+$ T-cell proliferation (P=0.732). Administration of the anti-CD83 antibody, 3C12C, partially abolished the inhibitory effect of KM-H2 supernatant (Figure 4D). 3C12C alone had no effect on T-cell proliferation (Figure 4E).

HL patient serum sCD83 declined to normal levels correlated with a complete or partial response by PET-CT scan

We monitored changes in circulating sCD83 in six HL patients during sequential chemotherapy. All assessments of response were made by positron emission tomography – computed tomography (PET-CT) scan using the Lugano classification system. All patients received 3-6 cycles of chemotherapy; five achieved a complete response (CR) and one patient a partial response (PR) by PET-CT scan (Figure 5, *Online Supplementary Table S2*). Serum sCD83 decreased, returning to normal levels when the patients had a CR to chemotherapy, as documented by PET-CT scan in patients #1 and #2. In patients #3 and #6, the serum sCD83 level was still elevated when the PET-CT scan showed CR but normalized after one further cycle of chemotherapy. Patient #4 showed a PR prior to cycle 5 by PET-CT-scan, however the serum sCD83 level only started to decrease during cycle #5 reaching a normal range in cycle 6, coinciding with CR. PET-CT scans in patient #5 showed progressive disease (PD) after cycle 2, but a PR after another two cycles of chemotherapy, when the corresponding sCD83 reduced to normal level.

3C12C and 3C12C-MMAE kills HL cell lines

The ADCC activity of the anti-CD83 mAb, 3C12C, was tested on the three HL lines: KM-H2, L428 and HDLM2. Whilst 3C12C killed KM-H2 and L428 efficiently, HDLM2 was relatively resistant to it (Figure 6A). To elucidate this, the stability of 3C12C binding on the HL cell surface were tested. HL lines were cultured in saturating concentration

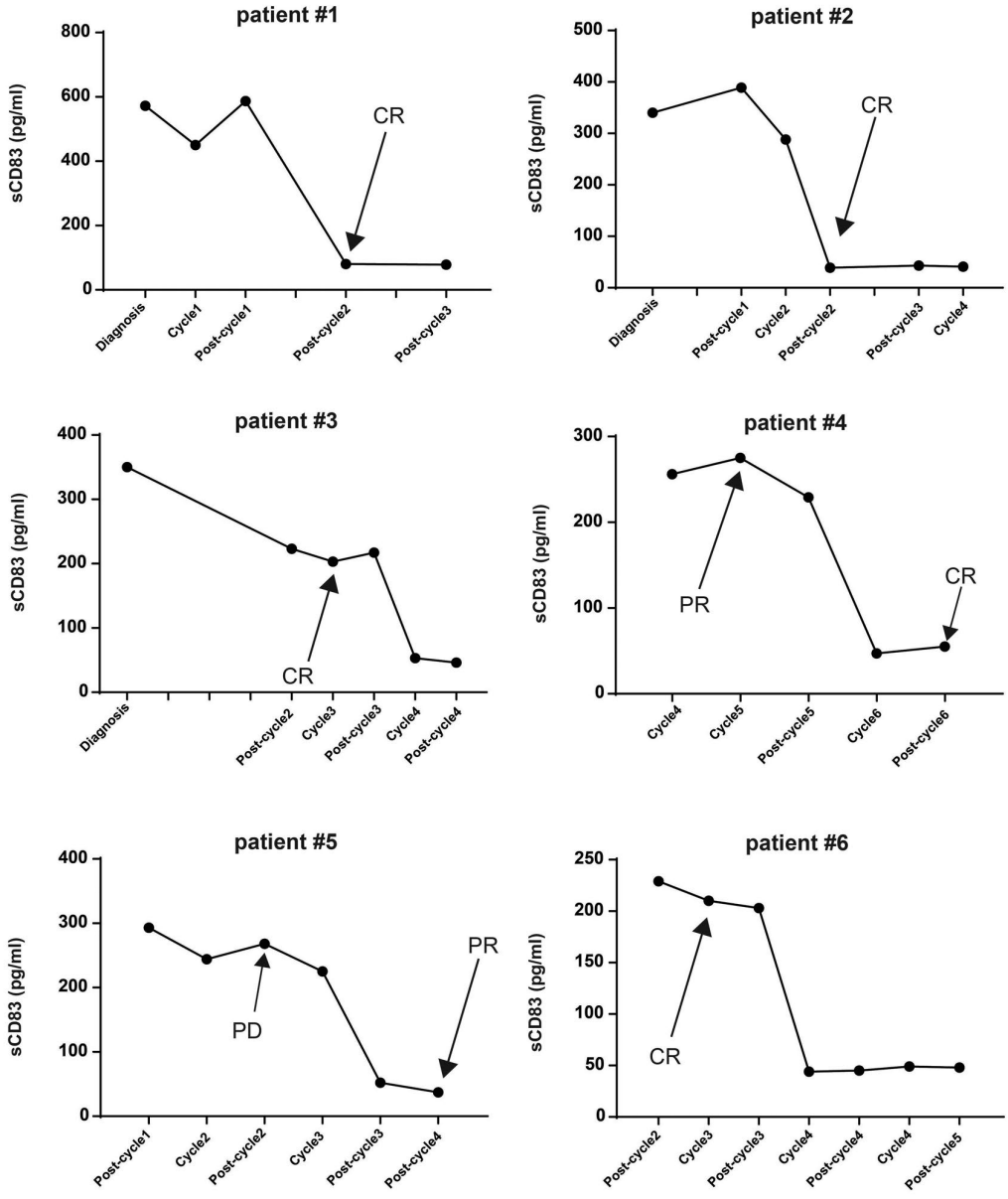

Figure 5. Time course of soluble CD83 (sCD83) in Hodgkin lymphoma patients during chemotherapy. The sCD83 level in the sera of six HL patients during different cycles of chemotherapy was examined by ELISA. Arrows indicate when PET-CT scans were performed and the results of complete response (CR), partial response (PR) or progressive disease (PD) are noted.

of 3C12C (10 μg/ml) on ice followed by washing off unbound antibody. Cells were then cultured without 3C12C for up to two hours. The remaining 3C12C bound on the cell surface were detected by a secondary anti-human antibody. Though L428 and HDLM2 have a lower level of surface CD83 expression compared to KM-H2, our analysis showed that the 3C12C level on the surface of HDLM2 reduced much faster than on L428, while the 3C12C bound to L428 were far more stable (Figure 6B). This suggested 3C12C was rapidly internalized in KM-H2 and HDLM2, while 3C12C was internalized slower in L428. To investigate further potential therapeutic applications, we generated a 3C12C toxin-conjugate (3C12C-MMAE). *In vitro*, 3C12C-MMAE killed CD83$^+$ KM-H2 cells most efficiently, followed by HDLM2 and L428, while CD83$^-$ HL-60 cells were the least sensitive to 3C12C-MMAE (Figure 6C and *Online Supplementary Figure S3*). In addition, the intracellular CD83 level in HDLM2 was much higher than L428, lending to more sensitivity of the HDLM2 to the killing of 3C12C-MMAE (*Online Supplementary Figure S1*).

Administration of 3C12C is safe in non-human primates

To "de-risk" the antibodies before advancing 3C12C into a clinical trial, we performed pre-clinical dose-escalation studies of 3C12C in non-human primates. Five baboons were injected intravenously with 3C12C (1, 5, 10 mg/kg on d0, 7, 14, and 21). No adverse clinical events were recorded during follow up for 84 days. We assessed blood counts and biochemistry weekly, and monitored different immune cell populations by flow cytometry or immune histology. Administration of 3C12C did not affect blood cell counts (white blood cells [WBC], red blood cells [RBC], and platelets), liver (ALP and AST) or kidney (creatinine) function (*Online Supplementary Figure S4*). The total T-cell number, and ratio of CD4$^+$/CD8$^+$ T cells all remained normal up to day 84 (*data not shown*). However, there was evidence of 3C12C efficacy in that CD1c$^+$DC counts were reduced. We found that baboon blood B cells expressed CD83 as human B cells (*data not shown*), and reductions in blood B cells were noted by flow cytometry (Figure 7A). In addition, B-cell areas in lymph nodes were reduced in the 3C12C-treated animals (10mg/kg) compared to the control animals (human IgG 10mg/kg) (Figure 7B).

Discussion

HL is driven by the malignant HRS cell, which are of B lineage origin.[31,32] A significant number of patients experience relapsed/refractory disease following first-line chemotherapy.[33] Less toxic treatments for relapsed/refractory HL would be highly desirable, as exemplified by the introduction of the anti-CD30 antibody drug conjugate (brentuximab).[10,34] In the study herein, we were able to identify sCD83 as a new potential biomarker for HL, and CD83 as a target for a therapeutic mAb and derivatives.

CD83 was first described on activated B cells and we originally detected CD83 on HL using frozen sections.[19] Our ability to stain paraffin embedded lymph node biopsy samples of HL patients encouraged this study and allows for the assessment of CD83 expression in routine clinical practice. CD83 was highly expressed on HRS cells with a different staining pattern to CD30. Thus, CD83 is potentially another diagnostic marker of HL. More importantly, this work suggests that the majority of HL patients might be suitable for a therapeutic mAb targeting CD83. An anti-CD83 mAb may also work synergistically with chemotherapy, which is similar to the treatment of stage III or IV HL with anti-CD30 antibody drug conjugate

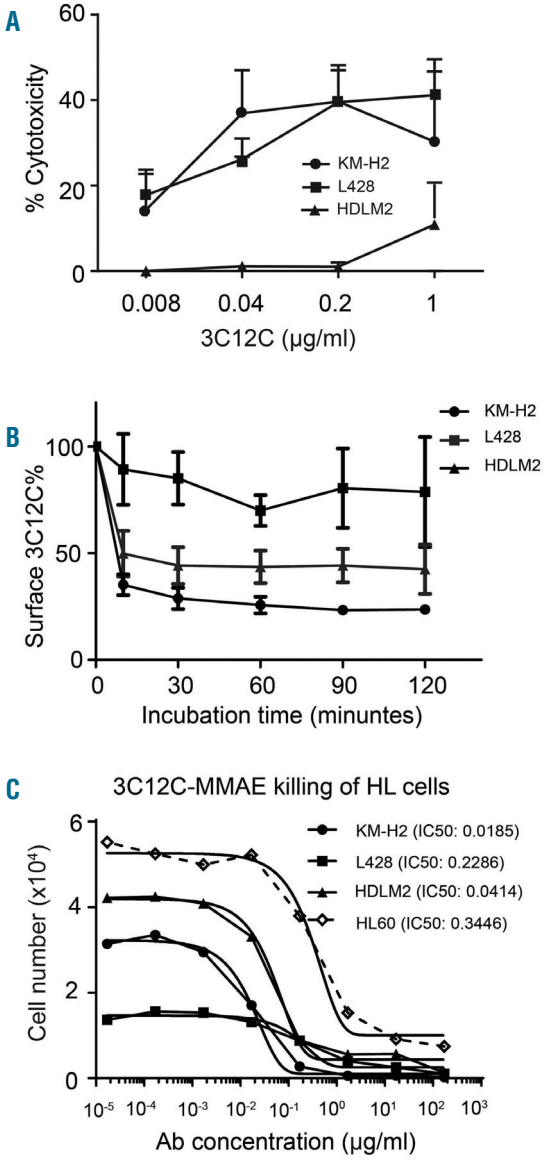

Figure 6. 3C12C and 3C12C conjugation with monomethyl auristatin E (3C12C-MMAE) kill Hodgkin lymphoma (HL) cell lines *in vitro*. (A) Target cells KM-H2, L428 or HDLM2, labeled with Calcein-AM were co-cultured with effector cells (human PBMC) at effector: target ratio of 25:1 with increasing 3C12C concentration from 0 μg/ml to 1 μg/ml at 37°C for three hours. Supernatant was collected for fluorescence reading (excitation 485nm, emission 538nm) of released Calcein. Antibody (Ab)-dependent cell cytotoxicity was calculated (n=3). (B) HL cells were cultured in 3C12C saturation concentration (10 μg/ml) on ice followed by intensive washing and culture without 3C12C from 0-2 hours. The remaining levels of 3C12C bound on the cell surface were detected by a secondary anti-human antibody with flow cytometry. The remaining surface level of 3C12C on KM-H2, L428 and HDLM2 was normalized to the level of time 0. (n=3). (C) CD83$^+$ KM-H2, L428, HDLM2 or CD83$^-$ HL-60 cells were cultured with different concentrations of 3C12C-MMAE for three days before determining viable cells by 7-amino-actinomycin D (7AAD) staining with flow cytometry. The half maximal inhibitory concentration (IC_{50}) is shown. Data was from one of four representative experiments.

(brentuximab).[10] As CD83 is inducible, it is possible that either drug induced or inflammatory activation would induce greater CD83 expression on HRS cells. We also identified serum sCD83 as a potential disease marker. Its immunosuppressive effect was reversed by anti-CD83 mAb at levels readily obtained *in vivo*. We predict that 3C12C would target HL cells directly through ADCC, but it has the additional therapeutic effect of reversing the inherent immunosuppressive effect of CD83. Such a synergistic response has the potential to have a significant clinical effect with limited toxicity.

Recent studies revealed the impact of tumor microenvironment on tumor progression and therapy. HL is a leading example. The low frequency malignant HRS cells secrete several factors and generate a surrounding infiltrate of immune cells that contribute to the pathogenesis of the disease.[35-37] CD83 appears to be involved in this process. We previously demonstrated that the transfer of membrane proteins on myeloma cells to T cells disrupted the immune response and was associated with poor prognosis.[38] We found that HL tumor cells express CD83 and can transfer surface CD83 molecules by trogocytosis. CD83 transfer from KM-H2 to T cells *in vitro* was consistent with the finding that some lymphocytes in the lymph node biopsy samples, especially in CD83 high expression patients, expressed CD83. The proportion of Treg in the trogocytosed $CD83^{+}CD4^{+}$ T cells was not increased, but $CD83^{+}$ T cells, especially $CD4^{+}$ T cells, expressed a higher level of PD-1 than $CD83^{-}$ T cells. PD-1 and PD-1L interaction contributes to the immunosuppressive microenvironment of HL.[39] Such PD-1^{high} $CD83^{+}$ T cells might become unresponsive in the tumor microenvironment.[40] A CD83 target therapy might be combined with brentuximab and PD-1 blockage to enhance the clinical response.

The serum of some hematopoietic malignancies have increased levels of sCD83.[20,30] The supernatant of HL cells inhibited T-cell proliferation, but this inhibitory effect was not related to Treg induction (*data not shown*). The anti-CD83 mAb, 3C12C, partially abolished the inhibition by KM-H2 supernatant. Thus, sCD83 from the supernatant

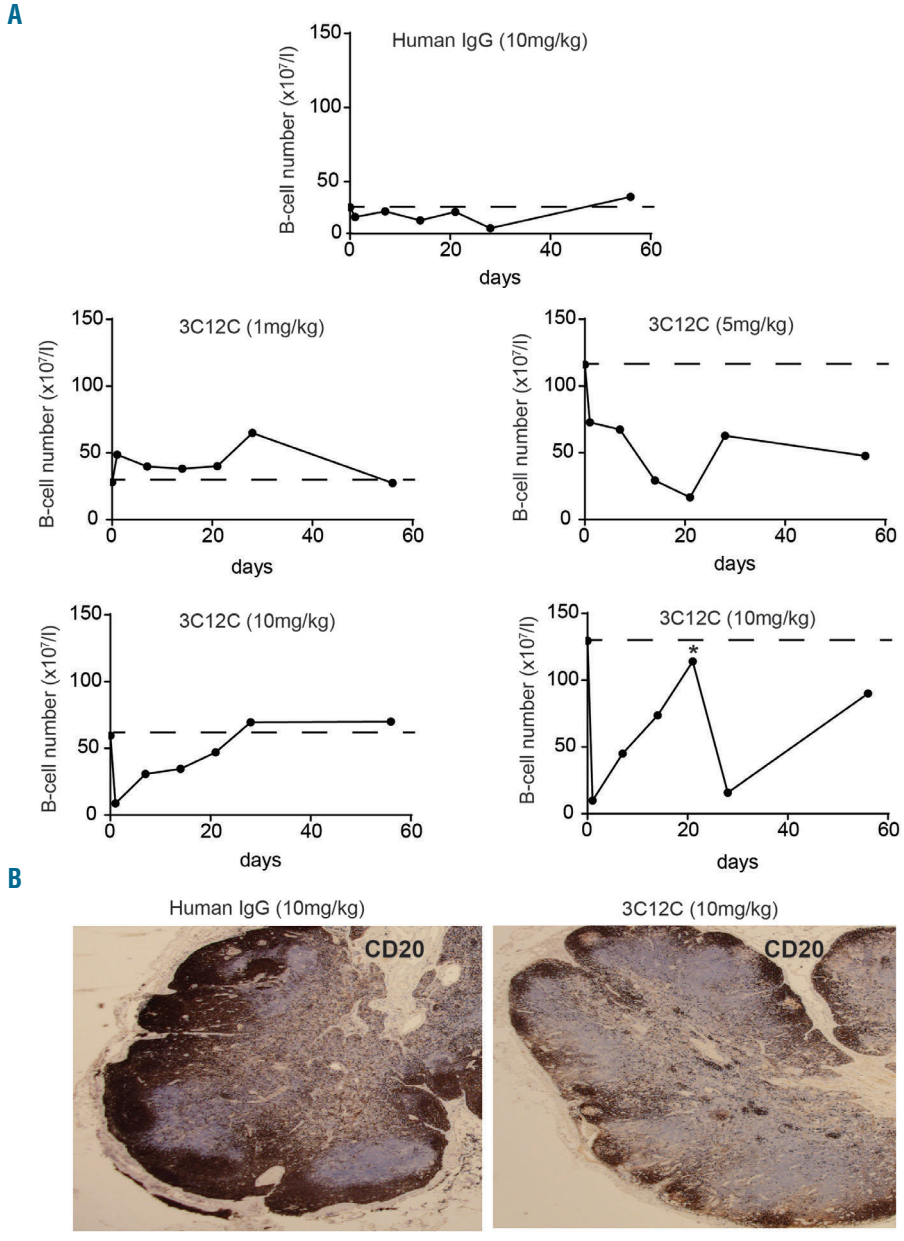

Figure 7. 3C12C reduced B cells in non-human primates. Five non-human primates were injected with 3C12C (1, 5, 10, 10 mg/kg, n=4) or human Immunoglobulin G (IgG; 10mg/kg, n=1) at days 0, 7, 14 and 21. Blood and serum samples were collected for cell counts (red cells, white cells and platelets), liver function (ALP and AST levels) and kidney function (creatinine level) analysis. (A) $CD19^{+}$ B cells were enumerated from PBMC of five animals by flow cytometry. Dashed lines indicate the base cell number at day 0. *indicates one time point when WBC was extremely high on that animal. (B) A lymph node biopsy was taken at day 28 from 3C12C (10mg/kg) and control-treated animals. B cells stained with anti-human CD20 mAb on paraffin-embedded lymph node biopsy samples are shown. One of the two similar results for the two animals receiving 10 mg/kg 3C12C showing reduced B-cell areas compared to the human IgG control animal.

plays a key inhibitory role on T cells. We also found that HL cell line supernatant inhibited normal B-cell proliferation (*data not shown*). Other cytokines or soluble factors from the KM-H2 supernatant may also contribute to the inhibitory effect, e.g., sCD30. An 85kDa soluble form of the CD30 molecule (sCD30) has been shown to be released by CD30$^+$ cells *in vitro* and *in vivo*. sCD30 was elevated in the serum of HL[41,42] and other CD30-expressing tumors, as well as inflammatory conditions with strong T- or B-cell activation. The CD30-Fc fusion protein inhibits T-cell proliferation,[43] whilst sCD30 is also involved in the pathogenesis of renal, islet transplant rejection.[44,45] The effect of brentuximab on sCD30 has not been investigated. The thymus and activation related chemokine (TARC; CCL17) is expressed by HRS cells.[46]

TARC is confirmed as a biomarker of HL, since elevated serum TARC reflected the disease activity and correlated with clinical response.[47,48] Herein, as well as confirming elevation of serum sCD83 in active HL, we monitored sCD83 on six HL patients who underwent sequential cycles of chemotherapy. A complete response shown by PET-CT scan correlated with the decreased sCD83 levels. Thus sCD83 may be another biomarker candidate for monitoring the potential clinical response. A much larger cohort of HL patients will be monitored prospectively in order to explore this further. Additional investigations regarding the effect of sCD83 on HL biology may well assist therapeutic development in HL. Natural sCD83 has proved difficult to obtain for functional studies, suggesting the sCD83 structure and/or function is sensitive to *in vitro* manipulation.[49]

We have developed a human anti-human CD83 mAb, 3C12C, to investigate in clinical trials. It kills HL cells through ADCC, but in order to enhance its activity, we developed a 3C12C toxin conjugate (3C12C-MMAE). The unconjugated parent anti-CD30 antibody SGN-30 had no effect on HL in a study of 38 patients.[50] Brentuximab vedotin SGN35, which is a drug conjugate of SGN-30 with MMAE, has, however, proven to be a highly promising drug with CD30$^+$ lymphoma.[10] We found 3C12C-MMAE kills CD83$^+$ HL cell lines KM-H2 and HDLM2 very efficiently. Although HDLM2 cells express less surface CD83 and are resistant to ADCC killing, the high killing efficiency of HDLM2 with 3C12C-MMAE is likely related to the rapid antibody internalization and high intracellular CD83 turnover. 3C12C binding on L428 is relatively stable, rendering it sensitive to killing by ADCC, but less likely to be killed with 3C12C-MMAE, which is mediated *via* antibody internalization. Further improvements in the 3C12C-MMAE conjugate preparation are planned.

Finally, we tested the safety of 3C12C in non-human primates. We saw no clinical toxicity, abnormalities of blood count, liver or renal function or a decrease in the target CD1c$^+$DC population (*data not shown*). By monitoring B cells, we saw depletion in the blood and lymph nodes. The depletion of activated CD83$^+$ B cells led to a significant reduction in the B-cell area of lymph nodes. This early evidence of CD83 target cell depletion in non-human primates is most encouraging, suggesting an ADCC effect that should translate readily to the clinic.

Taken together, these data demonstrate that CD83 is a new potential diagnostic HL marker and serum sCD83 levels are likely to reflect HL disease load. CD83 is a target for therapeutic mAb development as well as CD83 target derivatives. The potential therapeutic human anti-CD83 antibody, 3C12C, kills HL cells efficiently *in vitro*. It is safe in non-human primates, and depletes CD83+ target cells. Further development of 3C12C in human studies merits serious consideration.

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Supplementary Methods

Hodgkin lymphoma (HL) cell lines

Three HL cell lines KM-H2, L428 and HDLM2 (gift from Prof Volker Diehl, University of Cologne, Germany) were used. Control cell line HL-60 was from the Christchurch Haematology Research Group.

Flow Cytometry

The following antibodies were used: CD3-Alexa Fluor (AF)700 or CD3-Phycoerythrin (PE), CD4-PE-CF594, CD15-Violet (V)450, CD19-V450, CD20-V421, CD30-PE, CD40-PE-Cy7, CD279 (PD-1)- Brilliant Violet (BV)786, CD274 (PD-L1)-PE-Cy7 (all from BD Biosciences), CD25-BV421 and CD107-PE-Cy7 (Biolegend). Mouse anti-human CD83 monoclonal antibodies (mAbs), HB15a-Fluorescein Isothiocyanate (FITC) was obtained from Beckman and Coulter, and HB15e-FITC from BD Biosciences. 3C12C is a human IgG1 anti-human CD83 mAb made in house. Isotype control antibodies included mouse IgG1 Kappa- FITC, mouse IgG2b-FITC (BD Biosciences) and human IgG1 Kappa (Sigma Aldrich). For intracellular CD83 expression, cells were fixed and permeabilized with Foxp3 intracelluar staining kit (Ebioscience). Data were collected on a Fortessa X20 flow cytometer (BD Biosciences) and analyzed with FlowJoV9&10 software (TreeStar).

Immunofluorescence staining

KM-H2, L428 or HDLM2 cells (10^5 cells) were cytospun onto lysine coated microscope slides. Cells were fixed and permeabilized with acetone at -20°C overnight. This was followed by rehydration in PBS/1% BSA and blocking with 10% goat serum (Sigma Aldrich). Cells were stained with primary antibodies: HB15a (Beckman and Coulter), HB15e (STEMCELL Technologies) or 3C12C anti-CD83 antibodies, followed with goat anti-mouse IgG-AF647 (for HB15a, HB15e) or goat anti-human IgG-AF488 (for 3C12C) (Thermo Fisher Scientific). Nuclei

were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Thermo Fisher Scientific). Cells were visualized using a laser scanning confocal microscope (Leica SP8) and composite images produced using Image J (National Institutes of Health).

In situ hybridization

Epstein-Barr virus encoding small RNA (EBER) in situ hybridization was performed using Bond EBER probe, Bond RNA negative control probe and Bond RNA positive control probe with Bond Anti-fluorescein antibody (Leica Biosystems). Staining was performed on 3µm sections of formalin fixed paraffin embedded tissue. Antigen retrieval, hybridisation and immunohistochemical staining was performed on an automated Leica Bond III (Leica Microsystems). Enzyme 1 (Leica Biosystems) was used for antigen retrieval for 15 minutes. A Bond Polymer Refine Detection system with 3,3'-diaminobenzidine (DAB) was used for chromogenic detection.

Trogocytosis analysis

Venous blood was collected from healthy donors (HD) under approval of SLHD HREC. Human PBMC were isolated by centrifugation on Ficoll-Paque-PLUS (GE Healthcare). T cells were isolated from PBMC using EasySep Human T cell Isolation Kit (STEMCELL Technologies) according to the supplier's instructions. KM-H2 cells were cultured with purified $CD3^+$ T cells from human PBMC for 4 hours at a ratio of 5:1. CD83 expression on T cells was analyzed by flow cytometry using HB15a mAb. For fluorescent imaging, KM-H2 cells were labelled with CellVue Claret Far Red Fluorescent Cell Linker Kits (Sigma-Aldrich) and co-cultured with $CD3^+$ T cells for 4 hours at ratio of 5:1. Cells were then stained with biotinylated mouse anti-human CD3 mAb (BD Bioscience) and Strepdavidin-AF488 (Thermo Fisher Scientific). In some experiments, 0.4µm transwell insert (Corning) were used to separate T cells from KM-H2 cells

during culture. CD83 expression on T cells was analyzed by flow cytometry after 4 hours of culture.

T cell proliferation analysis

Purified T cells of human PBMC were labelled with 5nM Carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE ; Sigma-Aldrich) and stimulated with anti-CD2/CD3/CD28 T cell activation/expansion kit (Miltenyi) in the presence of supernatant from KM-H2 cells for 5 days. Cells were analysed by flow cytometry on a Fortessa (BD Bioscience). The proliferation Index (PI), that is defined as total number of divisions divided by the number of cells that went into division, and Division Index (DI), that is the average number of cell divisions that a cell in the original population has undergone, were analysed with FlowJo V9 (TreeStar).

PCR analysis

RNA was extracted with TRIzol (Life Technologies) and cDNA was transcribed from 100 ng RNA using SuperScript® III First-Strand Synthesis kit and random hexamers primer (Thermo Fisher Scientific) following the manufacturer's protocol. cDNA from the specified immune populations were amplified by PCR using human *CD83* exon 2 forward primer 5'-AGGTTCCCTACACGGTCTCC-3' and exon 5 reverse primer 5'-AAGATACTCTGTAGCCGTGCAAAC-3'. Primers to the *GAPDH* housekeeping gene 5'-ATGGGGAAGGTGAAGGTCGGA-3' (forward) and 5'-AGGGGCCATCCACAGTCTTCTG -3' (reverse) were used as an endogenous contol. Amplified fragments were separated on 2% agarose (Thermo Fisher Scientific) gel.

Stability of 3C12C antibody binding on the surface of HL lines.

KM-H2, L428 and HDLM2 cells were incubated with a saturated concentration of 3C12C (10µg/ml) for 30 minutes on ice. Cells were intensively washed, then cultured at 37°C 5% CO_2

from 0 minutes to 2 hours. Cells were stained with goat anti- human IgG (H+L)-AF488 (Invitrogen) and analyzed for the remaining surface 3C12C antibody level by flow cytometry. The geometric mean fluorescence (MFI) was analyzed using FlowJoV9&10 software (TreeStar). The remaining level of 3C12C on the cell surface was calculated as percentage of MFI at time 0.

Table S1: CD83 staining details in 35 HL patients:

Patient	Diagnosis	Stage	CD83 staining (Strong/Moderate/Weak)	% CD83+ HRS cells	CD30 staining	EBV (EBER)	Treatment response
1	NS	II	W	80	+	-	CR
2	NS	II	S	90	+	-	CR
3	MC	II	W	10	+	-	CR
4	MC	IV	W	70	+	-	CR
5	MC	II	S	80	+	+	CR
6	NS	II	M	70	+	-	CR
7	NS	II	M	70	+	-	NA
8	MC	II	S	20	+	-	CR
9	NS	II	S	<10	+	-	CR
10	NLP	II	S	70	+	-	CR
11	NLP	I	W	30	+	-	CR
12	CHL-U	III	S	90	+	+	CR
13	NS	II	S	80	+	-	CR
14	NS	IV	W	10	+	-	Relapsed -CR
15	MC	IV	W	<10	+	-	CR
16	MC	II	M	60	+	-	CR
17	NS	I	S	90	+	-	CR
18	NS	II	S	10	+	+	CR
19	LR	II	S	90	+	-	CR
20	NLP	I	S	>90	+	-	CR
21	NS	III	S	90	+	-	CR
22	NS	II	S	20	+	-	CR
23	NS	IV	W	<10	+	-	CR
24	NS	IV	W	80	+	+	CR
25	NS	II	W	10	+	-	CR
26	NS	II	M	<10	+	-	CR
27	MC	IV	S	20	+	+	Died
28	NS	III	M	<10	+	+	CR
29	NS	II	S	40	+	-	Died
30	NS	III	W	10	+	-	CR
31	NLP	IV	S	80	+	-	Relapsed
32	NS	II	S	>90	+	-	CR
33	NS	IIa	S	>90	+	-	Relapsed, CR after auto-HCT
34	cHL-U	IVb	M	40	+	+	CR
35	NS	IIIb	W	<10	+	-	CR

Table S2: Characteristics of 6 HL patients for analysis serum sCD83 level

Patient	Age	Diagnosis	Stage	Treatment	PET-CT
1	69	MC	IV	CHIVPP (cy1, 2, 3)	Post-cy2 (CR)
2	42	NS	III	ABVD (cy1, 2, 3, 4)	Post-cy2 (CR)
3	34	NS	II	ABVD (cy1, 2, 3, 4)	Post-cy2 (CR)
4	45	NS	III	ABVD (cy1, 2), BEACOPP (cy3, 4, 5, 6)	cy5 (PR), Post-cy2 (CR)
5	32	NS	II	ABVD (cy1, 2), BEACOPP (cy3, 4)	Post-cy2 (PD), Post-cy4 (PR)
6	36	NS	III	ABVD (cy1, 2, 3, 4, 5)	Post-cy2 (CR)

CR: complete response; PR: partial response; PD: progressive disease.

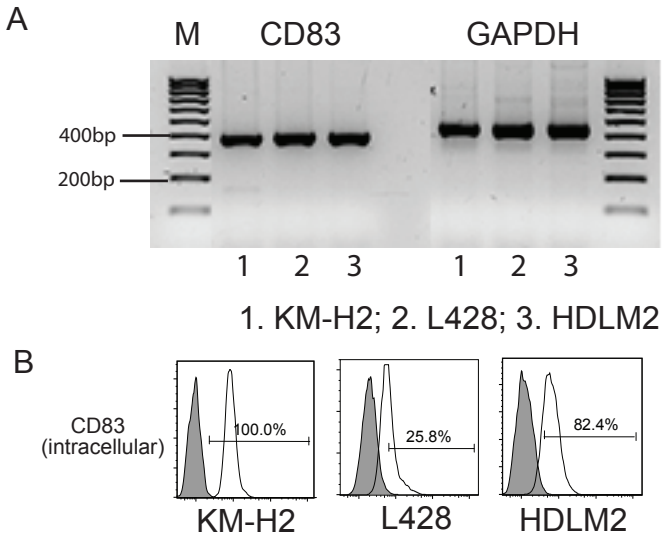

Fig. S1. CD83 transcript and intracellular expression in Hodgkin lymphoma cell lines.

(A) Total RNA was extracted from KM-H2, L428 and HDLM2 cells. cDNA was transcribed from 100 ng RNA. Expression of CD83mRNA and the house keeping gene GAPDH in KM-H2, L428 and HDLM2 were analysed by PCR. Amplified fragments were separated on 2% agarose gel. (B) KM-H2, L428 and HDLM2 cells were fixed and permeabilized, cells were stained with CD83 antibody (HB15e) and analysed for the intracellular CD83 expression by flow cytometry (n=3).

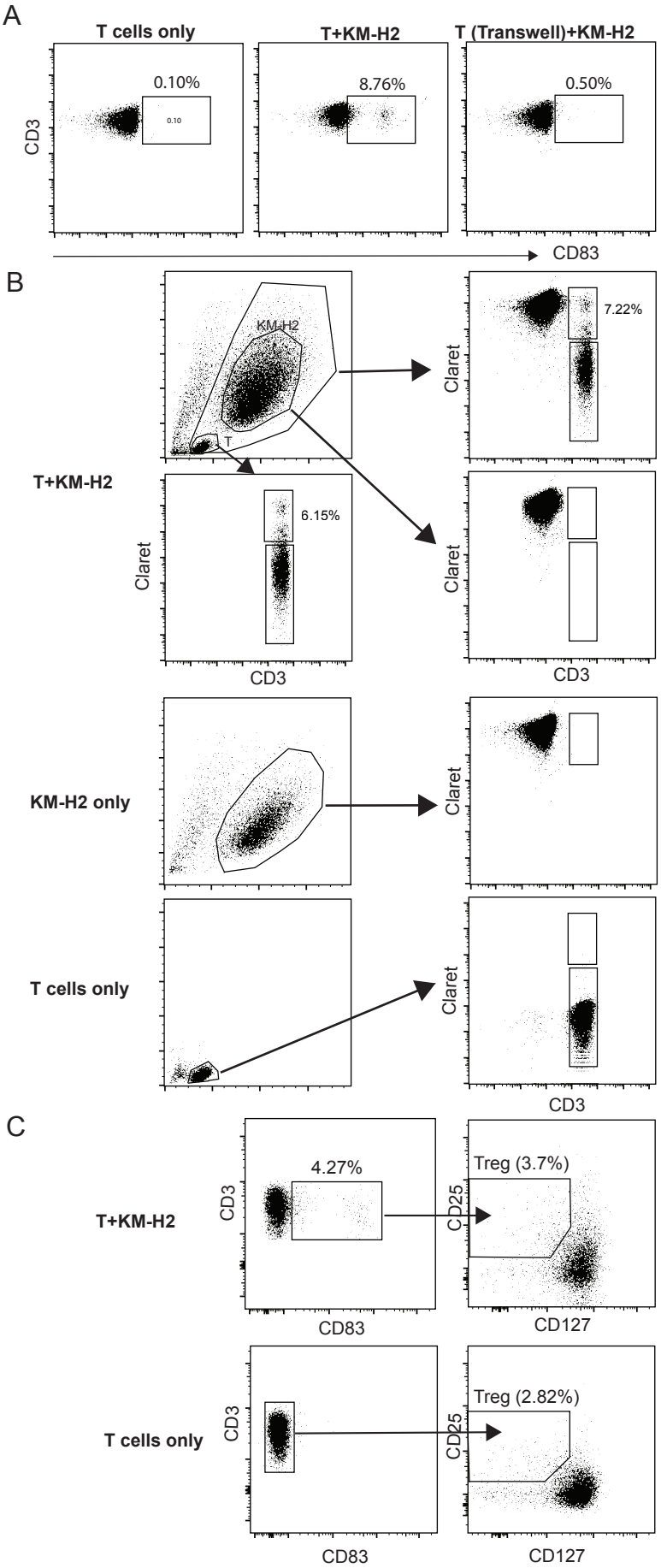

Fig. S2. Characterization the trogocytosis of Hodgkin and Reed-Sternberg (HRS) cells to T cells.

(A) T cells and KM-H2 cells were co-cultured with or without transwell, for 4 hours, CD83 expression on T cells was analyzed by flow cytometry, one of three reprsentative experiments shown. (B) KM-H2 cells were labelled with CellVue Claret and co-cultured with T cells at ratio of 5:1 for 4 hours. Claret expression were analysed on gated KM-H2, T cells or whole population. KM-H2 or T cells only used as control (n=3). (C) The proportion of $CD25^{high}CD127^{low}$ (Treg) in trogocytosed $CD83^{+}$ T cells were analysed, one of three representative experiments was shown.

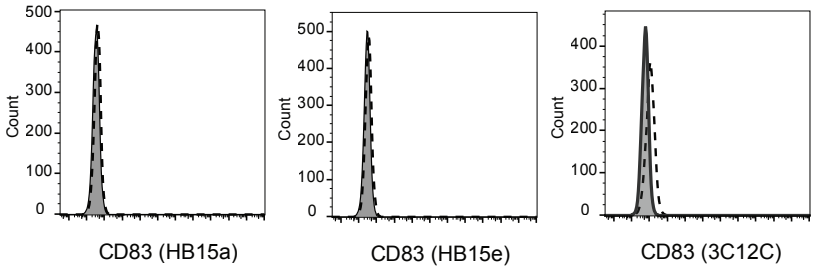

Fig. S3. CD83 expression on HL60 line.

CD83 expression on HL60 was analysed with mouse anti-human CD83 mAb HB15a, HB15e or human anti-human CD83 mAb 3C12C by flow cytometry. Grey filled histograms were isotype controls for CD83 antibodies.

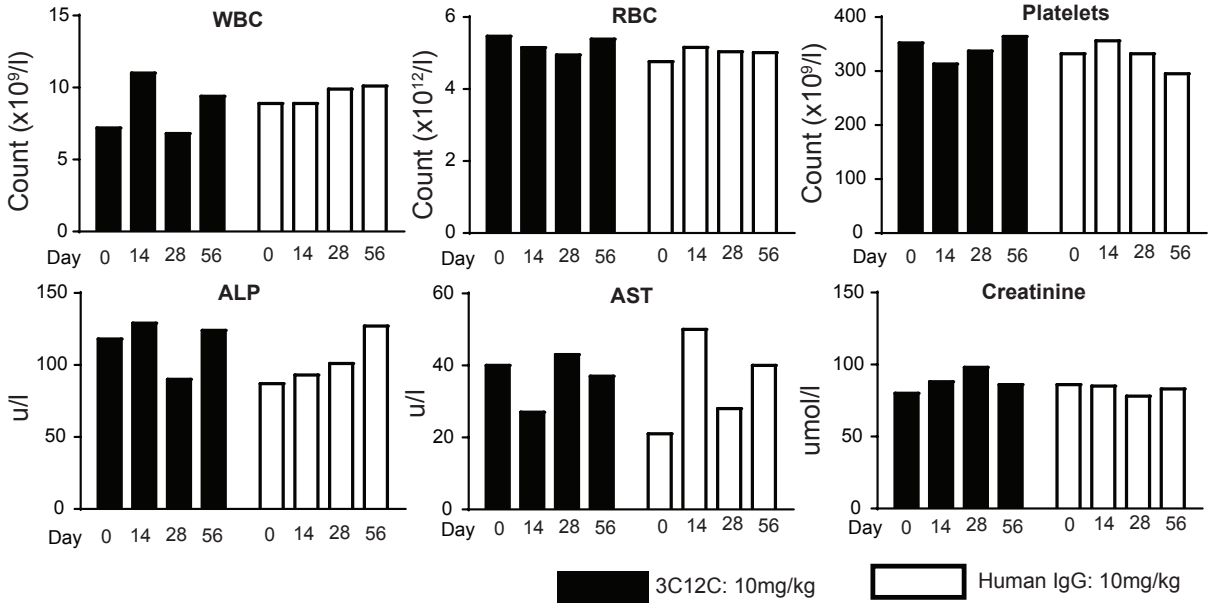

Fig. S4. 3C12C is safe in non-human primates.

Five non-human primates (Baboon) were injected with 3C12C (1, 5, 10, 10 mg/kg, n=4) or human IgG (10mg/kg, n=1) at day 0, 7, 14 and 21. Blood and serum samples were collected for blood cell counts (red cells, white cells and platelets), liver (ALP, AST level) and kidney function (Creatinine level) analysis. Data from the two animals receiving 10mg/kg of 3C12C or human IgG are shown.

Appendix C: Targeting CD83 in Mantle cell lymphoma with anti-human CD83 antibody

Li Z, Abadir E, Lee K, Clarke C, Bryant C, Cooper W, Pietersz G, Silveira PS, Hart DN, Ju X, Clark G. Targeting CD83 in Mantle cell lymphoma with anti-human CD83 antibody. Under Review (Clinical and Translational Immunology)

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Targeting CD83 in Mantle cell lymphoma with anti-human CD83 antibody

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Keywords:	CD83, Mantle cell lymphoma, Antibody-drug-conjugate, Immunotherapy < Immunology, NF-κB
Abstract:	**Objectives** Effective antibody-drug-conjugates (ADC) provide potent targeted cancer therapies. CD83 is expressed on activated immune cells including B cells and is a therapeutic target for Hodgkin lymphoma. Our objective was to determine CD83 expression on non-Hodgkin lymphoma (NHL) and its therapeutic potential to treat mantle cell lymphoma (MCL) which is currently an incurable NHL. **Methods** We analysed CD83 expression on MCL cell lines and the lymph node/bone marrow biopsies of MCL patients. We tested the killing effect of CD83 ADC in vitro and in an in vivo xenograft MCL mouse model. **Results** CD83 is expressed on MCL and CD83 ADC kills MCL in vitro and in vivo. The combination of CD83 ADC with Doxorubicin and Cyclophosphamide which are included in the current treatment regimen for MCL has synergistic killing effect of MCL. **Discussion** CD83 upregulation in MCL is corelated with the NF-kB activation. Doxorubicin and Cyclophosphamide enhance the NF-kB activity and increased CD83 expression on MCL cell lines **Conclusion** This study provides evidence that a novel immunotherapeutic agent

	CD83 ADC that, in combination with chemotherapy, has the potential to enhance the efficacy of current treatments for MCL.

Targeting CD83 in Mantle cell lymphoma with anti-human CD83 antibody

Running Title: Antibody target for mantle cell lymphoma

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Conflict of Interest: G.J.C. is a Director of DendroCyte BioTech Pty Ltd and Kira BioTech Pty Ltd. There are no other conflict of interests to disclose.

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Figures: 6

Table: 1

ABBREVIATIONS

ADC	Antibody Drug Conjugate
CP	Cyclophosphamide
DC	Dendritic Cell
DOX	Doxorubicin
HL	Hodgkin Lymphoma
MCL	Mantle Cell Lymphoma
MMAE	Monomethyl auristatin E
NHL	Non-Hodgkin Lymphoma

ABSTRACT

Objectives

Effective antibody-drug-conjugates (ADC) provide potent targeted cancer therapies. CD83 is expressed on activated immune cells including B cells and is a therapeutic target for Hodgkin lymphoma. Our objective was to determine CD83 expression on non-Hodgkin lymphoma (NHL) and its therapeutic potential to treat mantle cell lymphoma (MCL) which is currently an incurable NHL.

Methods

We analysed CD83 expression on MCL cell lines and the lymph node/bone marrow biopsies of MCL patients. We tested the killing effect of CD83 ADC *in vitro* and in an *in vivo* xenograft MCL mouse model.

Results

CD83 is expressed on MCL and CD83 ADC kills MCL *in vitro* and *in vivo*. The combination of CD83 ADC with Doxorubicin and Cyclophosphamide which are included in the current treatment regimen for MCL has synergistic killing effect of MCL.

Discussion

CD83 upregulation in MCL is corelated with the NF-κB activation. Doxorubicin and Cyclophosphamide enhance the NF-κB activity and increased CD83 expression on MCL cell lines

Conclusion

This study provides evidence that a novel immunotherapeutic agent CD83 ADC that, in combination with chemotherapy, has the potential to enhance the efficacy of current treatments for MCL.

Keywords: CD83; Mantle cell lymphoma; Antibody-drug-conjugate; NF-κB; Immunotherapy

INTRODUCTION

Mantle cell lymphoma (MCL) has an incidence of 0.5 per 100,000 in western countries and accounts for 2.5-6% of all non-Hodgkin lymphomas (NHL) (1, 2) Most MCL cases have an aggressive clinical course and develop resistance to treatment resulting in relapse after a variable period of remission. The median overall survival is 4-5 years (3).

The MCL cells originate from mature B-cells within the mantle zone of lymphatic follicles. The pathogenic hallmark and potential initiating oncogenic mutation is the up-regulation of Cyclin D1 (CCND1) which is predominantly due to the translocation of t (11;14) (q13; q32). In addition, other signalling molecules such as ataxia telangiectasia mutated, members of the nuclear factor κB (NF-κB) pathway or NOTCH family display constitutive activation in MCL (4-6).

Antibody drug conjugates (ADC), which combine monoclonal antibodies (mAbs) with small molecules or toxins have the advantage of highly specific killing and reduced toxicity compared with conventional chemotherapy (7). The recent approvals of ADC for the treatment of haematological malignancies, such as brentuximab vedotin and inotuzumab ozogamicin (8, 9), demonstrates high efficacy and low toxicity in the clinical practice (10, 11). New ADC in MCL including antibodies targeting novel antigens such as receptor tyrosine kinase-like Orphan Receptor 1 (ROR1) and novel payloads for already known targets, such as CD19 targeted therapy are under assessment in clinical trials (clinicaltrials.gov: NCT02669017, NCT02669017, NCT03424603).

CD83 is a transmembrane immunoglobulin superfamily member with a regulatory function on lymphocyte maturation, activation and homeostasis (12-14) in the immune system. CD83 is expressed by mature dendritic cells and activated lymphocytes but not by other peripheral blood cells (11, 15). Some tumor cells have high expression of CD83 on the cell surface, such as Hodgkin lymphomas (HL), diffuse large B cell lymphoma, adult acute lymphoblastic leukemias, gastric extra-nodal marginal zone lymphomas and lung cancer cells (15-20). We identified CD83 as a potential therapeutic target in HL, in which an anti-CD83 antibody drug conjugate effectively killed $CD83^+$ HL cells (21). A soluble version of CD83 protein (sCD83) with immune suppressive function has been reported. Serum sCD83 concentration is increased in some haematology

malignancies but returns to normal in patients who responded to chemotherapy (22, 23). Whether CD83 can be a therapeutic target for MCL has not been investigated. In this study, we analyzed the CD83 expression on MCL and tested the ability of CD83 ADC to kill MCL *in vitro* and *in vivo*.

RESULTS

CD83 is expressed on some MCL cell lines and in lymph node/bone marrow biopsies of MCL patients

CD83 expression was analyzed on MCL cell lines. Mino cells and Rec-1 cells expressed the CD83 on their cell surface, whilst the Z138 and Jvm2 lines did not. The HL cell line KM-H2 expressed the highest amount of cell surface CD83 (Figure 1A). Cell surface CD83 was expressed on 30.3% of the $CD19^+/CD5^+$ population of PBMC from one primary MCL (MCL01) patient (Figure 1B). As expected, CD83 mRNA transcripts was more abundant in $CD83^+$ MCL lines (Mino and Rec-1) than $CD83^-$ MCL cells (Z138 and Jvm2). Similarly, primary MCL cells had increased CD83 mRNA transcription (Figure 1C).

CD83 expression was analyzed on the FFPE lymph node biopsies from 18 MCL patients. Patient clinical characteristics are detailed in Table 1. The average age of patients was 65.7 years and most samples were from men (16/18, 88.9%). 83.3% (15/18) of the patients were assessed to be stage IV based on Ann Arbor Stage criteria (24). The Average Mantle Cell Lymphoma International Prognostic Index (MIPI) scores of high risk, intermediate or low risk were 55.6%, 22.2% and 22.2%, respectively. Among the 18 patients, 3/18 (16.67 %) biopsies expressed high levels of CD83 (>50% positive) on the MCL cells, 8/18 (44.44%) expressed moderate levels (10-50% positive), and 6/18 (33.33%) expressed low levels of CD83 (<10% positive) and 1/18 (5.55%) had no CD83 detection (Figure 1D). There is no correlation of CD83 expression level (cut-off 10% as low/high) with MIPI score or clinical stage.

Anti-CD83 antibody drug conjugate kills $CD83^+$ MCL cells *in vitro*

The CD83-MMAE drug activity was tested on four MCL lines (Mino, Rec-1, Z138 and Jvm2). $CD83^+$ HL cell line KM-H2 was included as control. Cells were exposed to 3C12C-MMAE for 72 hours, then the metabolic-based luciferase assay CellTitre-Glo was used to quantify viable cells. 3C12C-MMAE killed MCL $CD83^+$ cells and Mino cells efficiently. Although Mino cells expresses

less cell surface CD83 than KM-H2 cells, the IC50 level of both cell lines is similar (0.017 and 0.021μg/ml for KM-H2 and Mino respectively. Figure 1A). Both the $CD83^+$ Mino and Rec-1 cells were susceptible to naked 3C12C via NK mediated ADCC in dose dependent manner (Suppl Figure 1). Rec-1 had a higher IC50 level (>10μg/ml) when exposed to 3C12C-MMAE than Mino cells. Rec-1 MMAE resistance was confirmed when MCL lines were cultured with MMAE alone (Suppl. Figure 2). Since MMAE conjugated antibody has been reported to induce G2/M phase arrest (25), we tested the effect of 3C12C-MMAE on the cell cycle of $CD83^+$ Mino cells. About 45% Mino cells were arrested in G2/M phase after 18- hour exposure to 3C12C-MMAE and this was accompanied with a concomitant decrease in G0/G1 phase cells. The control ADC Herceptin-MMAE did not change the cell cycle of Mino cells (Figure 2B).

Anti-CD83 antibody drug conjugate effectively kills MCL in a xenograft model

To evaluate the efficacy of our ADC *in vivo*, we established a xenograft mouse model in which NSG mice were subcutaneously injected with Mino cells (Figure 3A). On day 18 post injection of Mino cells when tumors were palpable, 3C12C-MMAE or control vehicle was injected (i.p.). Mice were sacrificed and engrafted tumor cells were harvested for flow cytometry analysis. Engrafted tumors were $CD5^+CD19^+$ and expressed CD83 at similar level to the *in vitro* cultured Mino cells (Suppl. Figure 3). 3C12C-MMAE inhibited the tumor growth and increased the survival in tumor engrafted mice compared with control antibody-MMAE conjugate (Figure 3B&3C).

CD83 upregulation correlates with NF-κB activation in MCL

The *CD83* promoter contains NF-κB binding sites. Activation of NK-κB in normal B-cells and some B-cell malignancies induces CD83 expression (26, 27). It has been reported that the canonical NF-κB pathway is activated in some MCL cell lines and primary samples (5, 28). To reveal the potential relationship between CD83 expression and NF-κB activation status in MCL, we extracted cytosol and nuclear protein from MCL cell lines and analyzed NF-κB activation by western blot. Although activation of NF-κB in both $CD83^+$ and $CD83^-$MCL lines was detected, $CD83^+$ MCL cells, Mino and Rec-1, showed elevated p50 and RelA in the nuclear fraction, indicating strong canonical NF-κB pathway activation. In $CD83^-$ cell lines, p52 and RelB levels were high in the cytosol and nuclear fractions indicating non-canonical NF-κB pathway activation (Figure 4A). The primary MCL PBMC cell lysate (MCL01) had a similar canonical NF-κB

pathway activation pattern to Mino cells (Figure 4B). We then exposed $CD83^+$ cells to the canonical NF-κB pathway inhibitor, BAY11-7082. CD83 mRNA transcripts were reduced in both Mino and Rec-1 cells exposed to 1.25μM BAY11-7082 for 18 hours (Figure 4C). CD83 cell surface protein was also reduced by canonical NF-κB inhibitors (Figure 4D).

CD83 ADC has synergic killing effect with anthracyclines and alkylating agents

NF-κB activation can be induced in some malignancies by cytotoxic medications such as Doxorubicin (DOX) (29). Mino and Z138 cells were cultured with drugs included in standard chemotherapy regimens and novel treatment agents (Suppl. Table). Both canonical (p105/p50, RelA) and non-canonical NF-κB (P100/p52 and RelB) was increased in $CD83^+$ Mino cells, especially in the nuclear fractions, after treatment with DOX or CP. $CD83^-$ Z138 cells were more sensitive to death from exposure to DOX and CP and upregulated NF-κB molecules after short culture with DOX or CP (Figure 5 and Suppl. Figure 4). Furthermore, DOX and CP upregulated CD83 on $CD83^+$ Mino and $CD83^-$ Z138 cells (Figure 6A-B) whilst other chemotherapy drugs had no effect on CD83 expression (Suppl. Table). This renders the possibility of a synergistic interaction between CD83-MMAE with some chemotherapies. MCL cells were cultured with 3C12C-MMAE, DOX, CP alone or in combination. A combination index (CI) showed there was synergistic killing effect of 3C12C-MMAE and DOX on Mino and Z138 cells (Figure 6C).

DISCUSSION

CD83 expression has been reported in some lymphomas and leukemias. We showed here that CD83 is upregulated in 50% of primary MCL samples and some MCL cell lines. Interestingly, the immunohistochemical staining of primary MCL cells showed a pattern of heterogeneous expression, which reflects the heterogeneity of sub-clonal populations within the MCL tumor tissue, which has been reported by others (30). We observed CD83 upregulation by DOX and CP in MCL which possibly results from NF-κB activation induced by the DNA damage-independent stress from chemotherapy (31-33).

We tested anti-CD83 Ab as a single ADC agent in the treatment of $CD83^+$ MCL *in vitro* and in a xenogeneic mouse model. Interestingly, though CD83 expression in Mino cells is not as high as that on the classical Hodgkin lymphoma cells, KM-H2, they have a similar sensitivity to the anti-CD83 ADC. This could be the hyper-sensitivity of Mino cells to toxin MMAE and/or fast internalization of anti-CD83 Ab by MCL cells. Similar phenomena has been observed in ADC targeting cells with low antigen expression (7). Kovtun et al found that conjugates of ADC drug killed not only the target antigen-positive cells but also the neighboring antigen-negative cells which depends on the nature of the reducible disulfide bond linker and the release of the payload into adjacent antigen negative cells (34). An important factor that effects naked therapeutic antibody efficacy is the expression level of targeted antigen. ADCs have shown to be more effective than naked antibodies over a wider range of antigen expression levels (7). For example, while CD33-positive acute myelogenous leukemia tumors express relatively low levels of target antigen (5,000–10,000 receptors per cell), an ADC that targets the CD33 antigen still shows meaningful clinical response rates.(35). Although $CD83^+$ Rec-1 cells could be killed by CD83 antibody via ADCC, similar to KM-H2 and Mino cells (Figure 1S), they are not sensitive to 3C12C-MMAE which is likely due to their resistance of MMAE (36).

Current treatment of younger patients with MCL often includes DOX and CP. Given these drugs were capable of inducing CD83 expression in MCL, and our data showing the synergistic effect of anti-CD83 ADC with a conventional chemotherapy (DOX and CP), these patients may benefit from combination regimens of chemotherapy and anti-CD83 ADC.

MCL is a group of matured B cell lymphomas comprising two possible molecular pathogenesis. The pre-malignant B cells within the classic and most common subgroup of MCL originate from mantle zone and do not enter germinal centers. This subgroup does not have hypermutated Ig but are SOX11^{+}. The pre-malignant cells of the minor subgroup enter into the germinal center. These cells have hypermutated Ig and are SOX11^{-} (37). From limited sample size, we could not determine the correlation between CD83 and SOX11 expression on MCL. The CD83 gene promoter contains NF-κB binding sites (26, 38). Several types of lymphomas, such as HL, diffuse larger B cell lymphoma and chronic lymphocytic lymphoma, which have been reported as CD83^{+}, also showed NF-κB activation as a hallmark in their pathogenesis (39-42). MCL depends on NF-κB signalling for continued growth and proliferation and showed distinct NF-κB activation (5, 6). Our results showed that CD83 expression correlated with canonical NF-κB activation in MCL.

Future direction of targeting CD83 on MCL patients requires identification of CD83^{+} MCL patients, application of the most recent ADC techniques to generate CD83 ADC and the combination of ADC with current chemotherapy. In addition, targeting multiple pathways including NF-κB pathway potentially overcomes the resistance of targeting a single pathway to treat MCL. In conclusion, our research demonstrated CD83 expression in MCL cell lines and primary tumor cells and showed the anti-CD83 ADC is a possible therapy for CD83^{+} MCL treatment.

METHODS

Patient sample collection

MCL patient samples were collected with informed consent approved by the Sydney Local Health District Human Research Ethics Committee (X15-0464&LNR15/RPAH/615), consistent with the declaration of Helsinki. Archival formalin fixed paraffin embedded (FFPE) lymph node biopsies were obtained from 18 MCL patients at initial diagnosis (Table 1). Fresh peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque (GE Healthcare) density gradient from one MCL patient's sample (MCL01) collected at initial diagnosis.

Human anti-CD83 antibody and antibody drug conjugation

Human anti-CD83 antibody 3C12C and 3C12C-Monomethyl auristatin E (MMAE) conjugate were produced in house as described (21, 43).

Cell culture

MCL cell lines (Mino, Rec-1, Jvm2, Z138, purchased from ATCC), HL cell line KM-H2 (gift from Prof Volker Diehl, University of Cologne, Germany) and the Erythro-Leukemia cell line HEL (Purchased from ATCC) were cultured in complete RPMI-1640 medium containing 10% human fetal calf serum, 2mM glutaMAX™, 100U/ml penicillin, 100µg/ml streptomycin, 1mM sodium pyruvate,10mM HEPES, 10µM β Mercaptoethanol (Thermo Fisher Scientific) at 37°C, in 5% CO_2. BAY11-7082 (Sigma-Aldrich), Doxorubicin (DOX, DBL Pharmaceuticals), cyclophosphamide (CP, Slade Health) were added to cell cultures as described.

Immunohistochemical staining

Immunohistochemical staining was performed on 3µm sections of FFPE biopsy tissue of lymph node or bone marrow from MCL patients as described previously (21, 43). The primary CD83 antibody used was the F5 clone (Santa Cruz Biotechnology). Staining was performed on a Leica Bond III Autostainer (Leica Biosystems) using a Bond Polymer Refine Detection kit for visualization with 3,3'-diaminobenzidine (DAB).

Flow cytometry

The following antibodies were used: anti-human CD5-APC (BD Biosciences, clone L17F12), anti-human CD19-PE (BD Biosciences, clone HIB19), anti-human CD45-AF488 (BioLegend, clone HI30), anti-mouse CD45-PerCP Cy5.5 (BD Biosciences, clone 30-F11), anti-human CD83-FITC (Beckman and Coulter, clone HB15a) or 3C12C-FITC (made in house) (44). Data was collected using a Fortessa X-20 or Accuri C6 (BD Biosciences) flow cytometer and the data analyzed with FlowJo software (TreeStar).

Cell viability assays and cell cycle measurement

Cells (5000 per well) were cultured for 72 hours in 200µl complete RPMI-1640 medium containing various concentrations of 3C12C-MMAE, DOX, CP alone or combined. The cell viability was analyzed with the CellTiter-Glo Luminescent Cell viability kit according to the

manufacturer's instruction (Promega) and half maximal inhibitory concentration (IC50) was calculated. For cell cycle measurement, cells were cultured in complete RPMI-1640 medium containing 3C12C, 3C12C-MMAE, or irrelevant Ig-MMAE (Herceptin-MMAE conjugated with the same chemistry as 3C12C-MMAE in house) for the indicated times and then cells were fixed in 70% cold ethanol for 2 hours. Cells were stained with propidium iodide (PI) in the presence of 0.2mg/ml DNase-free RNase A (Sigma-Aldrich) and analyzed by flow cytometry.

Western blot

MCL cell lines and cells from one primary MCL sample treated with or without DOX (0.2μg/ml), CP (0.2mg/ml) for different time points were lysed and the nuclear and cytoplasmic fractions were isolated using NE-PER kit (Thermo Fisher Scientific) according to the manufacturer's instruction. The protein concentration of each fraction was measured using a BCA protein assay reagent kit (Thermo Fisher Scientific). 5-10 μg of protein were separated by SDS-PAGE through a 4-12% Bis-Tris Plus gel (Thermo Fisher Scientific) and transferred onto nitrocellulose membranes using an Iblot blotting system (Thermo Fisher Scientific). Following blocking with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 2 hours at room temperature and washing with 0.1% Tween 20 TBS (TTBS), the membranes were incubated overnight at 4 °C with primary antibody in TTBS containing 5% BSA. The primary antibodies included: rabbit anti-p105/p50 (ab32360:Abcam), mouse anti-p100/p52 (05-361:EMD Millipore), rabbit anti-p65 (8242S: Cell Signalling Technology), rabbit anti-RelB (ab18027: Abcam), rabbit anti-alpha-Tubulin (T6074, Sigma-Aldrich), mouse anti-human beta-actin (Bio-rad) and rabbit anti-human Histone (#9715: Cell Signalling Technology). Secondary anti-mouse or anti-rabbit antibody-horseradish peroxidase conjugate (1:3000 dilution) was incubated with membranes for 1 hour at room temperature and washed with TTBS. The blots were detected with the enhanced chemiluminescent (ECL) (Bio-Rad) reagents on Bio-Rad ChemiDoc MP imaging system (Gladesville, NSW Australia) according to the manufacturer's instructions. The relative protein expression level was analysed with Image Lab 4.1 Software (Bio-Rad).

MCL xenograft mouse tumor model

Five to six week female NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were purchased from Australian Bioresources and housed under specific pathogen-free conditions. Experimental

procedures were approved by the Sydney Local Health District (SLHD) animal welfare committee. Each mouse received $5x10^6$ Mino cells injected subcutaneously on right flank area. On day 18 post cell injection, mice were treated with a single dose of 3C12C-MMAE (2.5mg/kg), Herceptin-MMAE (2.5mg/kg) or saline only (i.p.). Mice were monitored for tumor growth and the tumor volume was measured by digital caliper (tumor volume=1/2(length × width^2)). The engrafted tumors were harvested, passed through a 100μm nylon cell strainer (BD Falcon) to prepare a single cell suspension, washed with PBS and analyzed by flow cytometry.

Statistical Analysis

Statistical analyses were performed using Prism 6.0 (GraphPad Software). Standard error of the mean is shown unless otherwise stated. Unpaired two-tailed student t-test or log-rank (mantel-cox) test were used as described. Differences with $p<0.05$ were considered significant. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. The combination index (CI) was analysed with CompuSyn (ComboSyn, Inc) (45).

AUTHOR CONTRIBUTIONS

Z.L. and X.J. designed, performed experiments, analyzed data and wrote the manuscript. D.N.J.H. and G.J.C. designed experiments, analyzed data and edited the manuscript. E.A., C.B. and W.C. recruited patients. K.L. and C.C. performed the immunohistochemistry staining and interpretation. G.P. designed 3C12C-MMAE and Herceptin-MMAE. P.A.S. guided mouse experiments and edited the manuscript.

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Table 1: Characteristics of 18 Mantle Cell Lymphoma patients

Age at enrolment (mean, range)	65.7±8.7 years
Sex: Male; n (%) Female; n (%)	 16（88.9%） 2 （11.1%）
Histologic subtype: Typical Blastoid	 16 (88.9%) 2 (11.1%)
Ann Arbor Stage I/III VI Unidentified	 2 (11.1%) 15 (83.3%) 1 (5.6%)
MIPI High risk Intermediate risk Unidentified	 10 (55.6%) 4 (22.2%) 4 (22.2%)
Medium survival after diagnosis, (years)	6 years

FIGURE LEGENDS

Figure 1. Mantle cell lymphoma (MCL) cells express of CD83

(A) CD83 expression on four MCL cell lines (Mino, Rec-1, Z138 and Jvm2) was analyzed by flow cytometry with human anti-CD83 antibody 3C12C-FITC (n=4). (B) One primary blastoid non-nodal MCL patient (MCL01) blood sample was stained with CD19-V450, CD5-APC and 3C12C-FITC and analyzed by flow cytometry for the CD83 expression on $CD19^+/CD5^+$ cells. (C) Quantitative real time PCR analysis of CD83 transcripts in MCL cell lines and MCL primary samples. Data were normalized by the amount of HPRT cDNA and calculated as fold change compared with CD83 level in Minami-1 and PBMC from healthy donors, respectively. (D) FFPE biopsies from 18 primary MCL patients were stained with anti-CD83 antibody. Percentage of CD83 positive cells is shown.

Figure 2. 3C12C conjugation with monomethyl auristatin E (3C12C-MMAE) kill Mantle Cell Lymphoma (MCL) lines in vitro

(A) $CD83^+$ KM-H2, Mino, Rec-1 or $CD83^-$ Z138, Jvm2 cells were cultured with different concentrations of 3C12C-MMAE for 72 hours before determining viable cells by CellTiter-Glo Luminescent Cell Viability assay. Data from one representative experiment (left) and the mean± SEM of half maximal inhibitory concentration (IC50) from three experiments (right) were shown. (B) Mino cells were cultured in 3C12C-MMAE (antibody concentration of 0.2µg/ml) for 18 hours, then cells were fixed for cell cycle analysis by flow cytometry. Data from one representative experiment (upper panel) and the mean ± SEM of three experiment data (bottom panel) is shown.

Figure 3. CD83 ADC kills MCL in xenograft mice

(A) Schematic design of the MCL xenograft NSG mouse model. (B) Quantification of tumor volume from MCL engrafted mice treated with 3C12C-MMAE, isotype control-MMAE or saline is shown (n =6 mice per group). One of three representative experiments is shown. (C) Kaplan-Meier survival curves of mice implanted with Mino cells (n =6 mice per group). Data was analyzed with log-rank test. ***: $p<0.001$. One of three representative experiments is shown.

Figure 4. Activation of NF-κB increases CD83 expression in MCL

(A) Western blot analysis of canonical pathway NF-κB proteins (p105/p50 and RelA) and non-canonical pathway NF-κB proteins (p100/p52 and RelB) levels in the cytosolic and nuclear extracts of CD83+ MCL cells (Mino and Rec-1) and CD83- MCL cells (Z138 and Jvm2). (B). Western blot analysis of canonical NF-κB proteins (p105/p50 and RelA) and non-canonical NF-κB proteins (p100/p52 and RelB) levels in the cytosolic and nuclear extracts of CD83+ MCL cells (Mino) and primary MCL cells (MCL01). (C) $CD83^+$ cells were treated with either a DMSO control or canonical NF-κB inhibitor BAY-11-7082 at different concentrations (0.25μM or 1.25μM) for 4 or 24 hrs. Real-time PCR (qPCR) analyses of CD83 cDNA from Mino (left) and Rec-1 (right) cells is shown. *: $p<0.05$. (D) $CD83^+$ cells were treated with either a DMSO control or BAY-11-7082 (0.25μM) for 24 hrs. Cell surface CD83 expression on Mino (left) and Rec-1 (right) was analysed by flow cytometry and mean fluorescence intensity (MFI) normalized to the untreated samples were shown (n=3). *: $P<0.05$; **: $p<0.01$. Data from one representative experiment is shown in (E). Filled histogram: isotype control. Open histogram with solid line: CD83 staining on cells treated with DMSO. Open histogram with dash lines: cells treated with BAY-11-7082 (0.25μM).

Figure 5. Doxorubicin and Cyclophosphamide activated NF-κB in MCL lines

Mino or Z138 cells were cultured in the presence of DOX (0.2ug/ml) or CP (0.5mg/ml) for 30 minutes, 2 hours, 6 hours and 24 hours. Cytoplasmic and nuclear proteins were isolated. Immunoblot analysis of cell lysate was performed with anti-NF-κB antibodies.

Figure 6. 3C12C ADC synergized with Doxorubicin/Cyclophosphamide

Mino or Z138 cells were cultured in the presence of DOX, (0.02μg/ml) or CP (0.2mg/ml) for 48 hours, cell surface CD83 expression were analyzed by flow cytometry. Mean fluorescent intensity ± SEM of three experiments were shown, (A) and one representative data (B) was shown. (C) Mino or Z138 cells were cultured with serial diluted 3C12C-MMAE (0.176, 0.088, 0.044, 0.022, 0.011, 0.0055, 0.00275 μg/ml), DOX (0.064, 0.032, 0.016, 0.008, 0.004, 0.002, 0.001 μg/ml), CP (1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025 μg/ml) or the combination of 3C12C-MMAE/DOX (0.176/0.064, 0.088/0.032, 0.044/0.016, 0.022/0.008, 0.011/0.004, 0.0055/0.002, 0.00275/0.001 μg/ml), the combination of 3C12C-MMAE/CP (0.176/1.6, 0.088/0.8, 0.044/0.4, 0.022,/0.2 0.011/0.1, 0.0055/0.05, 0.00275/0.025 μg/ml for 72 hrs. CellTitre-Glo Luminescent cell viability assay was used to determine the killing effect. Data was from one of three independent experiments. Combination index (CI) was analyzed with CompuSyn.

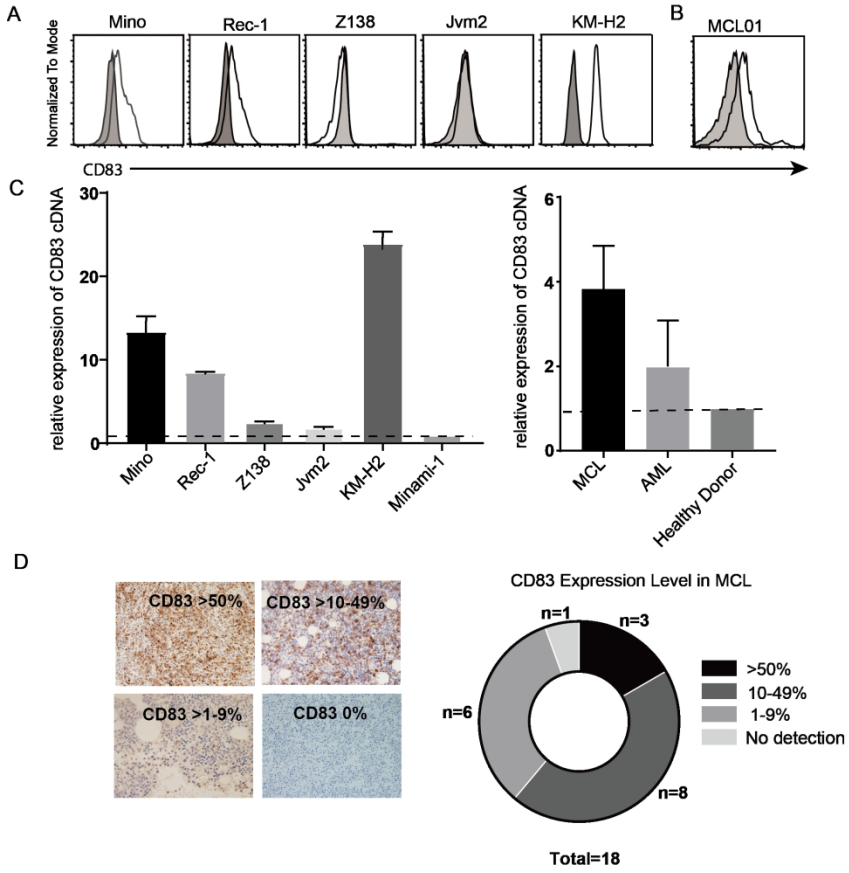

Figure 1

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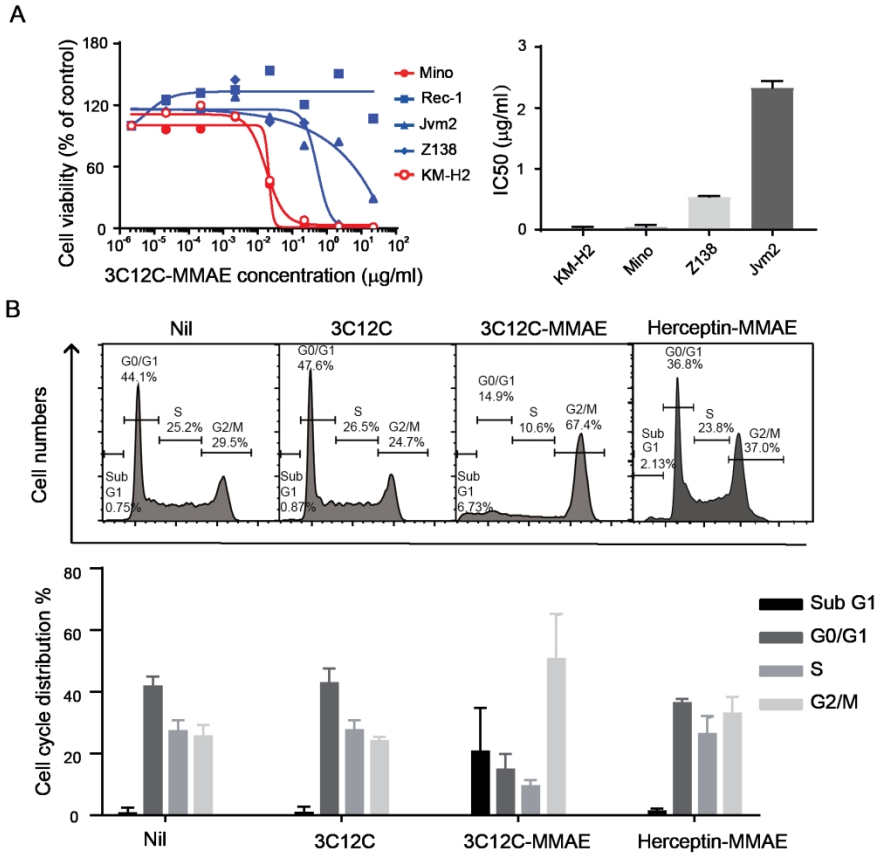

Figure 2

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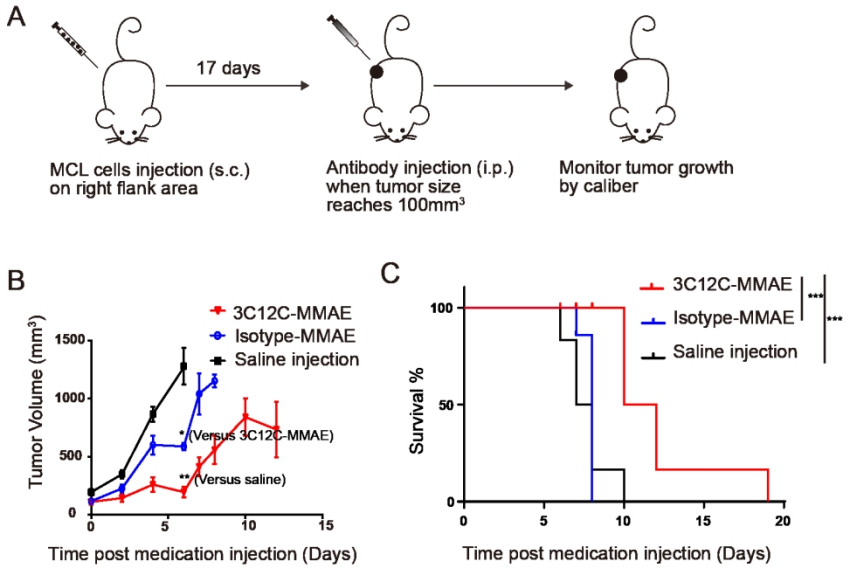

Figure 3

Figure 3. CD83 ADC kills MCL in xenograft mice
(A) Schematic design of the MCL xenograft NSG mouse model. (B) Quantification of tumor volume from MCL engrafted mice treated with 3C12C-MMAE, isotype control-MMAE or saline is shown (n =6 mice per group). One of three representative experiments is shown. (C) Kaplan-Meier survival curves of mice implanted with Mino cells (n =6 mice per group). Data was analyzed with log-rank test. ***: $p<0.001$. One of three representative experiments is shown.

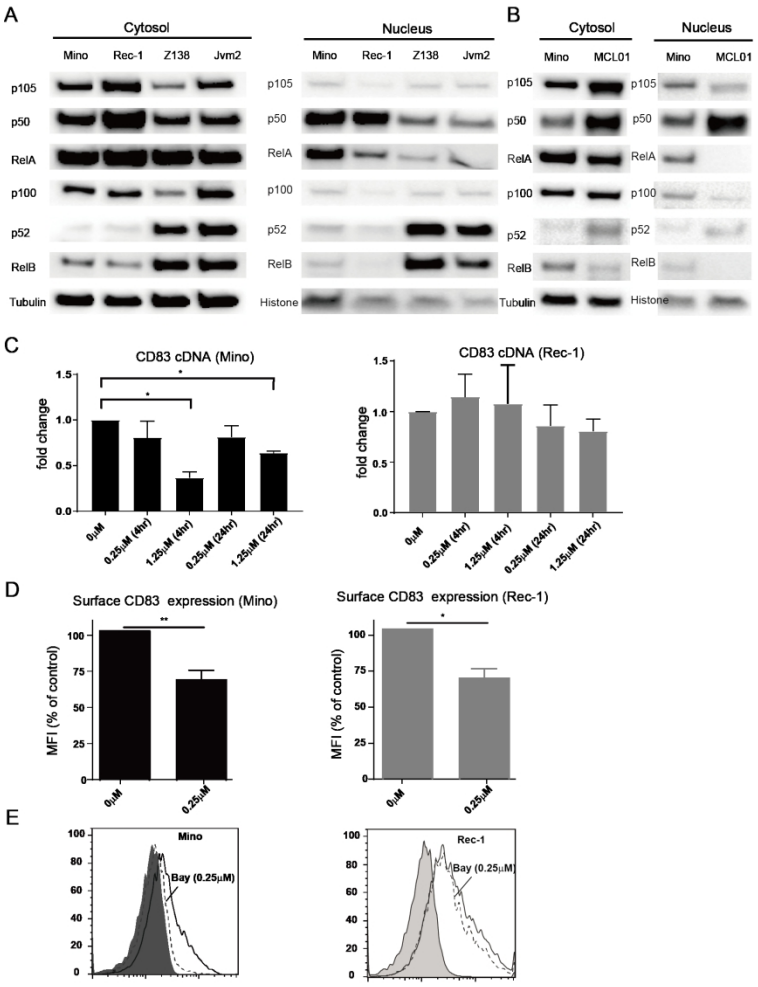

Figure 4

Figure 4. Activation of NF-kB increases CD83 expression in MCL

(A) Western blot analysis of canonical pathway NF-kB proteins (p105/p50 and RelA) and non-canonical pathway NF-kB proteins (p100/p52 and RelB) levels in the cytosolic and nuclear extracts of CD83+ MCL cells (Mino and Rec-1) and CD83- MCL cells (Z138 and Jvm2). (B). Western blot analysis of canonical NF-kB proteins (p105/p50 and RelA) and non-canonical NF-kB proteins (p100/p52 and RelB) levels in the cytosolic and nuclear extracts of CD83+ MCL cells (Mino) and primary MCL cells (MCL01). (C) CD83+ cells were treated with either a DMSO control or canonical NF-kB inhibitor BAY-11-7082 at different concentrations (0.25μM or 1.25μM) for 4 or 24 hrs. Real-time PCR (qPCR) analyses of CD83 cDNA from Mino (left) and Rec-1 (right) cells is shown. *: $p<0.05$. (D) CD83+ cells were treated with either a DMSO control or BAY-11-7082 (0.25μM) for 24 hrs. Cell surface CD83 expression on Mino (left) and Rec-1 (right) was analysed by flow cytometry and mean fluorescence intensity (MFI) normalized to the untreated samples were shown (n=3). *: $P<0.05$; **: $p<0.01$. Data from one representative experiment is shown in (E). Filled histogram: isotype control. Open histogram with solid line: CD83 staining on cells treated with DMSO. Open histogram with dash lines: cells treated with BAY-11-7082 (0.25μM).

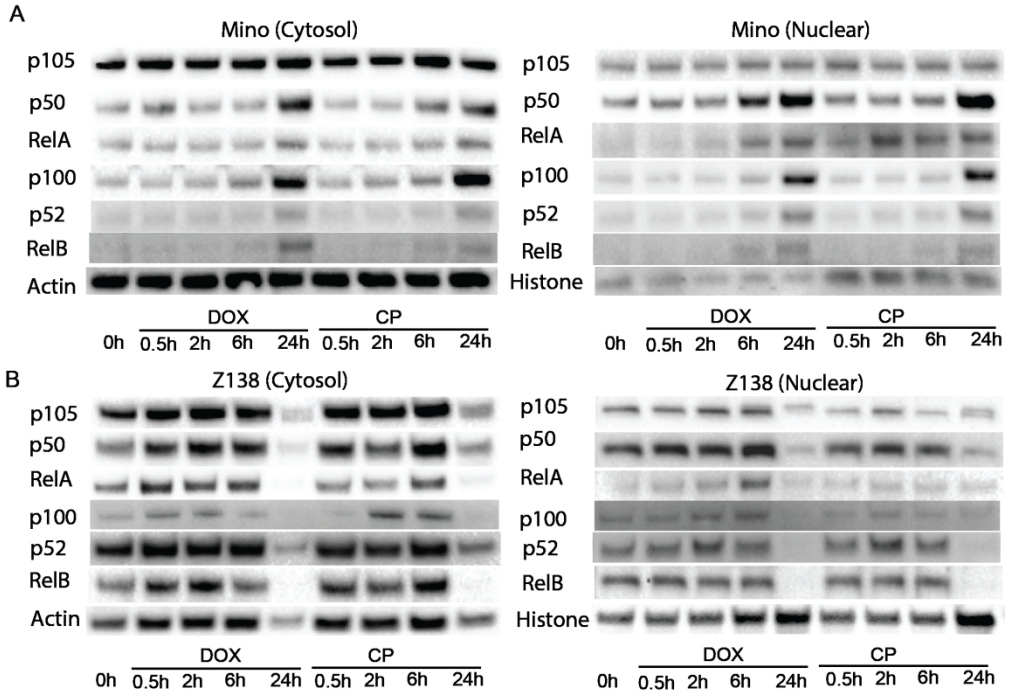

Figure 5

Figure 5. Doxorubicin and Cyclophosphamide activated NF-kB MCL lines
Mino or Z138 cells were cultured in the presence of DOX (0.2ug/ml) or CP (0.5mg/ml) for 30 minutes, 2 hours, 6 hours and 24 hours. Cytoplasmic and nuclear proteins were isolated. Immunoblot analysis of cell lysate was performed with anti-NF-kB antibodies.

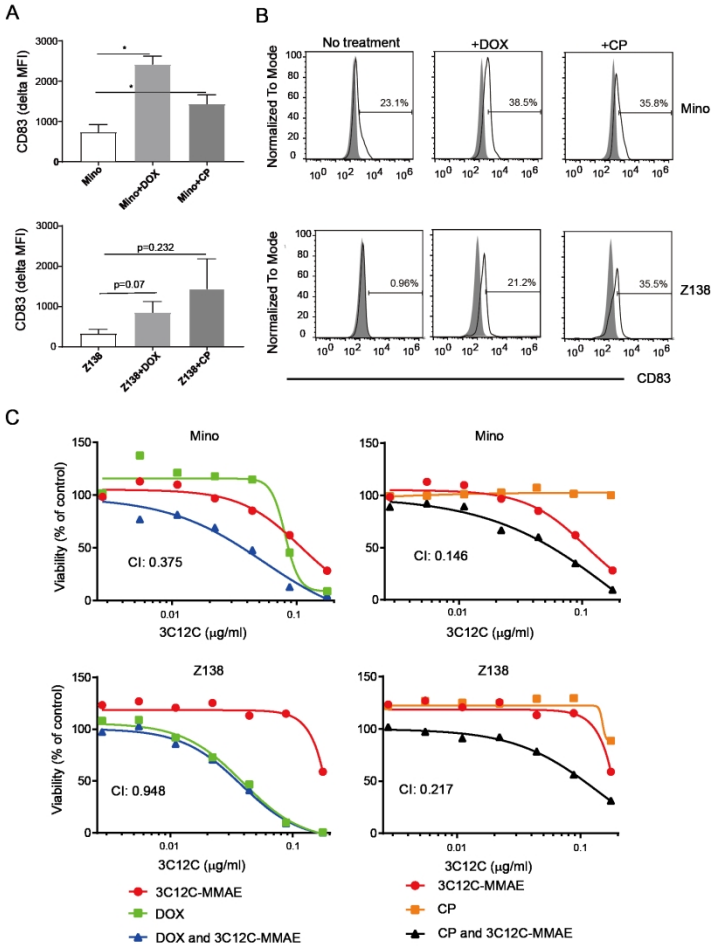

Figure 6

Figure 6. 3C12C ADC synergized with Doxorubicin/Cyclophosphamide
Mino or Z138 cells were cultured in the presence of DOX, (0.02μg/ml) or CP (0.2mg/ml) for 48 hours, cell surface CD83 expression were analyzed by flow cytometry. Mean fluorescent intensity ± SEM of three experiments were shown, (A) and one representative data (B) was shown. (C) Mino or Z138 cells were cultured with serial diluted 3C12C-MMAE (0.176, 0.088, 0.044, 0.022, 0.011, 0.0055, 0.00275 μg/ml), DOX (0.064, 0.032, 0.016, 0.008, 0.004, 0.002, 0.001 μg/ml), CP (1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025 μg/ml) or the combination of 3C12C-MMAE/DOX (0.176/0.064, 0.088/0.032, 0.044/0.016, 0.022/0.008, 0.011/0.004, 0.0055/0.002, 0.00275/0.001 μg/ml), the combination of 3C12C-MMAE/CP (0.176/1.6, 0.088/0.8, 0.044/0.4, 0.022,/0.2 0.011/0.1, 0.0055/0.05, 0.00275/0.025 μg/ml for 72 hrs. CellTitre-Glo Luminescent cell viability assay was used to determine the killing effect. Data was from one of three independent experiments. Combination index (CI) was analyzed with CompuSyn.

Table 1: Characteristics of mantle cell lymphoma patients

Age at enrolment (mean, range)	65.7±8.7 years
Sex: Male; n (%) Female; n (%)	 16（88.9%） 2 （11.1%）
Histologic subtype: Typical Blastoid	 16 (88.9%) 2 (11.1%)
Ann Arbor Stage I/III VI Unidentified	 2 (11.1%) 15 (83.3%) 1 (5.6%)
MIPI High risk Intermedium risk Unidentified	 10 (55.6%) 4 (22.2%) 4 (22.2%)
Medium survival after diagnosis	6 years