A blood dendritic cell vaccine for acute myeloid leukemia expands anti-tumor T cell responses at remission

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ABSTRACT

Only modest advances in AML therapy have occurred in the past decade and relapse due to residual disease remains the major challenge. The potential of the immune system to address this is evident in the success of allogeneic transplantation, however this leads to considerable morbidity. Dendritic cell (DC) vaccination can generate leukemia-specific autologous immunity with little toxicity. Promising results have been achieved with vaccines developed in vitro from purified monocytes (Mo-DC). We now demonstrate that blood DC (BDC) have superior function to Mo-DC. Whilst BDC are reduced at diagnosis in AML, they recover following chemotherapy and allogeneic transplantation, can be purified using CMRF-56 antibody technology, and can stimulate functional T cell responses. While most AML patients in remission had a relatively normal T cell landscape, those who had received fludarabine as salvage therapy have persistent T cell abnormalities including reduced number, altered subset distribution, failure to expand, and increased activation-induced cell death. Furthermore, PD-1 and TIM-3 are increased on CD4 T cells in AML patients in remission and their blockade enhances the expansion of leukemia-specific T cells. This confirms the feasibility of a BDC vaccine to consolidate remission in AML and suggests it should be tested in conjunction with checkpoint blockade.

INTRODUCTION

Acute myeloid leukemia (AML) is the most common adult acute leukemia. Intensive chemotherapy can induce complete remission (CR) but the majority of patients relapse. Removing residual disease after conventional chemotherapy is a major challenge. The potential of immune therapy to address this is demonstrated by the clinical efficacy of allogeneic haematopoietic cell transplant (ATx), which provides a graft-versus-leukemia (GVL) response through donor T cells. Natural anti-AML immunity is demonstrated by the success with checkpoint inhibitor therapy in elderly AML patients. Enhancing this T cell immunity is the goal of dendritic cell (DC) vaccination.

DC trials performed to date for AML have used “first generation” vaccines, predominantly composed of manufactured monocyte-derived DC (Mo-DC) resulting in detectable expansions of tumor antigen-specific T cell responses and some clinical efficacy. The use of pre-formed blood DC (BDC) as a tool for DC vaccination has begun to garner interest, with development of new methodologies for their isolation and reporting of landmark Phase I clinical trials. There are significant transcriptional and functional differences between BDC and Mo-DC. BDC present RNA encoded antigen more effectively than Mo-DC, stimulate better allogeneic T cell IFN-γ responses, induce stronger CD4 T cell proliferation and have superior migratory capacity. However, the rarity of BDC (<1% of leukocytes) has limited their use in clinical vaccines.

A mixture of BDC including CD1c+ and CD141+ myeloid DC (mDC), the most potent cross-presenting DC, can be purified in a single-step immune selection using a chimeric monoclonal antibody (mAb), hCMRF-56 mAb, and loaded with antigen to expand functional antigen-specific CD8+ T cells. We sought to determine if this approach were applicable in AML patients after chemotherapy or ATx, when disease burden is low and there is an opportunity to target residual disease after haematological recovery. We determined that BDC are deficient at diagnosis but return at CR, can be purified using hCMRF-56 mAb and drive the in vitro expansion of autologous viral and Wilms tumor 1 (WT1) specific T cell responses. We add that whilst the T cell landscape is not altered in AML patients after standard chemotherapy, patients who have received fludarabine show persistent abnormalities associated with an inability to respond to vaccination. Finally, we describe the expression of immune checkpoint molecules by T cells from...
AML patients in CR, providing a rationale to combine BDC vaccination with checkpoint blockade, and demonstrate that this can enhance BDC-induced tumor antigen-specific responses.

**Results**

**BDC are superior to Mo-DC at processing and cross-presenting long peptide antigen**

Cross-presentation of antigen is a fundamental function of DC as professional antigen presenting cells, presenting exogenous protein as peptide antigen in the context of HLA class I. We assessed the capacity of Mo-DC and CD1c+ mDC (as they are the most numerous DC in the CMRF-56 vaccine) to cross-present antigen. Both Mo-DC and CD1c+ mDC showed sustained presentation of the short, surface loaded FMP58 peptide, in the context of HLA class I after 16 hours of culture (Fig. 1A, B). CD1c+ mDC were able to present the peptide at significantly higher density than Mo-DC (p < 0.0001). Most notably, CD1c+ mDC had a strikingly increased capacity to cross-present a long FMP54–74 peptide into short FMP58–66 and A2 complexes at 16 hours compared with Mo-DC (Fig. 1A, B; p = 0.029).

**BDC are depleted at diagnosis but return in CR**

Markers used to identify BDC including HLA-DR, CD11c, CD123 and ILT3, are often present on leukemic blasts, and standard Lineage+ HLA-DR+ gating may be insufficient to identify BDC in patients with circulating blasts, resulting in over-estimation of BDC levels in AML patients at diagnosis.15-21 To investigate this, we sorted Lineage− HLA-DR− cells from three patients with circulating blasts and analysed their morphology. The great majority of the sorted cells were myeloblasts rather than typical mDC (Fig. 2A). Further examination of this population by flow cytometry revealed that these cells were CD45loCD34− (Fig. 2A). Further examination of this population by flow cytometry revealed that these cells were CD45loCD34− (Fig. 2A). The great majority of these cells were myeloblasts rather than typical mDC (Fig. 2A). We therefore developed a gating strategy to aid in the elimination of blasts from the BDC gate by including CD45, CD34 and CD304 and using strict definitions of BDC subsets (detailed in Figure S1B). Comparison of the previous gating strategy to our more stringent strategy, demonstrated contamination of the Lineage− HLA-DR− gate with myeloblasts accounting for the elevated “BDC” frequencies (Figure S1C).

Next we validated our gating strategy using ViSNE,22 which allowed us to directly compare healthy and patient BDC populations by visualising high dimensional data structures in two dimensions (Fig. 2B). All orthodox BDC subsets were identified in HD and AML CR patients but were reduced in active AML (Fig. 2B).

We then performed a Trucount analysis23 assessing AML patients (patient characteristics Table S1) at new diagnosis (ND, n = 8), relapse (RR, n = 6), in CR post-chemotherapy (CT, n = 18) or ≥12 months post-ATx (ATx, n = 7) along with age-matched healthy controls (HD, n = 15, Fig 2C). This revealed reduced numbers of CD1c+ mDC, CD141+ mDC and plasmacytoid (pDC) at diagnosis (p = 0.0001, p < 0.0001, p = 0.0013) and at relapse (p < 0.0001, p < 0.0001, p < 0.0001). Within 12 weeks following chemotherapy, CD1c+ mDC recovered and CD141+ mDC and pDC showed partial recovery (p = 0.02 and p = 0.009 respectively) when compared to HD. In four patients with AML-specific molecular abnormalities (NPM1 and FLT3-ITD), there was no detectable molecular disease in the peripheral blood when BDC recovered at CR, confirming that BDC do not contain the molecular marker and are independent of the leukaemic clone.

**CMRF-56+ BDC can be purified from AML patients in CR**

We confirmed that CMRF-56 antigen is not expressed by AML blasts and does not up-regulate following culture, in contrast to its up-regulation by BDC from HD or AML patients in CR (n = 4 Fig. 3A). Up-regulation of CMRF-56 antigen by CD1c+ mDC from AML CR patients (n = 18) was similar to age-matched HD (n = 14, p = 0.82, Fig. 3B). CD1c and CD141 BDC upregulated CMRF-56 similarly (Figure S2). This was accompanied by CD83 up-regulation with a trend to increased expression in the AML CR patients (p = 0.054, Fig. 3C).

After enrichment, CMRF-56 purity was 79.6+/- 3.9% and BDC purity was 28.2 p = 0.12 +/- 5.3% (n = 14) in AML CR patients, which was similar to HD (n = 31, p = 0.8) BDC purity (Fig. 3D-F). Importantly, this would generate adequate mDC numbers for therapeutic vaccination. As the gross yield of CMRF-56+ BDC from total PBMC was 0.7%, >5 x 10⁶ could be isolated from a leukapheresis collection of 1 x 10⁹ cells, sufficient for at least 5 vaccinations.

Following activation, CMRF-56+ BDC further up-regulated CD83 (78.5+/-2.5% at purification, 89.4+/-1.9% with

![Figure 1](image-url) Cross-Presentation of Long FMP Peptide by CD1c+ mDC and Mo-DC. Fresh, highly purified CD1c+ mDC and Mo-DC generated by 6 days in GM-CSF and IL-4 were pulsed with control (WT126-134), short (FMP58-66) or long (FMP54-74) peptides in equimolar amounts for 16 hrs. The presence of HLA-A2:FMP58-66 peptide complexes was detected by flow cytometry using a HLA-A2:FMP58-66-specific antibody. (A) A representative experiment is shown. B) Presentation FMP58-66 or FMP54-74 as HLA-A2:FMP58-66 peptide complexes were calculated as a delta MFI of HLA-A2:FMP58-66 FITC from control peptide (WT126-134) pulsed cells. CD1c+ mDC n = 5; Mo-DC n = 5; Two-way ANOVA, Fishers LSD test. *p < 0.05, **p < 0.0001.
activation, n = 3, p = 0.013) and displayed a mature phenotype with co-expression of CD80 (77+/−6.7%, n = 3) and CD40 (95.3+/−1.9%, n = 3, data not shown) as we have observed previously in HD.9

**T cells are competent in AML patients in CR, who have not received fludarabine**

To determine whether the T cell landscape will permit an active vaccination strategy in AML CR patients, we assessed T cell number, phenotype and function. Fludarabine is known to affect T cell number and function24 so we compared patients receiving fludarabine-containing regimens25 (FR) with those receiving non-fludarabine regimens (NFR), those post-ATx and age-matched HD.

The number of CD4+ and CD8+ T cells and subset distribution in NFR patients did not differ significantly from HD (Fig. 4A, B). However, FR treated patients had significantly reduced numbers of CD4+ T cells (p = 0.04, Fig. 4A) due to a loss of CD25hiFoxP3+ Treg (p = 0.03),
naive CD28\(^+\)CD45RO\(^-\) (p = 0.002) and memory CD28\(^+\)CD45RO\(^+\) (p = 0.002) CD4\(^+\) T cell subsets (Figure S3A). The deficit in CD4\(^+\) T cells with normal CD8\(^+\) numbers led to an abnormally low CD4:CD8 ratio (Fig. 4A). Despite their normal total CD8\(^+\) T cell counts, FR treated patients had an abnormal CD8\(^+\) T cell subset distribution with a significant reduction in the memory CD28\(^-\)CD45RO\(^+\) (p = 0.02) and trend to reduction in naïve CD28\(^-\)CD45RO\(^-\) (p = 0.07) subsets, resulting in a predominance of effector CD28\(^-\)CD45RO\(^-\) and terminally differentiated CD28\(^-\)CD45RO\(^-\)CD57\(^+\) CD8\(^+\) T cells (Fig. 4B). Patients in long-term CR post ATx had significantly elevated CD8\(^+\) T cell counts (p = 0.0013, Fig. 4A) owing to increased effector CD28\(^-\)CD45RO\(^-\) (p = 0.0011) and terminally differentiated CD28\(^-\)CD45RO\(^-\)CD57\(^+\) subsets (p = 0.01, Fig. 4B).

We performed broad phenotyping of TBET and EOMES by flow cytometry of CD8T cells, to gauge functional cytotoxic potential and determine significant differences between patient groups. T-BET was significantly elevated in NFR and ATx patients’ CD8\(^+\)CD28\(^+\)CD45RO\(^+\) effector T cells (p = 0.017, p = 0.014 respectively). ATx patients also had a trend to increased T-BET (p = 0.08) coupled with reduced PD-1 (p = 0.015) in the CD28\(^-\)CD45RO\(^-\)CD57\(^+\) (Figure S3C and data not shown). The co-expression of T-BET and EOMES by CD8\(^+\) T cells, showed similar patterns in both NFR and ATx patients compared to healthy donors, suggestive of a normal functional effector capacity (Figure S3B). Conversely, patients receiving FR, had significantly increased co-expression of TBET and EOMES in naïve CD8\(^+\) T cells and increased TBET expression in effector CD8\(^+\) T cells (Figure S3C).

An independent mass cytometry analysis of ATx patient PBMC confirmed these findings, demonstrating a subset imbalance favoring more mature, effector CD4\(^+\) and CD8\(^+\) T cells with high cytotoxic potential, an elevation of PD-1, particularly in the CD4\(^+\) T cells, and an activated T cell signature indicated by elevated Ki67, CCR5 and HLA-DR (Figure S4).

Next, we assessed the direct functional response of T cells during CR. Whilst CD4\(^+\) and CD8\(^+\) T cells from all patients produced IFN-gamma in response to stimulation with PMA and ionomycin, responses were higher in CD8\(^+\) T cells from FR and ATx patients than in HD (p = 0.014, p = 0.041 respectively, Fig. 4C). Stimulation with anti-CD2/CD3/CD28 beads resulted in IFN-gamma production by CD8\(^+\) T cells, but was accompanied by markedly increased activation induced cell death (AICD) in T cells from FR patients (p = 0.008, Fig. 4D) compared to HD.

**CMRF-56\(^+\) BDC from AML patients in CR expand functional autologous T cell responses**

CMRF-56\(^+\) BDC expand viral and tumor-specific T cell responses in HD and myeloma patients,\(^9,12,14\) but the function of BDC in AML patients at CR was unknown. We explored the ability of CMRF-56\(^+\) BDC prepared in CR after chemotherapy (NFR, n = 6 or FR, n = 3) and after ATx (n = 5), to expand T cell responses in a single in vitro stimulation to model viral antigens and Wilms tumour antigen (WT1), a model leukemia associated antigen. Anti-viral (FMP58,66 and/or CEF peptide pool) CD8\(^+\) T cell responses could be expanded by CMRF-56\(^+\) BDC purified from 5/6 NFR, 0/3 FR and 5/5 ATx patients (Fig. 5A, C). WT1-specific CD8\(^+\) precursor frequencies varied greatly across the AML CR cohort with NFR patients having very low or undetectable precursors (median 0.005% of CD8T cells, range 0–0.08%), whereas most ATx patients had larger precursor pools (median 0.064%, range 0.012–1.48%). WT1-specific (WT1\(_{126,134}\) WT1 peptivator) responses were expanded by CMRF-56\(^+\) BDC from 2/6 NFR, 0/3 FR and 4/5 ATx patients (Fig. 5B, C). None of the FR patients had

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**Figure 3.** Immune Selection with hCMRF-56 mAb Enriches BDC in AML Patients Achieving CR. (A) Representative histograms showing hCMRF-56 mAb staining of BDC (top panels) in a healthy donor (HD) and an AML patient in complete remission (AML CR) following 16 hours of culture (solid line) versus secondary only (dashed line) contrasted with primary AML peripheral blasts (bottom panel, 3 representative plots shown). (B) Collated data of CMRF-56 antigen up-regulation by CD1c\(^+\) mDC after 16 hours of culture detected by hCMRF-56 mAb in HD (n = 14) and AML CR (n = 18) and (C) of CD83 up-regulation (HD, n = 8, AML CR n = 14). (D) Representative scatter plots of hCMRF-56 staining of cultured PBMC from an AML CR patient pre- and post- hCMRF-56 immune selection. (E) Comparison of hCMRF-56 and (F) BDC purities achieved by hCMRF-56 immune selection in AML CR patients (n = 14) versus HD (n = 31). Mann-Whitney test, not significant.
detectable WT1-specific CD8+ precursors, expanded anti-WT1 responses or even anti-viral responses, and in 3/3 patients, >95% of their T cells died within 5 days of stimulation.

**Blocking highly expressed T cell checkpoint molecules enhances BDC vaccine responses**

Combining BDC vaccination for AML with T cell checkpoint blockade is an attractive therapeutic option, given that expression of TIM-3 and PD-1 on T cells post-ATx precedes relapse,26 dual TIM-3 and PD-1 blockade enhances survival in murine AML models,27 and the clinical success of checkpoint inhibitors in recent trials.1,28 We therefore examined PD-1 and TIM-3 expression on T cells in peripheral blood and bone marrow to determine the potential for blocking these checkpoints in AML at the clinically relevant time point of CR after chemotherapy or ATx. We analysed bone marrow of AML patients after NFR (n = 6) and FR (n = 3), and compared them to HD marrows (n = 3). The proportion of effector CD8+ T cells (CD28+CD45RO+CD57-) in the marrow of FR patients was increased in the marrows of NFR treated patients (p = 0.009) with a trend observed in NFR treated patients (p = 0.061, Figure S5C).
In the peripheral blood, PD-1 and TIM-3 were elevated on CD4^+ T cells, particularly the memory CD28^−CD45RO^+ population (data not shown) in the FR (PD-1 p = 0.0005, TIM-3 p = 0.001) and ATx (PD-1 p = 0.044, TIM-3 p = 0.027) treated patients (Fig. 6A, B). In the NFR group there was a trend to increased PD-1 expression by CD4^+ T cells (p = 0.06, Fig. 6A). Peripheral blood CD8^+ T cells showed no significant increase in PD-1 or TIM-3 expression (Fig. 6A, B). These findings were consistent with the CyTOF data from ATx patients (n = 4) compared to HD (n = 3, Figure S4). However, in the peripheral blood, PD-1 and TIM-3 were elevated on CD4^+ T cells, particularly the memory CD28^−CD45RO^+ population (data not shown) in the FR (PD-1 p = 0.0005, TIM-3 p = 0.001) and ATx (PD-1 p = 0.044, TIM-3 p = 0.027) treated patients (Fig. 6A, B). In the NFR group there was a trend to increased PD-1 expression by CD4^+ T cells (p = 0.06, Fig. 6A). Peripheral blood CD8^+ T cells showed no significant increase in PD-1 or TIM-3 expression (Fig. 6A, B). These findings were consistent with the CyTOF data from ATx patients (n = 4) compared to HD (n = 3, Figure S4).

Finally, the ability of checkpoint blockade to enhance WT1-specific T cell responses following CMRF-56^+ BDC stimulation was assessed. PD-1 blockade enhanced WT1-specific CD4^+ responses in 3/3 and CD8^+ responses in 2/3 ATx patients (Fig. 6C, G). The addition of anti-TIM-3 mAb to PD-1 blockade further enhanced the response in 1/3 patients. PD-1 and TIM-3 blockade was most effective in patient 11 who showed the highest PD-1 (Fig. 6D) and TIM-3 (Fig. 6E) expression on the CD4^+ T cells at the start of culture, and had no effect on patient 18, who did not up-regulate PD-1 on T cells in culture. BDC from all patients expressed similar levels of PD-L1 (Fig. 6F).

**Discussion**

Preparing a vaccine composed of pre-formed BDC from AML patients is a novel concept. This study adds important new data on the inherent functional differences between BDC and in vitro generated Mo-DC, reinforcing indications that BDC are superior for therapeutic vaccination. To support the potential...
Figure 6. T Cell Checkpoint Blockade has the Potential to Enhance CMRF-56 BDC Vaccination in AML CR Patients Post-ATx. (A) Expression of PD-1 and (B) TIM-3 on unmanipulated CD4 and CD8 peripheral T cells in AML patients in CR following NFR (n = 6) or FR (n = 5) chemotherapy or post-ATx (n = 6) was compared to age-matched HD (n = 10). (C) The effect of anti-PD-1 (Nivolumab) and/or anti-TIM-3 (F38–2E2) antibodies on the CMRF-56 BDC-driven WT1-specific CD8 T cell responses in 3 post-ATx patients. CMRF-56 BDC were pulsed with WT1 peptivator, activated and added to autologous PBMC +/- PD-1 and TIM-3 blocking antibodies. Cultures were re-stimulated with autologous WT1 peptivator loaded CMRF-56 BDC on day 7 and assayed on day 15 for WT1 peptivator-specific IFN-gamma and CD107a. (D, E). On days 0 and 15, PD-1 and TIM-3 expression was assessed by flow cytometry on CD4 (solid line) and CD8 (dashed line) T cells against an isotype control (filled histogram). (F) PD-L1 expression was also assessed on CMRF-56 BDC (solid line) against an isotype control (filled histogram). (G) Summary of WT1-specific IFN-gamma from CD4 and CD8 T cells, and CD107a on CD8 T cells in each patient. Patient 05 (squares), Patient 11 (triangles), Patient 18 (circles). Data was analysed by one-way ANOVA with Dunnett’s multiple comparison test. # p < 0.1, *p < 0.05, **p < 0.01, ***p < 0.001.
of using a “second generation” BDC vaccine in AML, we showed that BDC recover in the peripheral blood of AML patients in CR, can be enriched by single-step immune selection using hCMRF-56 mAb, and function to expand leukemia antigen-specific T cells. We add to this a description of the T cell landscape at diagnosis and CR post-chemotherapy and ATx showing there is little perturbation of T cell phenotype post anthracycline and cytarabine based induction (NFR), but marked abnormalities if fludarabine is added (FR) suggestive of a poor clinical response to a vaccine in the latter group. Finally, we describe the expression of T cell checkpoint molecules at CR in AML and show that their expression within individual patients may predict the ability of blocking antibodies to enhance clinical leukemia antigen-specific responses.

To stimulate an effective cytotoxic T cell response to exogenous proteins, DC must be able to cross-present protein antigen. We have previously shown that the rare blood CD141⁺ mDC are highly efficient at cross-presenting antigen. We now add to this clear evidence of the superior cross-presenting ability of CD1c⁺ mDC over Mo-DC. Mo-DC had a poor ability to sustain class I peptide complexes, and a had a demonstrably weaker capacity to, process and present long peptide than CD1c⁺ mDC, which presented an internal CD8 epitope processed from FMP long peptide in the context of HLA class I at significantly higher density. We have previously demonstrated that in contrast to Mo-DC, antigen presentation by BDC is sustained for > 24 hours, suggesting an ability to provide an ongoing stimulus for specific T cell expansion. This reinforces the potential of a “second generation” DC vaccine composed of pre-formed BDC. However, application of such a vaccine in AML requires that BDC are present, can be purified and remain functional.

It has been reported that BDC are increased in AML patients at presentation. However, many of the markers used to identify BDC such as HLA-DR, CD11c, CD123 and ILT3, may also be expressed by circulating blasts in AML. Since the publication of these works there has been considerable progress in the identification of BDC, allowing for more accurate identification. We found that cells sorted from the peripheral blood of AML patients using a BDC gating strategy matching those published works have myeloblast morphology. Our refined identification strategy was validated through ViSNE analysis and identified discrete BDC subsets, clearly distinguished from other myeloid cells.

We confirm a deficiency of circulating CD1c⁺ mDC and pDC at diagnosis, and add that CD141⁺ mDC are also reduced. Importantly, BDC recovery begins as early as 5 weeks post-chemotherapy with normal BDC levels identified in many patients at 11 weeks post-chemotherapy. Interestingly, pDC do not recover in the circulation to levels equivalent to age-matched HD after AML, suggesting that their bone marrow niche may be affected. Importantly, CD1c⁺ and CD141⁺ mDC subsets recovered, albeit incompletely, in CR post-chemotherapy and post-ATx. AML patients’ CD1c⁺ and CD141⁺ DC upregulated CD83 and CMRF-56 after culture in a similar fashion to HD. This allowed for immune selection with hCMRF-56 mAb resulting in BDC enriched preparations of similar quality to HD. It has been suggested that BDC are dysfunctional in AML at diagnosis, based on experiments performed with sorted BDC, which may have been contaminated with circulating blasts. We have shown that BDC purified at CR expand autologous CD8⁺ T cells recognizing viral and leukemia-associated antigen, WT1, in a single in vitro stimulation. This supports the feasibility of the vaccine and confirms that BDC are functional in AML in CR.

The T cell landscape affected responses to DC stimulation in vitro. Patients who had received non-fludarabine containing chemotherapy had normal CD4 and CD8⁺ T cell subsets, and no elevation in Tregs at CR, and it was possible to expand antigen-specific T cell responses. Patients who had received fludarabine had profound perturbations of T cell immunity as previously described. They failed to expand in response to viral or tumor antigens, had an increase in terminally differentiated T cells, and underwent AICD after non-specific TCR ligation. Similar biology has been demonstrated in FR treated CLL patients, where memory cells were increased at the expense of naïve T cells and IFN-gamma production was increased. Increased susceptibility to AICD in T cells from patients that have received FR is a novel finding. We hypothesise that FR-induced DNA damage is detected increasingly as cells enter cycle after TCR ligation. The multiple T cell abnormalities in FR patients suggests they will not be good candidates for T-cell directed immunotherapy.

WT1-specific responses were expanded more frequently in patients after ATx than after chemotherapy. This may be partly because allogeneic grafted T cells do not undergo thymic central selection, while autologous T cells do. Therefore, autologous T cells with a high affinity for self-antigens, such as WT1 will be deleted, and only low affinity T cells will enter the peripheral pool. This does not apply after ATx, and in part explains why the precursor frequency was higher, and expansions more frequent. Other major differences in the T cell pool after ATx, particularly increased effector CD8⁺ T cells, increased effector TBE₂ expression and IFN-gamma production by CD4⁺ and CD8⁺ T cells suggests that the immune system post ATx is in a suitable state for immunotherapeutic intervention. As relapse remains the major cause of death after ATx, strategies to reinforce GVL without inducing graft-versus-host disease (GVHD) are highly appealing. Early pre-clinical data has suggested the potential of combining checkpoint blockade with DC therapy in AML, and clinical assessment of PD-1 blockade with a Mo-DC-based vaccine in AML has been undertaken. We demonstrated increased expression of PD-1 and TIM-3 on CD4⁺ T cells in AML at CR and after ATx. CD4⁺ T cell help is essential for expanding effective antigen-specific T cell responses. Hence, we hypothesized that PD-1 and TIM-3 blockade could enhance CD8⁺ T cell responses in vitro. The enhancement of CMRF-56⁺ BDC vaccine induced WT1 response by TIM-3 and PD-1 blockade varied, with the greatest increase seen in the patient with the largest up-regulation of PD-1 during culture. Taken together, our peripheral blood and bone marrow phenotyping together with our in vitro functional study suggests that checkpoint blockade has the potential to augment CMRF-56⁺ BDC induced responses in vivo.
BDC have an enhanced capacity to cross-present antigen compared to Mo-DC, further supporting their clinical evaluation as a "second generation" DC vaccine.\textsuperscript{7,8} To determine whether this approach is feasible in AML, we confirmed that BDC can be purified from patients in CR, either after induction chemotherapy or ATx, and used to expand antigen-specific T cell responses. In addition, anti-leukemia T cell responses can be further enhanced in some patients by concomitant checkpoint blockade. In particular, this finding highlights the potential of active vaccination in AML patients after ATx. As it is possible to purify CMRF-56\textsuperscript{+} BDC in large numbers by leukapheresis,\textsuperscript{14} it may be practical to purify CMRF-56\textsuperscript{+} BDC directly from donor grafts. In the future, this could offer a means to vaccinate patients after ATx to enhance leukemia-specific immunity without promoting GVHD.

**Methods**

**Patients**

Venous blood (3ml) was collected from healthy donors (HD) and AML patients with newly diagnosed (ND) or relapsed disease (RR), or 30ml from those achieving CR post-chemotherapy (CT) or post-ATx attending The Royal Prince Alfred Hospital from January 2014 to October 2016. Blood was collected at diagnosis and/or a minimum of 6 weeks post-chemotherapy or 6–12 months post-ATx from patients receiving no immune suppression and with no evidence of GVHD. Blood was collected with informed consent consistent with the Helsinki declaration of 1975 and appropriate ethics approval (HREC/15/RPAH/485, HREC/11/RPAH/170). Patient characteristics are described in Tables S1 and S2. Remission of AML was determined based on standard guidelines.\textsuperscript{35} Bone marrow aspirates had an absence of abnormal blasts and <5% myeloblasts, and when documented, there was an absence of detectable molecular disease.

**Antigen presentation assay**

The capacity of CD1c\textsuperscript{+} mDC and Mo-DC to cross-present influenza matrix protein (FMP) long peptide was assessed using blood samples from HLA-A*0201\textsuperscript{+} HD, CD1c\textsuperscript{+} mDC purified by flow sorting\textsuperscript{36} were compared to day 5 to 6 Mo-DC generated from CD14 MACs bead (Miltenyi Biotec) purified monocytes cultured in 800U/mL GM-CSF (Miltenyi Biotec) and 1000 U/mL IL-4 (Life Technologies). DC were incubated with 10 μg/mL FMP\textsubscript{58–66} (GILGFVFTL) equimolar amounts of FMP\textsubscript{54–74} (PLTGILGVFTLTPVQGRL) or control WT1\textsubscript{126–134} (RMFPNAPYL) peptide (Mimitopes) for 16 hrs. Presentation of surface HLA-A2 FMP\textsubscript{58–66} peptide complexes were detected by flow cytometry using a FITC-labelled antibody recognizing HLA-A2:FMP\textsubscript{58–66} (Dendritics).

**Flow cytometry and BDC enumeration**

BDC were enumerated in whole blood using Trucount methodology as previously described.\textsuperscript{23} In brief, 100μL fresh blood was placed into Trucount tubes containing fluorescently labelled antibodies to CD45, Lineage (CD3, CD14, CD19, CD20, CD34, CD56, CD235a), HLA-DR, CD11c, CD16 (BD Biosciences), CD304, CD141 (Miltenyi Biotec) and CD1c (eBiosciences), before addition of Pharmlyse (BD Biosciences) and acquisition on a FACS Canto II cytometer (BD Biosciences). Visualised Stochastic neighbour embedding (ViSNE)\textsuperscript{22} analysis was performed using Cytobank\textsuperscript{37} as described\textsuperscript{38} on Lineage negative HLA-DR\textsuperscript{+} cells using non-lineage markers as clustering channels. The morphology of cells within the BDC gate was determined by sorting Lin- HLA-DR\textsuperscript{+} cells on an Influx cell sorter (BD Biosciences). Cells were cytospun (Thermo-Shandon), May-Grunwald-Giemsia stained using a Cell-Dyn Slide maker stainer (Abbott Diagnostics) and photographed using an Olympus BX40 microscope camera with Labsens software (Olympus).

**CMRF56 immune selection**

CMRF-56\textsuperscript{+} BDC were purified as previously described.\textsuperscript{9} In brief, PBMC were incubated in X-VIVO 15 media (Lonza) for 14–16 hours, before purification with biotinylated hCMRF-56 mAb, anti-biotin beads and MACS technology (Miltenyi Biotec). Purity was confirmed by flow cytometry using mAbs to CD45, HLA-DR, Lineage (CD14, CD19, CD20, CD34, CD56) and CD83.

**T cell assays**

T cell subsets were assessed by flow cytometry (FACS Canto II cytometer, BD) using CD3, CD4, CD8, CD28, CD45RO, CD57, CD25, T-bet, TIM-3 (all from BD), and/or FoxP3 (eBiosciences) and confirmed by CyTOF (see Figure S4). For functional assays, PBMC were cultured with 50ng/mL PMA and 1ug/mL Ionomycin (Sigma-Aldrich) or anti-CD2/CD3/CD28 beads (Miltenyi Biotec, 1:2 ratio PBMC ratio) with breflidin A, monensin and CD107a PE-Cy7 (BD Biosciences) added for the final 4 hours before labelling with the indicated antibodies. Activation-induced cell death (AICD) was determined by comparing the percentage of viable CD3\textsuperscript{+} cells via Fixable Live/Dead Violet (ThermoFisher) in anti-CD2/CD3/CD28 bead-stimulated versus unstimulated cultures.

**Expansion of antigen-specific T cells**

CMRF-56\textsuperscript{+} BDC were pulsed with CEF (Cytomegalovirus, Epstein-Barr Virus, Influenza, HLA class I peptides, JPT), CMV peptivator or WT1 peptivator (Miltenyi Biotec, 1 ug/ml of each peptide/mL) for 2 hours in X-VIVO-15 media (Lonza) at 37°C 5% CO\textsubscript{2} then activated with 1000 U/mL GM-CSF (Miltenyi Bitech) and 300 ng/mL Pam3CSK4 (Invivogen) for 2 hours. For HLA-A*2\textsuperscript{+} patients, CMRF-56\textsuperscript{+} BDC were activated then pulsed for 2 hours with nonamer HLA-A*0201-restricted peptides, FMP\textsubscript{58–66} (2 ug/ml) or WT1\textsubscript{126–134} (10 ug/ml) and cultured with CMRF-56\textsuperscript{+} PBMC (1 BDC:10 PBMC). Where indicated, 20 ug/ml Nivolumab (Bristol-Myers-Sqibb), 10 ug/ml Nivolumab, or 10 ug/ml anti-TIM-3 (BioLegend, F38–2E2) were added. Cultures were supplemented with IL-15 (20 ng/mL), IL-7 (10 ng/mL) and...
IL-2 (25 U/mL) on day 3 and media was replaced with fresh IL-2 at 25 U/mL on days 5 and 7. Responses were detected on day 9/10 by dextramer (FMP58,6–134, 112-134, Immudex) for HLA-A2+ patients, and/or by co-culturing with autologous peptide-pulsed PBMCs for 6 hours before detecting intracellular IFN-gamma as described above for patients of all HLA types.

**Statistical analyses**

Statistical analyses were performed using Prism version 6 (GraphPad Software Inc.) using the Mann-Whitney, one-way ANOVA or Kruskal-Wallis test as appropriate.

**Disclosure of interest**

D.N.J.H. and G.J.C. are both Directors of DendroCyte BioTech Pty Limited, which holds the patent for the humanized anti-CMRF-56 mAb. The remaining authors declare no conflict of interest.

**Acknowledgments**

We wish to thank the patients who were involved in this study, our volunteer healthy blood donors, Elizabeth Swinnerton, the blood collection team, Louise Kerr and the transplant team at Royal Prince Alfred Hospital, and Francesca Springall in the department of molecular haematology at Royal Prince Alfred Hospital.

**Funding**

This work was supported by the Australian National Health and Medical Research Council under Grant Number 543727; Cancer Institute New South Wales under Grant Numbers 11/TPG/3–012; Anthony Rothe Memorial Trust under Grant Number RRE/700; Cancer Australia under Grant Number 543713; Sydney Catalyst, The Ainsworth Foundation, Enid Mem Rev. 2015;67:73–51. doi:10.1124/jci.110.009456. PMID:26240218

7. Prue RL, Van Vidi, R, Radford KJ, Tong H, Hardy MY, D’Rozario R, Waterhouse NJ, Rossetti T, Coleman R, Tracey C, et al. A Phase I Clinical Trial of CD1c (BDCA-1)+ Dendritic Cells Pulsed With HLA-A*0201 Peptides for Immunotherapy of Metastatic Hormone Refractory Prostate Cancer. J Immunother. 2013;36:1266–71. doi:10.1097/CJI.0b013e31827d38f0.

8. Tel J, Aarnitzen EH, Baba T, Schreiber GT, Schulte BM, Benitez-Ribas DB, Boerman OC, Croockewit S, Ogren WJ, Van Rossum M, et al. Natural human plasmacytoid dendritic cells induce antigen-specific T-cell responses in melanoma patients. Cancer Res. 2013;73:1063–75. doi:10.1158/0008-5472.CAN–12-2583. PMID:23345163

9. Fromm PD, Padamadisitrus MS, Hsu J, Van Kooten Losio N, Verma ND, Lo TH, Silveira PA, Bryant CE, Turtle CJ, Prue RL, et al. CMRF-56(+) blood dendritic cells loaded with mRNA induce effective antigen-specific cytotoxic T-lymphocyte responses. Oncoimmunology. 2015;4:e4114655. doi:10.1007/s00277-011-1231-2. PMID:21520003

10. Robbins SH, Walzer T, Dembele D, Thibault C, Defays A, Bessou G, Xu H, Vivier E, Sellars M, Pierre P, et al. Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. Genome bio. 2008;9:R17. doi:10.1186/gb-2008-9-1-r17. PMID:18218067

11. Osugi Y, Vuckovic S, Hart DN. Myeloid blood CD11c(+) dendritic cells and monocyte-derived dendritic cells differ in their ability to stimulate T lymphocytes. Blood. 2002;100:2858–66. doi:10.1182/blood.V100.8.2858. PMID:12351396

12. Freeman JL, Van Vidi, F, Hart DN. CMRF-56 immunoselected blood dendritic cell preparations activated with GM-CSF induce potent anti-myeloma cytotoxic T-cell responses. Journal of immunotherapy. 2007;30:740–8. doi:10.1097/JCIb0031381f4b26. PMID:17893566

13. Jongbloed SL, Kassianos AJ, McDonald KJ, Clark GJ, Ju X, Angel CE, Chen CJ, Dunbar PR, Waldey RB, Jeet V, et al. Human CD141(+) (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. J Exp Med. 2010;210:1247–60. doi:10.1084/jem.20092140. PMID:20479116

14. Fonseca RD, Garrison L, Prue RL, TMW, Smith RM, Silveira PA, Bryant CE, Turtle CJ, Prue RL, Hart DN. Practical blood dendritic cell vaccination for immunotherapy of multiple myeloma. Br J Haematol. 2008;143:374–7. doi:10.1111/j.1365-2141.2008.07346.x. PMID:18729856

15. Moby M, Isnardon D, Blaise D, Mozziacconci MI, Lafage-Pochitaloff M, Briece F, Gastau TA, Olive D, Gaugler B. Identification of precursors of leukemic dendritic cells differentiated from patients with acute myeloid leukemia. Leukemia. 2002;16:2267–74. doi:10.1038/sj. leu.2402706. PMID:12399972

16. Moby M, Jarrossay D, Lafage-Pochitaloff M, Zandotti C, Briece F, de Lambarlier XN, Isnardon D, Sainty D, Olive D, Gaugler B. Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairments. Blood. 2001;98:3750–6. doi:10.1182/blood.V98.13.3750. PMID:11739182

17. Rickmann M, Krauter J, Stamler K, Heuser M, Salguero G, Mischak-Weissinger E, Ganser A, Stripecke R. Elevated frequencies of leukemic myeloid and plasmacytoid dendritic cells in acute myeloid leukemia with the FLT3 internal tandem duplication. Ann Hematol. 2011;90:1047–58. doi:10.1007/s00277-011-1231-2. PMID:21520003

18. Rickmann M, Macke L, Sundarasetty BS, Stamler K, Figueiredo C, Blasczyk R, Heuser M, Krauter J, Ganser A, Stripecke R. Monitoring dendritic cell and cytokine biomarkers during remission prior to relapse in patients with FLT3-ITD acute myeloid leukemia.
Ann Hematol. 2013;92:1079–90. doi:10.1007/s00277-013-1744-y. PMID:23616009

19. Ehninger A, Kramer M, Rollig C, Thiede C, Bornhauser M, von Bonin M, Wermke M, Feldmann A, Bachmann M, Ehninger G, et al. Distribution and levels of cell surface expression of CD33 and CD123 in acute myeloid leukemia. Blood Cancer J. 2014;4:e218. doi:10.1038/bcj.2014.39. PMID:24927407

20. Dobrowska H, Gill KZ, Serban G, Ivan E, Li Q, Qiao P, Suciu-Foca N, Savage D, Alobad B, Bhagat G, et al. Expression of immune inhibitory receptor ILT3 in acute myeloid leukemia with monocytic differentiation. Cytometry B Clin Cytom. 2013;84:21–9. doi:10.1002/cyto.b.21050. PMID:23027709

21. Bahia DM, Yamamoto M, Chaffaille Mde L, Kimura EY, Bordin JO, Filgueiras MA, Kerbauy J. Aberrant phenotypes in acute myeloid leukemia: a high frequency and its clinical significance. Haematologica. 2001;86:801–6. PMID:11522535

22. Amir E-ad, Davis KL, Tadmor MD, Simonds EF, Levine JH, Bendall SC, Shenfeld DK, Krishnaswamy S, Nolan GP, Pe'er D, viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nat Biotech. 2013;31:545–52. doi:10.1038/nbt.2594.

23. Vuckovic S, Gardiner D, Field K, Chapman GV, Khalil D, Gill D, Marlton P, Taylor K, Wright S, Pinzon-Charry A, et al. Monitoring dendritic cells in clinical practice using a new whole blood single-platform TruCOUNT assay. J Immunol Methods. 2004;284:73–87. doi:10.1016/j.jim.2003.10.006. PMID:14736418

24. Gassner FJ, Weiss L, Geisberger R, Hofbauer JP, Egle A, Hartmann TN, Greil R, Tinhofer I. Fludarabine modulates composition and function of the T cell pool in patients with chronic lymphocytic leukemia. Cancer Immunol Immunother. 2011;60:75–85. doi:10.1007/s00262-010-0920-3. PMID:20857100

25. Parker JE, Pagliuca A, Mijovic A, Czepulkowski B, Rassam SM, Samarutunga IR, Grace R, Gover PA, Mufﬁt GJ. Fludarabine, cytarabine, G-CSF and idarubicin (FLAG-IDA) for the treatment of AML patients who Achieve a Chemotherapy-Induced Remission. Blood Cancer J. 2015;5:e330. doi:10.1038/bcj.2015.58. PMID:26230954

26. Kong Y, Zhang J, Claxton DF, Ehmann WC, Rybka WB, Zhu L, Zeng H, Schell TD, Zheng H, PD-1(hi)TIM-3(+) T cells associate with and predict leukemia relapse in AML patients post allogeneic stem cell transplantation. Blood Cancer J. 2015;5:e330. doi:10.1038/bcj.2015.58. PMID:26230954

27. Zhou Q, Munger ME, Veenstra RG, Weigel BJ, Hirashima M, Munn DH, Murphy WJ, Azuma M, Anderson AC, Kuchroo VK, et al. Coexpression of Tim-3 and PD-1 identiﬁes a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. Blood. 2011;117:4501–10. doi:10.1182/blood-2010-10-310425. PMID:21385853

28. Davids MS, Kim HT, Bachireddy P, Costello C, Liguori R, Savell A, Lukez AP, Avigan D, Chen YB, McSweeney P, et al. Iplimumab for Patients with Relapse after Allogeneic Transplantation. N Engl J Med. 2016;375:143–53. doi:10.1056/NEJMoa1601202. PMID:27410923

29. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, Leenen PJ, Liu YJ, MacPherson G, Randolph GJ, et al. Nomenclature of monocytes and dendritic cells in blood. Blood. 2010;116:e74–80. doi:10.1182/blood-2010-02-258558. PMID:20628149

30. Boldt DH, Von Hoff DD, Kuhn JG, Hersh M. Effects on human peripheral lymphocytes of in vivo administration of 9-beta-D-arabino-furanosyl-2'-fluoroadenine-5'-monophosphate (NSC 312887), a new purine antimetabolite. Cancer Res. 1984;44:4661–6. PMID:6205751

31. Barrett AJ, Battivalla M. Relapse after allogeneic stem cell transplantation. Expert Rev Hematol. 2010;3:429–41. doi:10.1586/ehm.10.32. PMID:21083034

32. Zhong RK, Loken M, Lane TA, Ball ED. CTLA-4 blockade by a human mAb enhances the capacity of AML-derived DC to induce T-cell responses against AML cells in an autologous culture system. Cytotherapy. 2006;8:3–12. doi:10.1080/14653240500499507. PMID:16627340

33. Rosenblatt J, Stone RM, Avivi I, Uhl L, Neuberg D, Joyce R, Tzachanis D, Levine JD, Boussiotis VA, Zwick J, et al. Clinical Trial Evaluating DC/AML Fusion Cell Vaccination Alone and in Conjunction with PD-1 Blockade in AML Patients Who Achieve a Chemotherapy-Induced Remission. Blood. 2011;118:948.

34. Laidlaw BJ, Craft JE, Kaech SM. The multifaceted role of CD4(+) T cells in CD8(+) T cell memory. Nat Rev Immunol. 2010;10:162–11. doi:10.1007/nri.2010.10. PMID:26781939

35. Dohner H, Estey EH, Amadori S, Appelbaum FR, Buchner T, Burnett AK, Dombret H, Fenaux P, Grimwade D, Larson RA, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115:453–74. doi:10.1182/blood-2009-07-235558. PMID:19880497

36. Kassianos AJ, Jongbloed SL, Hart DN, Radford KJ. Isolation of human MAb enhances the capacity of AML-derived DC to induce T-cell responses against AML cells in an autologous culture system. Cytotherapy. 2006;8:3–12. doi:10.1080/14653240500499507. PMID:16627340

37. Lo TH, Silveira PA, Fromm PD, Verma ND, Vu PA, Kupresanin F, Adam R, Kato M, Cogger VC, Clark GJ, et al. Characterization of the Expression and Function of the C-Type Lectin Receptor CD302 in Mice and Humans Reveals a Role in Dendritic Cell Migration. J Immunol. 2016;197:885–98. doi:10.4049/jimmunol.1600239. PMID:27316686