Targeting the arginine metabolic brake enhances immunotherapy for leukaemia

Mussai, Francis; Wheat, Rachel; Sarrou, Evgenia; Booth, Sarah; Stavrou, Victoria; Fultang, Livingstone; Perry, Tracey; Kearns, Pamela; Cheng, Paul; Keehsan, Karen; Craddock, Charles; De Santo, Carmela

DOI: 10.1002/ijc.32028
License: Creative Commons: Attribution (CC BY)

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Mussai, F, Wheat, R, Sarrou, E, Booth, S, Stavrou, V, Fultang, L, Perry, T, Kearns, P, Cheng, P, Keehsan, K, Craddock, C & De Santo, C 2018, 'Targeting the arginine metabolic brake enhances immunotherapy for leukaemia', International Journal of Cancer. https://doi.org/10.1002/ijc.32028

Link to publication on Research at Birmingham portal

General rights
Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

• Users may freely distribute the URL that is used to identify this publication.
• Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
• Users may use extracts from the document in line with the concept of ‘fair dealing’ under the Copyright, Designs and Patents Act 1988 (?).
• Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy
While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Download date: 10. Mar. 2020
Short Report

Targeting the arginine metabolic brake enhances immunotherapy for leukaemia

Francis Mussai, Rachel Wheat, Evgenia Sarrou, Sarah Booth, Victoria Stavrou, Livingstone Fultang, Tracey Perry, Pamela Kearns, Paul Cheng, Karen Keehsan, Charles Craddock and Carmela De Santo

Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom

Paul O’Gorman Leukaemia Research Centre, College of Medicine, Veterinary Life Sciences, Institute of Cancer Sciences, University of Glasgow, United Kingdom

Institute of Cancer and Genomic Sciences, University of Birmingham, Birmingham, United Kingdom

Bio-cancer Treatment International Ltd, Hong Kong

Therapeutic approaches which aim to target Acute Myeloid Leukaemia through enhancement of patients’ immune responses have demonstrated limited efficacy to date, despite encouraging preclinical data. Examination of AML patients treated with azacitidine (AZA) and vorinostat (VOR) in a Phase II trial, demonstrated an increase in the expression of Cancer-Testis Antigens (MAGE, RAGE, LAGE, SSX2 and TRAG) on blasts and that these can be recognised by circulating antigen-specific T cells. Although the T cells have the potential to be activated by these unmasked antigens, the low arginine microenvironment created by AML blast Arginase II activity acts a metabolic brake leading to T cell exhaustion. T cells exhibit impaired proliferation, reduced IFN-γ release and PD-1 up-regulation in response to antigen stimulation under low arginine conditions. Inhibition of arginine metabolism enhanced the proliferation and cytotoxicity of anti-NY-ESO T cells against AZA/VOR treated AML blasts, and can boost anti-CD3 Chimeric Antigen Receptor-T cell cytotoxicity. Therefore, measurement of plasma arginine concentrations in combination with therapeutic targeting of arginase activity in AML blasts could be a key adjunct to immunotherapy.

Introduction

T cell immunity plays a key role in the body’s defence through the recognition of foreign antigens, expansion in T cells, and signalling through cytokines and cell surface molecules.

Key words: arginine, T, CTAG, AML, immunotherapy

Additional Supporting Information may be found in the online version of this article.

Conflict of interest: The authors declare no conflict of interest exists.

Grant sponsor: Bloodwise and Cancer Research UK; Grant sponsor: Treating Children with Cancer, University of Birmingham Alumni donors; Grant sponsor: Amber Phillpott Trust

DOI: 10.1002/ijc.32028

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

History: Received 20 Aug 2018; Accepted 13 Nov 2018;
Online 28 Nov 2018

Correspondence to: Francis Mussai, Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom. Tel: 0121 414 7047, E-mail: francis.mussai@nhs.net

However, in cancer patients, despite large numbers of abnormal cells being present, immune surveillance is subverted such that T cells fail to recognise the malignant cells or are inhibited in their proliferation or function. A resurgence of cellular immunotherapy strategies has shown the potential for T cells to eradicate leukaemia. Indeed, even relatively crude immune approaches such as allogeneic stem cell transplant in patients with high risk or relapsed AML demonstrate the clinical benefit of T cell activity, despite the risk of Graft Versus Host Disease.

Only a few new drugs have been developed for AML that have significantly altered patient outcomes—of these epigenetic modifiers such as azacitidine and vorinostat appear amongst the most promising. One proposed mechanism of action is the upregulation of MHC-restricted previously undetectable cancer-testis antigens (CTAG) allowing antigen-specific T cells to recognise and kill AML blasts. However, attempts to trigger autologous T cell responses have led to only limited clinical improvements, with the mechanism of failure poorly understood. Furthermore, the mechanisms of how patients’ immune surveillance fails to control leukemic expansion, have not been well characterised.

Recently the interaction between cancer cell metabolism and immunity has become a focus in understanding cancer-immune evasion. The consumption of amino acids such as
Targeting arginine metabolism to enhance immunotherapy

**What's new?**

To date, cellular immunotherapy strategies against acute myeloid leukaemia (AML) have demonstrated limited efficacy. Here, arginine concentrations in AML patients were shown to be significantly lower than in healthy controls, both at diagnosis and during immunomodulatory treatment. Although T cells were able to recognise and be activated by antigens expressed on AML blasts, the low arginine microenvironment acted as a metabolic brake on T cell function and expansion. Targeting arginine metabolism in AML patients should be an adjunct to current treatment strategies in order to enhance host anti-leukaemia immunity and boost the activity of CAR-T or adoptive T cell therapies.

---

argonine have been shown to impact T cell responses in the laboratory setting, yet the translational importance of this mechanism in patients has not been explored. Here we identify how the failure to address the arginine metabolic microenvironment impacts immune-modulatory epigenetic therapy or CAR-T cytotoxicity against leukaemic blasts.

**Materials and Methods**

**Patient samples and study approvals**

Blood samples were obtained from 80 AML patients ineligible for intensive chemotherapy treated with either azacitidine or azacitidine and vorinostat in a multi-centre, randomised phase II trial (RAVVA; NCT01617226). Fresh Peripheral Blood Mononuclear Cells were separated using a Lymphoprep (Alere, Stockport, UK) gradient and stored in liquid nitrogen. Samples from healthy donors were obtained from the University of Birmingham. In accordance with the Declaration of Helsinki, all samples were obtained after written, informed consent prior to inclusion in the study. Regional Ethics Committee (REC Number 10/H0501/39) approval for the study was granted.

**RT-Q-PCR analysis**

RT-Q-PCR was used to detect Cancer-Testis Antigens and Arginase II in purified patient-derived AML blasts and cell lines. RNA was extracted using an RNeasy Mini kit (Qiagen, Venlo, Netherlands). cDNA was prepared using SuperscriptTM III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and the Applied Biosystems 7500 in duplicate using FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and the Applied Biosystems 7500 Fast Real-Time PCR system. Analysis of gene expression was calculated according to 2-ΔΔT method plotted as arbitrary units of mRNA relative to GAPDH.13

| Primer sequences (Eurofin) list | Forward | Reverse |
|-------------------------------|---------|---------|
| **Human:**                    |         |         |
| MAGE                          | 5′-AGTCCTCGGGAGGCCTCC-3′ | Forward |
|                               | 5′-ACTCACCTCTCCAGATT-3′  | Reverse |
| LAGE                          | 5′-CCAGAGTGGAGTTGCCC-3′  | Forward |
|                               | 5′-CTGCGACCTCTGCTGGGA-3′  | Reverse |
| TRAG3                         | 5′-CCAYAGGGTCCCAAGACA-3′  | Forward |
|                               | 5′-GCTCTCCCGAAAGAGACTGG-3′ | Reverse |

**Peptides List**

| Peptides List | SLLMWITQC | LKLSGVVRVL |
|---------------|-----------|------------|
| LAGE          |           |            |
| RAGE          |           |            |
| TRAG3         | ILLRDAGLV |            |
| SSX2          | KASEKIFYV |            |
| NYESO         | SLLMWITQC |            |
| SART3         | LLQAEAPR |            |
| MAGEA10       | GLYDGMHLE |            |
| MAGEA4        | GYVDGReHTV|            |
| MAGEA8        | KVAELVHL |            |
| MAGEA12       | FLWGPRLV |            |
| MAGEA1        | KVLEYVIK |            |

**IFN-γ ELISPOT**

HLA-A*02+ AML patients were identified by flow cytometry staining (BD Pharmingen, San Jose, CA) of the whole blood. To identify AML patients which contained CTAG responsive T cells IFN-γ release was measured by ELISPOT (eBioscience, San Diego, CA). In brief, 96-well PDVF plates were coated with IFN-γ capture antibody. PBMCs were thawed and plated in the presence of CTAG peptides (20 ng/ml). Influenza GIL and EBV YVL peptides were used as positive controls. After 36 hr, the plates were washed and biotinylated detection antibody added. Avidin–HRP solution was added and the plate incubated for 45 min after a further wash, substrate solution was added, and left to develop until spots developed, at which point the reaction was terminated by washing, and the plate was left to dry. Spots were counted using a ELISPOT reader.
**Statistics**

$t$-Tests (parametric) were used to determine the statistical significance of the difference in paired observations between two groups (GraphPad Prism, San Diego, CA). $p$ values are two-tailed and where values were $< 0.05$, they were considered statistically significant. A Kaplan–Meier survival curve was generated using GraphPad PRISM.

Methodological information regarding cell lines, flow cytometric analysis, mixed lymphocyte reactions, our AML murine models, ELISA and engineered T cells can be found in the supplements.

**Results**

T cells are functionally exhausted despite enhanced cancer-testis antigen expression on AML blasts

Epigenetic modifying drugs such as azacitidine and vorinostat may boost the anti-leukaemia T cell immune response by upregulating Cancer-Testis Antigens expression.5,6,7 First we showed that treatment of AML cell lines with Azacitidine and Vorinostat can upregulate MAGE, LAGE, RAGE, TRAG3, and SSX2 CTAGs on AML tumour cell lines (Supporting Information Fig. S1a). Recently we investigated the clinical efficacy of combining azacitidine with vorinostat in a Phase II trial in AML and high risk MDS patients.5 Although a number of clinical responses were seen, vorinostat provided no additional clinical benefit in terms of overall survival. We hypothesised that these drugs should have upregulated CTAGs in AML blasts making them a more attractive immune target. However, the mechanism of why the T cell response is inadequate to control the disease is unknown.

CTAGs are expressed in the context of HLA-A*02. Analysis of AML blasts from HLA-A*02 patients on the AZA/VOR clinical trial after three and six cycles of therapy identifies upregulation of the five families of CTA expression (MAGE, RAGE, LAGE, SSX2 and TRAG3) compared to before drug administration in individual patients (Fig. 1a) confirming our hypothesis. On recognition of target antigens, classically T cells respond through cell division, IFN-γ release and cytotoxicity. Patient-derived PBMC from 30 HLA-A*02 patients were isolated before and during treatment (Supporting Information Fig. S1b), and pulsed with relevant CTAG peptides ex vivo. Increased activated anti-CTAG T cells for MAGE, LAGE, and SSX2 antigens were particularly prominent as assessed by release of IFN-γ release after AZA/VOR (Fig. 1b, Supporting Information Fig. S1c and d). However, although the drugs led to significant increase in antigen-specific T cell activation compared to baseline, surprisingly, these responses are not greater than those of healthy donors (Supporting Information Fig. S2a).8

We hypothesised that T cells in patients may be functionally exhausted—a state characterised by a failure to expand in response to stimulation and upregulation of cell surface inhibitory receptors such as PD1, LAG3 and TIM3.16 First, we identified that in the majority of patients T cells had a significantly reduced ability to proliferate ($p = 0.0078$) compared to those from healthy donors (Fig. 1c). Phenotyping demonstrated upregulated PD1 expression ($p = 0.0001$) throughout treatment, with no changes in LAG3 or TIM3 (Fig. 1d, Supporting Information Fig. S2b and c). Thus, the expected physiological expansion of activated T cells in response to antigens on AML blasts is absent.

The failure to address the low arginine microenvironment impairs anti-CTAG clinical responses

Arginine metabolism is a pathway which is aberrant in AML blasts and can influence the immune microenvironment.4,9 Using an immunocompetent, syngeneic model of AML, we showed that significant numbers of AML blasts (CD45.2+) were detectable in the bone marrow (median 80%) and spleens (median 40%) of mice, analogous to presentation of AML in patients (Supporting Information Fig. S2d and e). AML bearing mice showed significant reductions in the percentage of T cells in the bone marrow ($p = 0.0002$) and spleens ($p = 0.0002$; Fig. 1e) which also expressed increased PD1 (bone marrow $p = 0.0002$, spleens $p = 0.0011$) consistent with our human findings (Fig. 1f). The mice had significant reductions in plasma arginine compared to controls ($p = 0.0031$; Fig. 1g).

Nonself-antigen presentation by an allogeneic MHC is a profound model of T cell stimulation and proliferation. To investigate the specific impact of plasma arginine concentrations, we first identified in vitro that T cell expansion in response to allogeneic dendritic cells was profoundly inhibited by the addition of recombinant human arginase to mimic AML blast activity (Fig. 2a) with a corresponding reduction in activation-induced IFN-γ release (Fig. 2b). Next equal numbers of human T cells ($10 \times 10^5$) were engrafted into NOD-SCID mice. T cells rapidly engrafted in the spleen ($n = 14$ days). Analogous to the arginine deplete microenvironment found in patients at diagnosis, administration of recombinant arginase led to a significant reduction in serum arginine ($p = 0.001$; Fig. 2c) and a failure of T cells to expand ($p = 0.011$; Fig. 2d). Notably overall survival was prolonged by the effects of arginine deprivation on T cells in these mice which otherwise develop Graft Versus Host Disease lethality from T cell responses to allo-antigens ($p = 0.049$; Fig. 2e).

Next we investigated the contribution of arginine metabolism on T cell responses in the trial patients. Arginine II can be regulated by histone deacetylases in nonmalignant cells, therefore we hypothesised that the AZA/VOR may also down-regulate arginase activity in AML blasts.10 Culture of AML cell lines with azacitidine and vorinostat did not decrease arginase
Figure 1. Azacitidine and Vorinostat induce an upregulation of Cancer-Testis Antigens in AML blasts. (a) AML patients treated with azacitidine and vorinostat have increased expression of MAGE, LAGE, RAGE, TRAG3 and SSX2 Cancer-Testis Antigens in AML blasts at Cycle 3 and Cycle 6 compared to at the time of study enrolment. Expression assessed by qRT-PCR in n = 40 patients. Baseline expression is shown by the red line (fold change 1). (b) Antigen-specific T cells from patients treated with azacitidine and vorinostat demonstrated increased IFN-γ release in response to CTAG peptide stimulation ex vivo. IFN-γ positive T cells were measured by ELISPOT. (c) T cells from AML patients have a reduced proliferative capacity compared to those from healthy donors, in response to CD3/CD28 antibody stimulation. (d) Expression of exhaustion marker PD1 was assessed on CD3+ T cells from the blood of AML patients during treatment. (e) MLL-AF9 AML engrafted mice have significantly reduced T cell numbers in the bone marrow and spleen compared to healthy controls as assessed by flow cytometry. (f) T cells from the bone marrow and spleens of engrafted mice have increased PD1 expression compared to healthy controls, as assessed by flow cytometry. (g) AML mice have a significant reduction in plasma arginine concentrations compared to healthy controls.
Figure 2. Arginase activity inhibits allogenic T cell responses. (a) Recombinant arginase leads to inhibition of T cell proliferation in allogeneic mixed leukocyte reactions. Five representative donors shown. (b) Culture of T cells from healthy donors in complete media (R10%) or arginine depleted media leads to a significant reduction in IFN-γ release in response to CD3/CD28 antibody stimulation. (c) Administration of recombinant arginase to NOG-SCID mice engrafted with human lymphocytes leads to a significant reduction in plasma arginine. (d) Recombinant arginase leads to a significant reduction in the frequency of T cells in the spleens of NOG-SCID mice engrafted with human lymphocytes, as assessed by flow cytometry. (e) Kaplan–Meier survival curves showing NOG-SCID mice engrafted with human T cells have increased survival after treatment with recombinant arginase. (f) Arginase activity of AML cell lines after treatment with azacitidine and/or vorinostat. (g) Concentration of arginine in the supernatants of AML cell lines after treatment with azacitidine and/or vorinostat.

Mussai et al. Int. J. Cancer: 00, 00–00 (2019) © 2018 The Authors. International Journal of Cancer published by John Wiley & Sons Ltd on behalf of UICC
enzyme activity (Fig. 2f) or supernatant arginine concentrations (Fig. 2g). Consistent with this, analysis of patients’ AML blasts demonstrated no change in Arginase II over time (Fig. 3a). Patients maintained an increase in plasma Arginase II enzyme (Fig. 3b, $p = 0.0002$) and significant reduction in arginine concentrations (Fig. 3c, $p = 0.0001$) throughout the trial. Therefore, the arginine deplete environment remains throughout therapy and can inhibit antigen-dependent T cell responses in vitro and in vivo.

**Targeting arginine metabolism enhances T cell immunotherapy responses**

Arginine catabolism appears to be a critical factor in holding back the immune response to antigen on AML blasts. We therefore investigated if targeting Arginase II enzyme activity could enhance the T cell response in vitro. L-NMMA and NOHA are two arginine analogues which can lead to reversible inhibition of arginine metabolism in vitro. Human T cells and purified AML blasts were first co-cultured, in the presence of stimulatory allogeneic dendritic cells. AML blasts inhibited T cell proliferation replicating our murine findings. However, inhibition of arginase activity with L-NMMA and L-NOHA led to restoration of T cell proliferation. (Supporting Information Fig. S3a).

NY-ESO-1 is an established AML-associated cancer-testis antigen that can be presented by HLA-A2 positive blasts (Supporting Information Fig. S3b).$^6$ Patient antigen-specific CTAG responses can be modelled through the selection and expansion of anti-NY-ESO T cells ex vivo, a process latterly used to generate adoptive immunotherapy. In co-cultures engineered anti-NY-ESO T cells alone had no effect on K562 AML viability, including after K562 cells were pretreated with azacitidine and vorinostat (Fig. 3d). Although no additional upregulation of NY-ESO was detected on K562 (Supporting Information Fig. S3C), the arginase inhibitors enhanced engineered anti-NY-ESO T cell cytotoxicity, leading to a further significant reduction in the number of viable K562 cells ($p = 0.001$; Fig. 3d).

We hypothesised that arginine metabolism may be similarly contributing to the failure of other antigen-specific T cell immune therapies. Clinical responses with CAR-T cell therapy for AML have lagged behind the notable successes in Acute Lymphoblastic Leukaemia, despite the presence of viable antigenic targets.$^{11,12}$ The mechanism of failure is poorly understood. CAR-T cell proliferation was assayed in normal and low arginine conditions. Low arginine conditions significantly inhibited CAR-T cell proliferation (Fig. 3e). Incubation of anti-CD33 CAR-T cells with K562 led to a reduction in the number of viable AML cells ($p = 0.022$; Fig. 3f). Inhibition of arginine metabolism enhanced CAR-T cell cytotoxicity against the AML cells and led to a further significant reduction in viable AML cells ($p = 0.003$; Fig. 3f) Azacitidine and vorinostat did not affect CAR-T cell cytotoxicity. Therefore, like autologous immune responses, engineered T cells have the capacity to recognise and kill AML blasts but their full potential is not realised until arginine metabolism is targeted and inhibited.

**Discussion**

The aberrant expression of Cancer-Testis Antigens offers the potential to use a patient’s immune system, or engineered immune therapies, to target AML. A number of CTAs including SCPI, MAGE, SPAN-Xb, SLLP1 and PRAME CTAs have been identified in unmanipulated AML blasts and may correlate with an improved overall survival.$^{13–15}$ In the RAAVA Phase II trial patients, we showed that azacitidine and vorinostat increased expression of a number of CTAGs not previously reported such as LAGE, TRAG3 and SART3. Circulating T cells demonstrated the capacity to be activated in response to target antigen, thus epigenetic modifiers can act as immune modulators. However, as in previous clinical studies, two observations are pertinent which have not been adequately justified to date. The first is that the number of CTAG responding T cells is small despite the large blast and antigenic burden. In the case of MAGE, these antigen-specific T cells may constitute less than 1% of the total CD8+ T cell pool, an insignificant number considering the rapid rate of leukaemia cell division. The second observation is that despite the presence of antigen and circulating responsive T cells there is no correlation with overall response to epigenetic modifying drugs. For adoptive T cell combinations, epigenetic modulation has mostly been tested in solid tumours, with only one phase I trial reporting encouraging results using decitabine to boost a NY-ESO-1 vaccine for human ovarian cancer.$^{16–18}$

Our findings are the first to report the clinical impact of the low arginine microenvironment on immune therapy approaches in patients, and extend our preclinical findings on the role of tumour arginine metabolism in suppressing immunity.$^{4,19}$ In the case of AML blasts, arginine catabolism is a function of their Arginase II enzyme,$^9$ in combination with a failure to express a full complement of arginine recycling enzymes, making blasts reliant on extracellular sources of arginine.$^{20}$ The increased metabolic requirement for arginine by activated T cells is clear, with arginine also contributing to T cell survival through BAZ1B, PSIP1 and TSN signalling.$^{21}$ In our study, no correlation of T cell responses and patient clinical outcome was seen, suggesting that any immune responses are inadequate in the low arginine microenvironment. Attempts to reactivate autologous immunity or enhance cellular immunotherapy therefore require not only availability of antigen expression but must also address the immunosuppressive metabolic microenvironment which acts as constraint to cellular expansion. Although epigenetic modifying drugs may influence multiple pathways, the study demonstrated they cannot help to downregulate Arginase II expression.

Our study has important translational implications and help to explain the functional impairment in T cells seen in trials of peptide vaccines in AML patients or more recently with CAR-T cells.$^{22}$ A more direct approach to metabolic
Figure 3. Inhibition of arginine metabolism enhances T cell immunotherapy cytotoxicity against AML. (a) AML patients treated with azacitidine and vorinostat have no significant changes in Arginase II expression in AML blasts at Cycle 3 and Cycle 6 compared to at the time of study enrolment. Expression assessed by qRT-PCR. (b) Plasma Arginase II concentrations are elevated in AML patients, compared to healthy controls, and are not significantly altered after cycles of azacitidine and vorinostat treatment. (c) Plasma arginine concentrations are decreased in AML patients, compared to healthy controls, and are not significantly altered after cycles of azacitidine and vorinostat treatment. (d) Anti-NY-ESO antigen-specific T cells were cultured with K562 (target) AML cells. The numbers of viable K562 cells was significantly reduced if antigen-specific T cells are used in combination with inhibitors of AML arginine metabolism (+inhibitors: L-NMMA and NOHA). Pretreatment with azacitidine and vorinostat did not affect cytotoxicity. (e) Anti-CD33 CAR-T cell proliferation is inhibited when cultured in arginine depleted media. CAR-T cell numbers were counted by flow cytometry after 72 hr. (f) Anti-CD33 CAR-T cells were cultured with K562 (target) AML cells. The numbers of viable K562 cells were reduced by CAR-T cells alone. The numbers of viable K562 cells was further reduced if AML arginine metabolism is inhibited (+inhibitors: L-NMMA and NOHA). Pretreatment with azacitidine and vorinostat did not affect cytotoxicity.
modulating therapy would be to target the Arginase II enzyme’s active site. A multitude of novel inhibitors have sought to target arginase enzymes, mostly with limitations such as reversibility, a failure to cross the cell membrane, or the need to block both Arginase and INOS enzymes if present in cancer cells.\textsuperscript{23,24} The most clinically advanced arginase inhibitor is CB-1158 (Calithera), an orally active agent that is undergoing clinical trial alongside checkpoint inhibitors in patients with advanced solid tumours (NCT02903914). Alternatives could include screening of patients’ plasma for arginine concentrations to ensure T cell therapies are administered at an optimal time point, or administration of T cell therapies with an arginine supplemented diet. The later approach has been used with mixed results to enhance healing postsurgery, but may have untoward effects of feeding tumour metabolism. As a number of cancers which are targets for T cell immunotherapies deplete arginine through aberrant arginine recycling pathways, future strategies should seek to urgently address this significant metabolic challenge.\textsuperscript{25}

Acknowledgements
The authors thank the patients who contributed samples to the study. Thank you to Jane Cooper, Cay Shakespeare, Andrea Hodgkinson and research nurses for collection of patient samples. Thank you to Nemouela Gbandi and the CRUK Clinical Trials Unit team for coordination of clinical data. We thank BCTI for provision of BCT-100.

Author contributions
F.M. and C.D.S designed the study, performed research, analysed data and wrote the study. R.W., S.B., L.F., E.S., V.S., T.P., K.K. performed research. C.C. provided access to patient samples as Chief Investigator of the RAVVA trial. F.M., C.C., K.K., and P.K. secured ethical approval for the study.

REFERENCES
1. Dombret H, Seymour JF, Butrym A, et al. International phase 3 study of azacitidine vs conventional care regimens in older patients with newly diagnosed AML with >30% blasts. Blood 2015;126:291–9.
2. Garcia-Manero G, Tambaro FP, Bekele NB, et al. Phase II trial of vorinostat with idarubicin and cytarabine for patients with newly diagnosed acute myelogenous leukemia or myelodysplastic syndrome. J Clin Oncol 2012;30:2204–10.
3. Atanackovic D, Luetkens T, Kloth B, et al. Cancer-testis antigen expression and its epigenetic modulation in acute myeloid leukemia. Am J Hematol 2011;86:918–22.
4. Mussai F, De Santis C, Abu-Dayyeh I, et al. Acute myeloid leukemia creates an arginase-dependent immunosuppressive microenvironment. Blood 2013;122:749–58.
5. Craddock CF, Houlton AE, Quek IS, et al. Outcome of Azacitidine therapy in acute myeloid leukemia is not improved by concurrent Vorinostat therapy but is predicted by a diagnostic molecular signature. Clin Cancer Res 2017;23:6430–40.
6. Goodyear O, Agathanggelou A, Novitzky-Basso I, et al. Induction of a CD8+ T-cell response to the MAGE cancer testis antigen by combined treatment with azacitidine and sodium valproate in patients with acute myeloid leukemia and myelodysplasia. Blood 2010;116:1908–18.
7. McLarnon A, Piper KP, Goodyear OC, et al. CD8 (+) T-cell immunity against cancer-testis antigens develops following allogeneic stem cell transplantation and reveals a potential mechanism for the graft-versus-leukaemia effect. Haematologica 2010;95:1572–8.
8. Nagorsen D, Scheibenbogen C, Schaller G, et al. Differences in T-cell immunity toward tumor-associated antigens in colorectal cancer and breast cancer patients. Int J Cancer 2003;105:221–5.
9. Zhang Q, Hosain DM, Duttagupta P, et al. Serum-resistant CpG-STAT3 decoy for targeting survival and immune checkpoint signaling in acute myeloid leukemia. Blood 2016;127:1687–700.
10. Pandey D, Sikka G, Bergman Y, et al. Transcriptional regulation of endothelial arginase 2 by histone deacetylase 2. Arterioscler Thromb Vasc Biol 2014;34:1556–66.
11. Kenderian SS, Ruella M, Shenvato O, et al. CD33-specific chimeric antigen receptor T cells exhibit potent preclinical activity against human acute myeloid leukemia. Leukemia 2015;29:1637–47.
12. Wang QS, Yang Y, Lv HY, et al. Treatment of CD33-directed chimeric antigen receptor-modified T cells in one patient with relapsed and refractory acute myeloid leukemia. Mol Ther 2015;23:184–91.
13. Lim SH, Austin S, Owen-Jones E, et al. Expression of testicular genes in haematological malignancies. Br J Cancer 1999;81:1162–4.
14. Wang Z, Zhang Y, Mardal A, et al. The spermatid-specific Sellarf1, a novel cancer-testis antigen in hematologic malignancies. Clin Cancer Res 2004;10:6544–50.
15. Greiner J, Schmitt M, Li L, et al. Expression of tumor-associated antigens in acute myeloid leukemia: implications for specific immunotherapeutic approaches. Blood 2006;108:4109–17.
16. Kunert A, van Brakel M, van Steenbergen-Langeveld S, et al. MAGE-C2-specific TCRs combined with epitopic drug-enhanced antigenicity yield robust and tumor-selective T cell responses. J Immunol 2016;197:2541–52.
17. Terracina KP, Graham LJ, Payne KK, et al. DNA methyltransferase inhibition increases efficacy of adoptive cellular immunotherapy of murine breast cancer. Cancer Immunol Immunother 2016;65:1061–73.
18. Oudsi K, Matsuzaki I, James SR, et al. Epigenetic potentiation of NY-ESO-1 vaccine therapy in human ovarian cancer. Cancer Immunol Res 2014;2:37–49.
19. Mussai F, Egan S, Hunter S, et al. Neuroblastoma Arginase activity creates an immunosuppressive microenvironment that impairs autologous and engineered immunity. Cancer Res 2015;75:3043–53.
20. Mussai F, Egan S, Higginbotham-Jones J, et al. Arginase dependence of acute myeloid leukemia blast proliferation: a novel therapeutic target. Blood 2015;125:2386–96.
21. Geiger R, Reckmann JC, Wolf T, et al. L-arginine modulates T cell metabolism and enhances survival and anti-tumor activity. Cell 2016;167:829–42.e13.
22. Utenthal B, Martinez-Davila I, Ivey A, et al. Wilms’ tumour 1 (WT1) peptide vaccination in patients with acute myeloid leukaemia induces short-lived WT1-specific immune responses. Br J Haematol 2014;164:366–75.
23. Santilli G, Pietrowska I, Cantileana S, et al. Polyphenon [corrected] E enhances the anti-tumor immune response in neuroblastoma by inactivating myeloid suppressor cells. Clin Cancer Res 2013;19:1116–25.
24. Pudlo M, Demougeot C, Girard-Thernier C. Arginase inhibitors: a rational approach over one century. Med Res Rev 2017;37:475–513.
25. Fultang L, Vardon A, De Santis C, et al. Molecular basis and current strategies of therapeutic arginine depletion for cancer. Int J Cancer 2016;138:501–9.