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Cathepsin G is broadly expressed in acute myeloid leukemia and is an effective immunotherapeutic target

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Immunotherapy is among the novel classes of therapy in development for acute myeloid leukemia (AML) and harnesses the specificity of the immune system to eliminate leukemia cells.[1] This approach builds upon the success of allogeneic hematopoietic stem cell transplant (allo-SCT) in AML, which can be curative in up to 50% of patients, but often at the cost of high rates of treatment-related toxicities, including graft-vs-host disease (GVHD).[2] To avoid this complication, one approach is to refine the potent graft-versus-leukemia (GVL) effect to target discrete leukemia antigens in the form of adoptive cellular therapy (ACT). Importantly, in order to overcome intratumoral heterogeneity, durable

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clinical efficacy will likely require targeting multiple leukemia antigens simultaneously, an approach that is currently limited by the number of known effective leukemia antigens.[3]

We previously reported that cathepsin G (CG), an azurophil granule protease, represents a novel leukemia-associated antigen (LAA).[4] CG is largely restricted to the myeloid lineage and like proteinase 3 (P3) and neutrophil elastase (NE), the sources of the well-established HLA-A2 (i.e., HLA-A*0201) LAA PR1,[5] CG is contained within the primary granules of maturing and mature neutrophils. However, CG is expressed later in myeloid differentiation, resides under a different promoter than NE and P3, and demonstrates a distinct pattern of expression, suggesting that targeting both PR1 and CG could be synergistic.[6] In our prior work[4], we demonstrated that CG is highly expressed in primary patient AML blasts, AML cell lines, and, critically, in leukemia stem cells (LSCs). We showed that CG is localized outside azurophil granules and is ubiquitinated, favoring antigen presentation. Additionally, we identified an HLA-A2 restricted epitope within the leader sequence of CG, designated as CG1 (amino acid sequence FLLPTGAEA), and showed that targeting CG1 resulted in lysis of AML blasts *in vitro*. Finally, we detected cytotoxic T-lymphocytes (CTLs) specific for CG1 in the peripheral blood of AML patients after allo-SCT[4].

In this study, we further investigate the therapeutic potential of targeting CG, specifically CG1, focusing on the anti-leukemia activity of CG1-specific-CTL *in vivo* and evaluating their toxicity against normal hematopoietic progenitor cells (HPCs). In addition, we analyze the expression of CG and its presentation by HLA-A2 in primary patient AML blasts through reverse-phase protein array (RPPA) and liquid chromatography (LC)/tandem mass spectrometry (MS/MS).

To determine the role of CG as an immunotherapeutic target *in vivo*, we first studied a patient-derived xenograft (PDX) treatment model of primary AML (UPN#1; Table 1). After confirming leukemia engraftment (~ 3 weeks), NSG mice were treated intravenously with either 0.5×10^6 CG1-CTL or HIV-CTL, or were left untreated. At the time of sacrifice, bone marrow (BM) was harvested and analyzed for leukemia burden. Mice treated with CG1-CTL had a significantly lower AML burden in the BM (24.94 % ± 4.451%; n = 13) compared with HIV-CTL-treated mice (61.46% ± 11.07%; n = 10) and untreated mice (87.9% ± 5.632%; n = 12) ($P < 0.01$; Figure 1a). The efficacy of targeting CG *in vivo* using CG1-CTL was also recapitulated in a murine model that incorporated the HLA-A2-transduced U937 myelomonoblastic leukemia cell line (U937-A2) (Supplementary Figures S1-S2).

We next sought to confirm that the CG1 peptide is presented on the HLA-$A2^+$ AML cell surface. We used W6/32 antibody to immunoprecipitate (IP) surface HLA Class I molecules and their bound peptides, which were isolated and analyzed via high sensitivity targeted LC-MS/MS. Blasts from seven newly diagnosed acute leukemia patients, including AML (n=4), ALL (n=2), and biphenotypic leukemia (n=1), as well as HL-60 and U937 cell lines and their HLA-A2 transfected counterparts were studied in this analysis. CG1 was identified in the eluted fraction in 3 of 4 patient AML cases (Table 1), including UPN #1 whose disease was used in the PDX model. CG1 was also identified on the surface of blasts from patient UPN #6 with precursor B-ALL, which is in agreement with two studies that confirmed CG expression and validated CG as an immunotherapeutic target in lymphoid malignancies.[7, 8]

In addition, CG1 was eluted from the surface of both HL-60-A2 and U937-A2[4] but not from either wild type cell line or from HLA-A2 negative UPN #5 (Supplementary Figure S3). Taken together, these data confirm that CG1 is naturally processed and presented on the surface of HLA*0201-positive leukemic blasts and cell lines, reinforcing its potential as a leukemia-associated antigen.

We previously demonstrated that primary AML blasts and $CD34^{+}38^{-}$ LSCs have higher expression of CG than normal HPC and are preferentially eliminated in *in vitro* cytotoxicity assays.[4] Nevertheless, since CG, like the LAAs NE and P3, is also expressed in healthy myeloid progenitor cells[6], we investigated the potential toxicity of CG1-CTL against the formation of typical colonies from healthy donor bone marrow (HDBM) progenitor cells using colony-forming unit (CFU) assays. HDBM (n=5) was cultured in methylcellulose semi-solid matrix either alone or in the presence of HIV-CTL or CG1-CTL (Figure 1b). After 7 days, the mean CFUs were similar among the untreated and the two treatment groups (195.6 ± 66.7 for untreated vs. 234.5 ± 109.5 for HIV-CTL vs. 259.1 ± 118.2 for CG1-CTL). The CFUs for HDs 3-5 were followed for an additional 7 days, and at day 14, the differences among the 3 groups remained non-significant. These data suggest that CG1-CTL, with a range of avidities, do not significantly impair normal hematopoiesis.

Next, given The Cancer Genome Atlas (TCGA) data demonstrating highest CG transcript expression across a diverse set of AML cases (Supplementary Figure S4), we utilized RPPA to study the expression of CG protein in 511 newly diagnosed AML patients, a cohort that has been described previously.[9] As shown in Figure 1c, CG was variably expressed across patients and the mean CG protein level was higher in APL and AML patients compared with normal $CD34^{+}$ HPC (mean Log_2 0.47 vs. mean Log_2 - 0.01 vs. mean Log_2 $^{-}$0.34, respectively; $P = 0.0375$ for AML vs. $CD34^{+}$ HPC). CG expression was higher in AML blasts than in HD $CD34^{+}$ cells in 230 samples, equal to normal $CD34^{+}$ cells in 234 samples and less than normal $CD34^{+}$ cells in 47 samples. We also specifically studied CG levels in the 47 patients from our cohort who experienced relapsed AML and for whom paired samples were available at initial diagnosis and at relapse. Overall, CG level was higher in relapsed disease than at diagnosis (mean Log_2 0.46 vs. mean Log_2 $^{-}$0.01, $P < 0.001$) and in 33 of the 47 (70%) patients with paired samples, CG levels were higher in the relapse sample (Figure 1d). These results confirm that CG is broadly expressed across AML cases and suggest that patients with relapsed disease may be candidates for CG-targeting therapies given their relatively high CG expression.

Although CG has been linked with aggressive behavior in solid tumors,[10] the prognostic role of CG in AML patients has not been reported. Analysis of our RPPA data revealed a significant association between CG levels and overall survival (OS) in our patient cohort. In 415 patients for whom survival information was available, patients with CG levels in the lower tertile (as well as those below the median) showed significantly better OS than those whose CG expression ranked in the upper 2/3 ($P = 0.04$) (Figure 1e). The survival advantage associated with low CG level was most pronounced in the subset of AML patients with intermediate cytogenetics and FLT3 mutations (Supplementary Figure S5), a prevalent subset of patients for whom the optimal treatment of leukemia in first remission is controversial.[11]

In this report, we validate CG as an immunotherapeutic target in myeloid leukemia. We demonstrate that targeting CG *in vivo* with CG1-CTL reduces the leukemia burden in NSG mice. In addition, even though CG is expressed in normal hematopoietic cells, similar to other LAAs[12, 13], targeting CG does not inhibit normal hematopoiesis. We postulate that CG1-CTL preferentially target leukemia over HPCs, in part, because CG has greater access to the MHC processing and presentation components within leukemic cells, since we previously showed that CG is located outside of azurophil granules and is ubiquitinated preferentially in AML.[4]

Critically, we also provide direct evidence that the HLA-A2 restricted peptide CG1 is naturally processed presented by primary AML. Lastly, we report that CG is expressed in a large cohort of AML patients and that high CG expression correlates with poor outcomes. We speculate that this association may be due to the potential interaction of CG with oncogenic proteins whose expression levels closely correlate with that of CG (Supplementary Figure S6). For example, there was a correlation between CG and the expression/phosphorylation of members of the Hippo pathway, TAZ and YAP1, which have been shown to play a role in cancer, including AML.[14, 15]

We have successfully targeted the LAA PR1 using various strategies including vaccination, T-cell based ACT, and a TCR-like antibody.[5] We intend to apply similar strategies in the clinic to target CG1, and potentially other CG peptides, ideally in the autologous setting to minimize the risk of GVHD. We recognize that the autologous setting may yield lower affinity CG1-CTL, which could provide a therapeutic advantage clinically in that these CG1-CTL may preferentially lead to killing of high CG1-expressing AML, while sparing normal tissues. However, we also recognize the value of high affinity tumor antigen specific CTL in that they provide potent tumor killing, albeit sometimes at the expense of unwanted toxicity. In conclusion, these data lay the foundation for development of CG-targeting immunotherapies in AML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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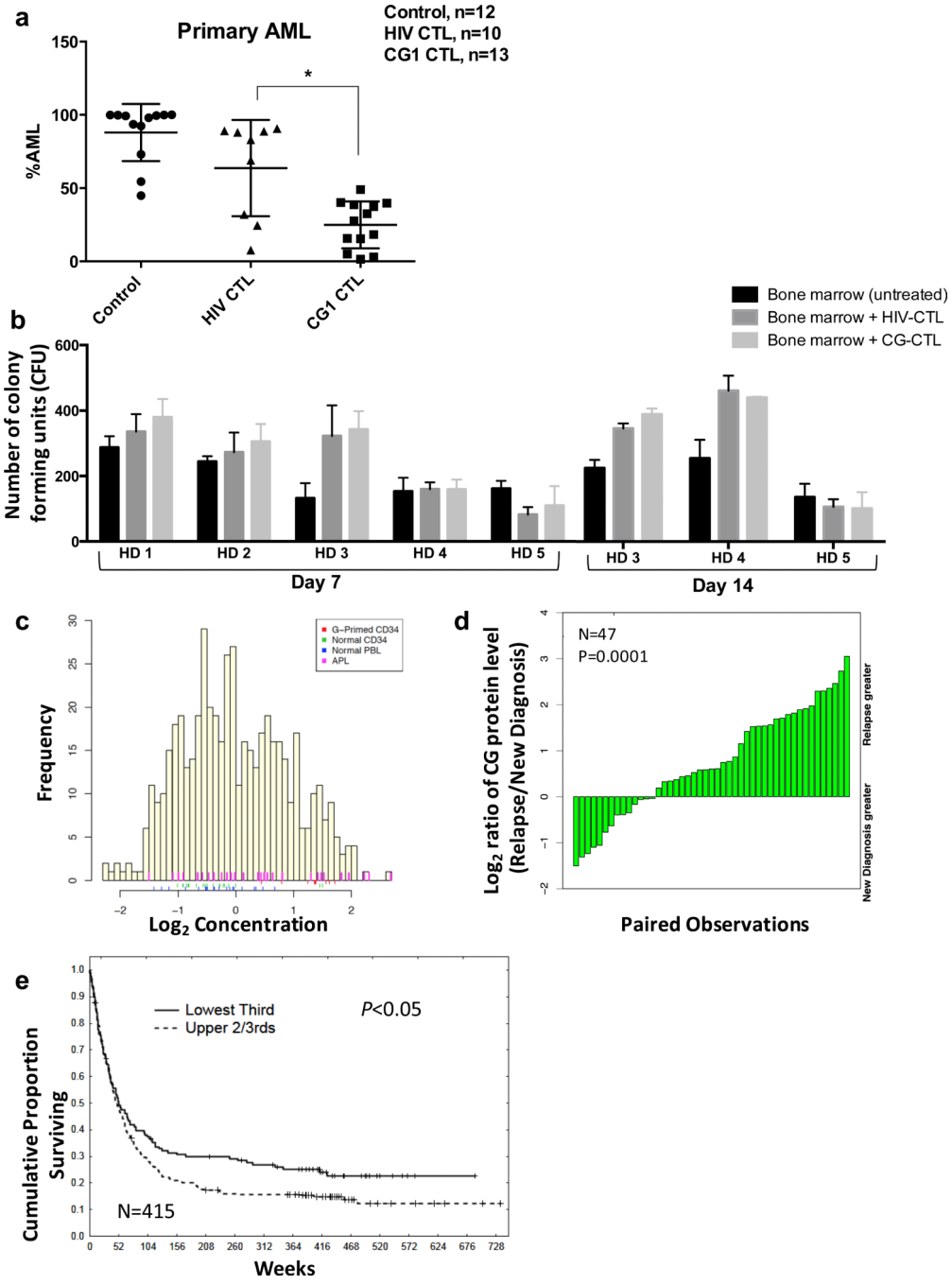

Figure 1.

Cathepsin G is an effective immunotherapeutic target *in vivo* and is broadly expressed in AML patients. (**a**) Irradiated NSG mice were injected intravenously with human primary AML blasts (UPN#1; 7×10^6 blasts) on day 0. After confirming leukemia engraftment (~ 3 weeks), mice were treated with either negative control HIV-CTL (0.5×10^6), CG1-CTL (0.5×10^6) or were left untreated. Mice were sacrificed for all groups when any mouse became moribund or during week 7. The results are expressed as percentage of $CD33^+/CD3^-$ cells from viable $hCD45^+/mCD45^-$ population within the bone marrow. Results reflect 4

independent experiments; *P<0.01. (**b**) Bone marrow from 5 healthy donors (HD 1-5) was cultured alone (untreated) or co-cultured with HIV-enriched T cells (HIV-CTL) or CG1-enriched T cells (CG1-CTL) at a 1:5 ratio for 4 hours in cell media. Cells were then resuspended in methylcellulose semi-solid matrix and co-cultured for 7 days. On day 7, colonies were counted and imaged; for HD 3, 4 and 5, colonies were again counted on day 14. Each group was cultured in triplicate and data represent 4 independent experiments. (**c**) Reverse-phase protein array (RPPA) was used to quantify protein levels of cathepsin G in blasts from 511 newly diagnosed AML patients (yellow bars) and 21 newly diagnosed APL patients (pink bars). Controls included healthy donor $CD34^+$ progenitor cells (n=21, green bars), healthy donor peripheral blood lymphocytes (n=21, blue bars), and GM-CSF-primed healthy donor $CD34^+$ progenitor cells (n=10, red bars). (**d**) Cathepsin G levels were assessed for 47 patients at diagnosis and relapse and were compared by paired t-test (P=0.0001). (**e**) Kaplan-Meier plots showing OS in AML patients (n=415) comparing patients with high CG protein expression by RPPA (upper 2/3) to patients with low CG expression (lowest 1/3). Results are significant by Cox univariate model testing (P= 0.04).

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Table 1

AML patient samples used in liquid chromatography tandem mass spectrometry (LC-MS/MS). UPN #1 patient blasts were utilized in the patient-derived xenograft (PDX) treatment model.

Sample	CG1	Leukemia	Cytogenetic/Molecular Abnormalities	HLA-A typing	Phenotype
UPN #1	yes	FAB-M1	Diploid 46 XX; FLT3-TD; NPM1$^+$; DNMT3A$^+$	A02:01, A24:02	CD13/33$^+$; MPO$^+$
UPN #2	yes	FAB-M1	Pseudodiploid 46XY; del (5q); CEBPA$^+$	A02:01, A03:01	CD33/34/38/117$^+$; HLA-DR$^+$, MPO$^+$
UPN #3	yes	FAB-M4/M5	not tested	A02:01	not tested
UPN #4	no	FAB-M4	Diploid 46XY; FLT3-TD; NPM1$^+$	A02:01; A32:01	CD13/33; MPO$^+$; HLA-DR$^+$
UPN #5	no	Biphenotypic	Diploid 46XY; t(9;22); BCR-ABL1	A24:02; A33:01	CD13/19d/33/34/38$^+$; MPO$^+$; HLA-DR$^+$
UPN #6	yes	B-ALL	Diploid 46XX; t(9;22); BCR-ABL1	A02:01; A24:02	CD10/13/19/33/34/38$^+$; HLA-DR$^+$
UPN #7	no	B/T-ALL	Hyperdiploid 50XY	A02:01, A03:01	CD13/19/33/34/38$^+$; HLA-DR$^+$
U937	no	Monocytic	Complex karyotype (>5 cytogenetic abnormalities)	A03:01; A31:12	CD13, CD33, CD15, CD11b, CD18
U937-A2	yes	Monocytic	Complex karyotype (>5 cytogenetic abnormalities)	A03:01; A31:12; A0201	CD13, CD33, CD15, CD11b, CD18
HL-60	no	APL	Complex karyotype (>5 cytogenetic abnormalities)	A01:01 *	CD13, CD33, CD38, CD117, MPO$^+$
HL-60-A2	yes	APL	Complex karyotype (>5 cytogenetic abnormalities)	A01:01; A02:01	CD13, CD33, CD38, CD117, MPO$^+$

Abbreviations: UPN, unique patient number; FAB, French-American-British; APL, acute promyelocytic leukemia; MPO, myeloperoxidase.

* Wild-type HL-60 cell line is missing a haplotype, hence only expresses one allele at the HLA-A locus.