A Novel HLA-A*0201 Restricted Peptide Derived From Cathepsin G Is An Effective Immunotherapeutic Target in Acute Myeloid Leukemia

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Abstract

Purpose: Immunotherapy targeting aberrantly expressed leukemia associated antigens (LAA) has shown promise in the management of acute myeloid leukemia (AML). However, because of the heterogeneity and clonal evolution that is a feature of myeloid leukemia, targeting single peptide epitopes has had limited success, highlighting the need for novel antigen discovery. In this study, we characterize the role of the myeloid azurophil granule protease cathepsin G (CG) as a novel target for AML immunotherapy.

Experimental Design: We used Immune Epitope Database and in vitro binding assays to identify immunogenic epitopes derived from CG. Flow cytometry, immunoblotting and confocal microscopy were used to characterize the expression and processing of CG in AML patient samples, leukemia stem cells and normal neutrophils. Cytotoxicity assays determined the susceptibility of AML to CG-specific cytotoxic T lymphocytes (CTL). Dextramer staining and cytokine flow cytometry were performed to characterize the immune response to CG in patients.

Results: CG was highly expressed and ubiquitinated in AML blasts, and was localized outside granules in compartments that facilitate antigen presentation. We identified five HLA-A*0201 binding nonameric peptides (CG1-CG5) derived from CG, and demonstrated immunogenicity of the highest HLA-A*0201 binding peptide, CG1. We showed killing of primary AML by CG1-CTL, but not normal bone marrow. Blocking HLA-A*0201 abrogated CG1-CTL mediated cytotoxicity, further confirming HLA-A*0201 dependent killing. Finally, we demonstrated functional CG1-CTLs in peripheral blood from AML patients following allogeneic stem cell transplantation.

Conclusion: CG is aberrantly expressed and processed in AML and is a novel
immunotherapeutic target that warrants further development.

**Statement of Translational Relevance**

Acute myeloid leukemia (AML) is an aggressive and clinically challenging malignancy. Although hematopoietic stem cell transplantation (HSCT) can be curative in AML, it carries a high rate of treatment-related mortality and morbidity. In order to minimize the toxicity of HSCT while taking advantage of the graft-versus-leukemia effect, leukemia-associated antigens (LAAs) have been identified and targeted successfully with immunotherapy. However, due to leukemia heterogeneity and tumor antigen loss, a mechanism of immune evasion by malignant cells, it is highly unlikely that any single antigen will be consistently expressed in all leukemias. This highlights the need for leukemia antigen discovery. In this study, we characterize a novel LAA, cathepsin G (CG). We show that CG is aberrantly expressed and processed by AML blasts and stem cells and that it can be targeted effectively in AML. These results provide preclinical evidence for the development of immunotherapeutic strategies to target CG in AML.
Introduction

Despite advances that have been made in the management of myeloid leukemia, the prognosis of patients with acute myeloid leukemia (AML) and blast-phase or tyrosine kinase inhibitor (TKI)-resistant chronic myeloid leukemia (CML) remains dismal (1, 2). This highlights the need for the identification of novel therapeutic strategies for the management of this group of aggressive diseases. Chemotherapy and TKIs remain the cornerstone therapies for the treatment of patients with AML and CML, respectively, while allogeneic stem cell transplantation (allo-SCT) is the main immunotherapeutic modality for patients with aggressive forms of AML and blast-phase or TKI-resistant CML. However, because of its significant toxicity, allo-SCT is reserved for patients with aggressive or relapsed disease. In addition to allo-SCT, immunotherapy in the form of vaccines and antibodies has demonstrated efficacy in the management of patients with AML and CML, but for the most part remains investigational.

In order to minimize the toxicity of allo-SCT while taking advantage of the graft versus leukemia (GvL) effect, numerous leukemia-associated antigens (LAAs) including PR1 (3), WT1 (4) and RHAMM (5), and leukemia-specific antigens such as BCR-ABL (6) have been identified and shown to elicit leukemia-specific immune responses. However, because of the heterogeneity of myeloid leukemia (7-9) and since cancerous cells can alter antigen expression to evade the immune system (10-12), it appears that targeting a single antigen is insufficient to completely eradicate malignantly transformed cells. Studies have been conducted targeting multiple epitopes from a single antigen (13-15) or simultaneously targeting different antigens (16, 17), with promising results. These data, together with the distinct susceptibility of myeloid leukemia to immunotherapy as
evidenced by the success of allo-SCT, emphasize the need to identify novel antigens that can be targeted individually or as a part of combination immunotherapeutic approaches.

Cathepsin G (CG) is a serine protease restricted to cells of myeloid lineage and is expressed within myeloid azurophil granules along with neutrophil elastase (NE) and proteinase-3 (P3); the latter two proteases are the source proteins for the PRI epitope. CG is involved in host immunity, cleavage of inflammatory mediators and receptors, and degradation of extracellular matrix components (18, 19). Like NE and P3, high level of CG transcription is seen at the promyelocytic stage of granulocyte development (20). Furthermore, CG is overexpressed in myeloid leukemia blasts (21), and was shown to be immunogenic in autoimmune disease (22), making it an ideal candidate immunotherapeutic target. These prior reports, including the study by Papadopoulos et al.(21) that demonstrated high expression and natural processing of the HLA-A*0201 restricted CG-derived peptide FLLPTGAEA on the surface of CD34+ blasts from one patient with CML, together provide the impetus for further investigating the immunotherapeutic potential of targeting CG in AML.

Since we previously showed that NE and P3 are aberrantly expressed by myeloid leukemia blasts (23), and because CG is also a component of azurophil granules, we investigated whether CG is aberrantly expressed in AML, which could make it an ideal target for leukemia immunotherapy. In this report, we identify CG1 (FLLPTGAEA), a naturally processed HLA-A*0201 restricted peptide derived from CG, in AML and demonstrate its high affinity to HLA-A*0201. We show that CG is aberrantly expressed in AML blasts and stem cells, and that CG1-CTLs specifically kill CG-expressing leukemia targets. Finally, we show functional CG1-CTLs in patients with AML.
following allo-SCT. Together, our data suggest an important role for targeting CG in the therapy of AML.
Materials and Methods

Patients, cells, and cell lines

Patient and healthy donor samples were obtained after appropriate informed consent through an institutional review board approved protocol at the University of Texas MD Anderson Cancer Center. The AML samples were collected between 2006 and 2012 and were selected based on their AML subtype, percent blasts, sample viability and HLA status. U-937 (myelomonoblastic leukemia) and T2 (B-cell/T-cell hybridoma) cell-lines were obtained from American Type Culture Collection (ATCC). Cell lines were cultured in RPMI-1640 with 25mM HEPES+L-Glutamine (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products), Penicillin (100 U/mL)/ Streptomycin (100 μg/mL) (Cellgro) and were kept in 5% CO₂ at 37 °C. Cell lines were authenticated by DNA finger-printing at MD Anderson Cancer Center within six months of use in experiments. Healthy individual and patient peripheral blood mononuclear cells (PBMC), bone marrow and granulocytes, were enriched using standard Histopaque 1077 or 1119 (Sigma) gradient centrifugation. Patient leukemia blast samples were collected at the time of original diagnosis.

Western blotting and immunoprecipitation (IP)

Whole cell lysates (WCL) from AML samples were generated using standard methodology with RIPA buffer (50 mM TrisHCl (pH 8); 150 mM NaCl; 1% NP-40; 0.1% SDS; 0.5% sodium deoxycholate). Purified CG (Sigma) and P3 (Athens Research and Technology) were used as positive controls; U-937 WCL was used as NE positive
control. For IP reactions, anti-CG antibody (Abcam) was added to pre-cleared WCLs and incubated overnight at 4°C. WCL and IP products were separated by electrophoresis on 10% SDS gels under reducing conditions, transferred onto PVDF membranes, blocked with 5% milk and probed with anti-CG (Abcam), anti-NE (Santa Cruz), anti-P3 (NeoMarkers), anti-ubiquitin (Santa Cruz) or anti-actin (Millipore) antibodies. Chemiluminescence was captured on Kodak film and digitally using Molecular Imager ChemiDoc XRS+ (BIO-RAD).

**CG peptide binding**

Immune epitope database (IEDB) binding algorithms were used to identify CG peptides with highest binding affinities [www.immuneepitope.org](http://www.immuneepitope.org) (24). Peptide binding was subsequently confirmed using standard peptide-T2 binding assays (25, 26). Briefly, T2 cells were washed and then incubated at 37 °C in serum-free media containing increasing concentrations of CG-peptides (BioSynthesis) (Supplementary Table S1) or PR1 peptide. After 90 minutes, cells were washed and stained with the FITC-conjugated anti-HLA-A*0201 antibody BB7.2 (Becton-Dickinson [BD]) to determine the stabilization of peptide/HLA-A*0201 on the T2 cell surface. Flow cytometry was performed using the BD FACSCanto II (BD). Data were analyzed using FlowJo software (Tree Star Inc.).

The affinity of CG1 for HLA-A*0201 and the relative stability of the peptide-HLA complexes were measured using the iTopia Epitope Discovery System (Beckman Coulter), following the manufacturer’s protocol. Both assays use a FITC-conjugated
anti-HLA antibody that binds to the correctly folded HLA-peptide complex and a positive control peptide (FLPSDFFPSV) for comparison with the test peptide. For the affinity assays, peptides were incubated in HLA-A*0201 coated wells at concentrations ranging from $10^{-4}$ to $10^{-8}$ M at 21°C overnight, in the presence of the anti-HLA antibody. After washing the wells to remove unbound antibody and peptide, fluorescence was read on a Synergy 2 microplate reader (BioTek) with the excitation set at 485 nm and emission detected at 528 nm. Results were graphed relative to the binding of the positive control peptide at $10^{-4}$ M, and the ED$_{50}$ was determined using GraphPad Prism’s nonlinear regression ‘log (agonist) versus response –variable slope (four parameter)’ curve. For the off-rate assay, peptides were incubated in HLA-A*0201 coated wells at a concentration of 11 μM at 21°C overnight, then washed and incubated at 37°C to allow the peptides to dissociate. Wells were washed again at the times indicated on the graph to remove dissociated peptide and antibody, and fluorescence was read on the microplate reader. Results were graphed relative to the positive control peptide as 100% binding at each time point. The t$_{1/2}$ was calculated using GraphPad Prism’s nonlinear regression, ‘dissociation – one phase exponential decay’ curve (27).

**Fluorescent confocal microscopy and flow cytometry analysis**

To determine intracellular CG expression, cells were washed, fixed with 4% paraformaldehyde, permeabilized and stained with Alexa-647 directly conjugated anti-CG antibody (Santa Cruz); we used the Alexa-647 conjugation kit (Invitrogen) to conjugate anti-CG to fluorophore. Aqua live/dead stain (Invitrogen) was used to assess viability in flow cytometry experiments. For confocal imaging, after staining, cells were
resuspended in ProLong Gold antifade reagent with dapi (Invitrogen). Confocal imaging was performed using Leica Microsystems SP2 SE confocal microscope (Leica) with 10x/25 air, 63x/1.4 oil objectives. Leica LCS software (version 2.61) was used for image analysis. Flow cytometry was performed using the Cytomation CyAn flow cytometer (Dako) and analyzed using FlowJo software (Tree Star Inc.).

Prior to intracellular CG staining of stem cells, leukemia and normal donor samples were stained with antibodies targeting CD34, CD38 and the lineage (Lin) markers CD3, CD14, CD16 (all from BD) and CD19 (eBioscience). Lin⁻ CD34⁺ CD38⁻ stem cells (28) were sorted using Influx cell sorter (BD). Because of their low frequencies, normal stem cells were FACS sorted from one normal donor bone marrow and pooled with sorted stem cells from three GM–CSF mobilized normal donor apheresis samples.

To determine the location of CG within AML and normal granulocytes (i.e. granular vs. extragranular) and the level of CG expression in stem cells, after staining for CG cells were imaged using BD Pathway 435 (BD) cell imager, 60x Olympus objective, and then analyzed using AttoVision software (BD). To determine the intracellular distribution of CG, regions of interest (ROI) were drawn around each cell. The ratio of the dimmest:brightest 10% of pixels per cell was calculated (29). To determine CG staining intensity, mean fluorescence intensity in each ROI was measured.

**Peptide-specific CTL lines**

Peptide-specific CTLs were expanded by stimulating PBMCs from healthy HLA-A*0201 individuals with peptide in vitro, as previously described (3, 30). Briefly, T2
cells were washed in serum-free RPMI 1640 medium and incubated with CG1 or negative control peptide PR1 at 20 µg/mL for 90 minutes at 37°C. Peptide-loaded T2 cells were irradiated with 7500 cGy, washed, and cultured with freshly isolated PBMCs at a 1:1 ratio in RPMI 1640 medium supplemented with 10% human AB serum. Cultures were re-stimulated with peptide-pulsed T2 cells on days 7, 14, and 21, and the following day 20 IU/mL of recombinant human interleukin-2 (rhIL-2; Invitrogen) was added.

**Cell-mediated cytotoxicity assay**

A standard calcein AM cytotoxicity assay was used to determine specific lysis as described previously (31, 32). Briefly, 1000 target cells in 10 µl (1.0 x10⁵ cells/ml) were stained with calcein-AM (Invitrogen) for 90 minutes at 37 °C, washed 3 times with RPMI-1640 and then co-incubated with 10 µl of peptide-specific CTL at varying effector to target (E:T) ratios. After a 4-hour incubation period at 37°C in 5% CO₂, 5 µl of Trypan blue was added to each well and fluorescence was measured using an automated CytoFluor II plate reader (PerSeptive Biosystems). For HLA-A*0201 blocking experiments, target cells were incubated with BB7.2 antibody prior to the addition of effector CTLs. Percent specific cytotoxicity was calculated as follows: (1-(Fluorescence_target+effector– Fluorescence_media)/(Fluorescence_target alone – Fluorescence_media)) x 100.

**Cytokine flow cytometry (CFC) and major histocompatibility (MHC)-dextramer staining**
T2 cells were pulsed with 20 μg/mL of CG1 peptide for 90 minutes, irradiated, and incubated with PBMC from HLA-A*0201 leukemia patients at a 1:1 ratio for 6 hours at 37°C in 5% CO₂. Brefeldin A (Sigma) was added after the first hour of incubation. After co-incubation of T2 and PBMC, media was removed, the cells were washed with PBS and stained with aqua live/dead stain (Invitrogen) for 20 minutes on ice. Cells were then washed with PBS, fixed and permeabilized using FACS Lyse and PermII solutions (BD). The following fluorescently conjugated MHC dextramer and antibodies were added to each sample: PE-CG1/HLA-A*0201 dextramer (Immudex); APC-PP65/HLA-A*0201 dextramer (Immudex); APC/H7-anti-CD8; lineage (Lin) markers including pacific blue-anti-CD4, CD14, CD16 (BD) and CD19 (Biolegend); PE-Cy7 anti-interferon (IFN)-γ and PerCP Cy 5.5-anti-tumor necrosis factor (TNF)-α (both from BD). The cells were washed, fixed and analyzed using a LSRII Fortessa flow cytometer (BD). Live, Lin⁻, CD8⁺, dextramer⁺ cells were then enumerated for IFN-γ and TNF-α production. Unpulsed T2 cells were used as negative stimulator controls. Fluorescence minus one (FMO) controls were performed for each sample and background staining was subtracted from each experimental group.

**Statistical Analysis**

GraphPad Prism 5.0 software was used to perform statistical analyses and P-values <0.05 were used to establish significance.
Results

CG is aberrantly expressed in primary AML and is ubiquitinated

We first examined the expression of CG by AML from patient samples with high peripheral blood blasts that were obtained at the time of original diagnosis. We initially examined 12 patient samples for CG expression and we present the immunoblots from 8 AML patient samples representing various AML subtypes. We performed western immunoblots on primary patient AML blasts, which demonstrated CG expression in a number of AML subtypes (Fig. 1A; Supplementary Table S2). We also examined these samples for expression of NE and P3, the two azurophil granule proteases from which the PR1-peptide is derived and that share a common promoter (3, 31, 32). Our data show lower expression of NE and P3 in the samples we used in our studies, compared with CG. Furthermore, there was no correlation between CG expression and NE or P3 expression. Because CG is located on a different chromosome (chromosome 14) than NE and P3 (33, 34), which are both located on chromosome 19 (35, 36), and since CG is expressed later than NE and P3 during the maturation of the myeloid progenitor under the regulation of a different promoter than NE and P3 (20, 37), it is not surprising that levels of NE and P3 did not correlate with CG expression. Furthermore, we demonstrate higher expression of CG in primary AML than in normal granulocytes (Fig. 1B).

Because protein ubiquitination facilitates proteasomal degradation of antigens for processing on MHC class I (38, 39), we investigated whether CG was also ubiquitinated in AML, thereby facilitating CG-derived peptide presentation on the leukemia cell surface. IP of AML WCLs with anti-CG antibody and subsequent probing with anti-ubiquitin demonstrate ubiquitination of CG in AML, but not in healthy granulocytes.
Furthermore, because cytosolic proteins are favored for antigen processing since they have direct access to the proteasome (40), we studied the subcellular localization of CG in AML and normal neutrophils. Our data show that CG is diffusely localized in AML in contrast with normal granulocytes, where it is located primarily in granules, as evidenced by distinct foci of staining in the normal granulocyte samples (Fig. 2A). We confirmed this observation using a high throughput bio-imaging system (BD Pathway 435 cell imager) that demonstrated the distribution of CG outside granules in a large number of leukemia blasts (AML #2 n=892 cells; AML #5 n=484 cells) (Fig. 2B and C). We show that 95% of normal granulocytes have a granular pattern demonstrated by a dim:bright ratio<0.6, in contrast to 1% and 9% of the blasts in AML #2 and AML #5 samples, respectively. Together, these data show aberrant diffuse localization of CG outside granules and ubiquitination in AML, suggesting preferential processing of CG in AML for presentation on MHC class I.

Multiple HLA-A*0201 binding epitopes are derived from CG

Five CG derived nonameric peptides were identified using IEDB and SYFPEITHI binding algorithms (Supplementary Table S1 and Fig. 3A). Although we identified five CG-derived peptides with high binding affinities to HLA-A*0201, we focused our experiments on CG1 peptide because of a prior report showing that CG1 is a naturally processed peptide in chronic myeloid leukemia (21), and because of our work confirming CG1 on the surface of leukemia cells (Supplementary Fig. S2). Using T2 binding and iTopia assays, the CG1 peptide (FLLPTGAEA) was confirmed to have a high binding affinity to HLA-A*0201 (CG1 IC₅₀=1.1 μM) in comparison with other CG derived
peptides and with the control peptide FLPSDFFPSV (Fig. 3A and 3B). Furthermore, using iTopia assays we measured the off-rate for CG1 and calculated the time for half-maximal dissociation of CG1 from HLA-A*0201 ($t_{1/2}$) to be approximately 14 hours.

**CG1-CTL lyse CG-expressing HLA-A*0201 AML**

To determine the ability of CG1-CTL to lyse primary AML blasts, we performed calcein AM cytotoxicity assays (31, 32). We first show dose-dependent specific killing of AML blasts by CG1-CTL, with minimal killing of HLA-A*0201 normal bone marrow (Fig. 4A). The low level of cytotoxicity seen in the HLA-A*0201 normal bone marrow sample may be due in part to a low level of expression of CG1 peptide by normal bone marrow myeloid cells. Since CG expression is highest in the early stages of myeloid cell development (20), CG1 may be presented by HLA-A*0201 on subsets of normal early myeloid progenitor cells in the bone marrow, as we previously reported for the LAA PR1(41); this low level expression could account for the low cytotoxicity seen in normal HLA-A*0201 bone marrow. There was no killing of HLA-A*0201 negative bone marrow consistent with CG1 being HLA-A*0201 restricted. Furthermore, we confirmed HLA-A*0201 dependency of CG1-CTL mediated killing since blocking HLA-A*0201 with the BB7.2 antibody abrogated CG1-CTL killing of HLA-A0201 AML and CG1-pulsed T2 cells (Fig. 4B).

Since we demonstrated HLA-A*0201 restricted specific cytotoxicity of AML blasts by CG1-CTL, we next examined CG1-CTL cytotoxicity using five HLA-A*0201 (Pt. 1-5) and one HLA-A*0201 negative AML patient samples (Pt. 6) (Fig. 4C). Our data show variable lysis of HLA-A*0201 AML samples by CG1-CTL, but not the HLA-A*0201
negative sample. Furthermore, at some of E:T ratios we observed higher specific lysis in some of the AML samples that had higher CG expression. However, there was some variability seen in killing of the AML samples independent of CG expression, suggesting that there may be differences in antigen presentation by the AML target cells that could account for differences in CG1/HLA-A*0201 presentation (Fig. 4C and Supplementary Fig. S3). Moreover, although we were unable to assess MFI of CG expression of Pt. 5 using flow cytometry due to sample limitation, immunoblotting of this patient sample demonstrated relatively high CG expression (see AML#8 Fig. 1A).

**CG expression is higher in CD34⁺ CD38⁻ LSC in comparison with normal stem cells**

Since the expression of target antigen is ideally absent or lower in normal cells compared to malignant counterparts, we investigated the expression of CG in normal and leukemia stem cells. We stained Lin⁻ CD34⁺CD38⁻ LSC (28) sorted from 2 different AML patients for intracellular CG. Because of their low frequency, sorted Lin⁻ CD34⁺ CD38⁻ stem cells from four different normal donors were combined and stained for CG. Confocal imaging shows higher expression of CG in LSC in contrast with normal stem cells (Fig. 5A). Using a high throughput bio-imaging system (BD Pathway 435 cell imager), we confirmed significantly higher expression of CG in cells from two different LSC samples (LSC 1: n=234 cells; LSC 2: n=570 cells) in comparison with normal stem cells (NSC) (n=357 cells) (Fig. 5B). Since we had to combine NSC from 4 different healthy donors, there may be variability in the expression of CG in NSC from different individuals, which may not be reflected in the mean values presented (Fig. 5B). However,
the median and range pixel intensity values of LSC 1 (median=620; range= 82-2474), LSC 2 (median=62; range= 46-2550) and NSC (median= 48; range= 45-497) together suggest higher expression of CG in LSC in comparison with NSC. Furthermore, a significant difference was also observed between the two LSC samples, which can be attributed to leukemia heterogeneity.

**Functional CG1-CTL are detected in AML patients following allo-SCT**

Since we showed CG expression in AML and lysis of CG-expressing AML by CG1-CTL in vitro, we next investigated whether immunity to CG1 can be detected in AML patients following allo-SCT. CG1/HLA-A*0201 dextramer was used to stain PBMC samples from patients with AML. We show the presence of CG1-CTL in AML patient peripheral blood following allo-SCT (Range, 0.07%-0.44%) at similar frequencies to what was previously detected for PR1- and WT1-CTL (Table 1) (3, 16). The gating strategy used and the specificity of CG1/HLA-A*0201 dextramer for CG1-CTL is demonstrated in Supplementary Fig. S4. Furthermore, we demonstrate functionality of patient CG1-CTL using a CFC assay measuring IFN-γ and TNF-α response following CG1-CTL stimulation with CG1-pulsed T2 cells. Although responses were detected in four of the five AML patient samples that were analyzed indicating functional CG1-CTL, they were highly variable ranging from 0.6% to 11%. The absence of a response in one of the samples (Patient 2) could be attributed to the lack of full immune reconstitution seen early following allo-SCT (day 30), since the other patient samples analyzed were collected at later time points following allo-SCT (Range, 205-1162 days).
Discussion

We report the discovery of a novel immunogenic epitope derived from the myeloid azurophil granule protease CG. We show high expression and ubiquitination of CG in AML blasts as well as aberrant CG localization outside azurophil granules, which could facilitate CG antigen presentation by leukemia. Additionally, CG is highly expressed in LSC but not normal hematopoietic stem cells. We identify five CG-derived nonameric peptides that bind with high affinity to HLA-A*0201 and show that the CG1 peptide (FLLPTGAEA) is a naturally processed immunogenic HLA-A*0201 epitope. Moreover, we show that CG1-CTL lyse HLA-A*0201-restricted CG-expressing targets, with minimal killing of HLA-A*0201 normal bone marrow. Importantly, we detected evidence of active immunity targeting CG in AML patients following allo-SCT.

Because of the heterogeneity of antigen expression in myeloid leukemia (42), targeting a single antigen is unlikely to provide adequate therapeutic efficacy unless a universal leukemia antigen is discovered. Moreover, expression of LAA by normal tissues, including hematopoietic stem cells, and the failure to elicit immune responses against LAA in some patients, together limit the clinical utility of targeting individual LAA and highlight the need for further antigen discovery. Considering these factors, CG is an ideal LAA since it is highly expressed by numerous AML subtypes in comparison with normal granulocytes (Fig. 1) and is a naturally processed peptide in myeloid leukemia (Supplementary Fig. S2). Although it is expressed by normal granulocytes and stem cells, we show a different pattern of expression in normal tissues. Specifically, we show lower expression in normal granulocytes and LSC, aberrant localization in myeloid leukemia outside granules and ubiquitination in AML blasts, but not normal...
granulocytes, together indicating preferential processing of CG in AML (43-45). The distinct expression and processing of CG in AML is further supported by the specific killing of HLA-A*0201 AML by CG1-CTL, with minimal killing of HLA-A*0201 normal bone marrow (Fig. 4). Together these data highlight the potential role for CG as a novel target in AML immunotherapy.

Our results corroborate two previous reports demonstrating a potential role for targeting CG in leukemia. In the first report by Papadopoulos et al., a high copy number of CG1 peptide was eluted from the surface of CD34+ blasts from one patient with CML using high-performance liquid chromatography (HPLC) purification and sequencing (21). Furthermore, they demonstrated higher expression of CG mRNA in CD34+ CML blasts versus normal CD34+ cells. However, the authors did not investigate immunity against CG1 in the patient that was studied. We expand on these findings by demonstrating that in AML, the CG protein is highly expressed and processed by AML blasts and Lin- CD34+ CD38- LSC and that the CG1 epitope is naturally presented on the cell surface. Furthermore, we demonstrate specific immunity targeting the CG1 peptide in vitro and in patients with AML. In the second report, Fujiwara et al. demonstrated a generalized IFN-γ response to P3, NE or CG in four patients with CML and one patient with AML using CD40 ligand (L)-activated B cells transfected with expression vectors encoding P3, NE and CG (46). In our study, we further characterize the anti-CG immune response in AML and show that CG1 is a naturally processed CG-derived epitope that is likely responsible for the immunity against CG. This epitope, along with CG2 and CG3 (Supplementary Table S1 and Fig. 3) are all derived from the signal peptide of CG. Because cleavage of the signal peptide occurs in the endoplasmic reticulum and golgi (47, 48), two
compartments that are involved in MHC class I antigen processing, the presence of the cleaved signal peptide in these compartments may facilitate processing for presentation on MHC class I. Furthermore, although ubiquitination is an important step in antigen degradation during the process of antigen presentation (38, 39), it may not be significant in the processing of CG1-CG3 peptides since these are naturally found within the signal domain of CG, which is normally cleaved during CG intracellular processing. Whether other CG-derived epitopes (i.e. CG2-CG5) are also immunogenic is the subject of ongoing research in our lab.

Furthermore, we studied whether there was a correlation between the expression of CG and the primary granule serine proteases NE and P3. This was investigated to determine whether targeting CG will add to the existing immunotherapeutic approaches that target PR1 peptide, which is a nonameric peptide derived from NE and P3 that has demonstrated clinical efficacy in vaccine trials of patients with AML, CML and MDS (16). The lack of a correlation between CG expression and NE or P3, as shown in Fig. 1, adds to the significance of CG as an immunotherapeutic target in AML. Unlike NE and P3, CG is located on chromosome 14 and has a promoter region that differs from NE and P3 (49, 50). Because of the distinct expression and regulation of CG that is independent of NE and P3 expression, CG may prove to be an effective target in AML that may be resistant to PR1 therapy because of low NE and P3 expression. Additionally, because of the heterogeneity among the leukemia subclones within an individual patient (9), which could account for variable expression of LAA by the leukemia cells, targeting CG could be used in combination with other LAA in a multipeptide immunotherapeutic approach to
broaden the immune response, as was previously shown for a combined WT1 and PR1 vaccine in myeloid leukemia (16).

Despite our promising data highlighting the immunogenicity of CG in AML, and since AML represents a heterogeneous group of diseases (9, 51), we realize that more samples representing the various AML subtypes, including AML subgroups expressing different mutations and cytogenetic abnormalities, are needed to more conclusively determine the broad applicability CG-targeting immunotherapy in AML. For example, Jin et al. demonstrated that the fusion protein AML1-ETO directly targets and suppresses CG in t(8;21) AML, suggesting a mechanism for leukemia escape from intracellular surveillance whereby the lack of CG prevents the cell from degrading abnormal proteins that may play role in leukemogenesis (52). This same mechanism of CG silencing in t(8;21) AML subtype may also contribute to leukemia evasion of the anti-CG immune response, which would also further promote leukemia escape as was shown for other tumor-associated antigens (53). Additional investigations are currently underway to more fully characterize CG expression in different subtypes of AML.

In addition to AML, which we show here, and CML (46), targeting CG may prove beneficial in non-myeloid hematopoietic malignancies. In a recent study, Gorodkiewicz et al. showed a significant amount of CG protein expression in three chronic lymphocytic leukemia (CLL) patient samples, which was lower than CG expression in two AML patient samples and higher than CG expression detected in leukemia cells from one chronic phase CML patient (54). In addition to CLL, Fujiwara et al. showed immunity to CG in three patients with acute lymphoblastic leukemia (ALL) (46). Collectively, these
two reports highlight a potential role for targeting CG in non-myeloid hematologic malignancies.

Taken together, our results identify CG as a novel target in AML. CG is highly expressed by some AML subtypes and is aberrantly located in cellular compartments that facilitate antigen presentation. Immunity targeting CG was elicited in vitro, was specific for malignant cells with minimal effects on normal cells, and was detected in AML patient samples following allo-SCT. Thus our findings show a promising role for CG targeting immunotherapy in AML.
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Table 1. Functional CG1-specific cytotoxic T lymphocytes are detected in AML patient peripheral blood following allogeneic stem cell transplantation

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<th>Sample</th>
<th>Diagnosis</th>
<th>Days post-SCT</th>
<th>% CG1 Dextramer post-SCT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Cytokine&lt;sup&gt;+&lt;/sup&gt; CG1-CTLs post-SCT&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>MDS RAEB-I</td>
<td>1162</td>
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<td>0.08</td>
<td>0</td>
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<tr>
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<td>AML-FAB M2</td>
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<td>0.20</td>
<td>ND</td>
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<tr>
<td>Patient 8</td>
<td>AML-M6</td>
<td>181</td>
<td>0.44</td>
<td>ND</td>
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</tbody>
</table>

<sup>a</sup>Gating strategy for CG1/HLA-A*0201 dextramer is shown in Supplementary Fig. S4.

<sup>b</sup>% cytokine<sup>+</sup> CG1-CTL refers to the sum percentages of dextramer positive CTLs that produced either IFN-γ, TNF-α, or both in response to CG1-pulsed T2 stimulator cells.

Abbreviations: SCT, stem cell transplantation; CTL, cytotoxic T lymphocytes; MDS, myelodysplastic syndrome; RAEB, refractor anemia with excess blasts; AML, acute myelogenous leukemia; FAB, French-American-British classification; NS, not specified; ND, not determined.
Figure Legends

**Figure 1.** Cathepsin G is overexpressed in myeloid leukemia. (A) Western blot (WB) showing CG expression in whole cell lysates (WCL) from 8 acute myeloid leukemia (AML) samples. Purified CG (15 ng), P3 (0.5 μg) and U-937 WCL (30 μg) were used as positive controls for CG, P3 and NE, respectively. (B) WB showing high CG expression in AML samples, in comparison with granulocytes (Gran) from four different normal individuals. WCL (35 μg) from each sample were loaded into each well; purified CG (15 ng) was loaded as positive control.

**Figure 2.** CG is located outside azurophil granules in AML. (A) Confocal microscopy staining of normal granulocytes and two patient leukemia samples show CG outside azurophil granules in AML blasts, in contrast with granulocytes where CG is located within granules. (B, C) Graphical representation of the granularity in leukemia blasts from two AML patients, AML #2 and AML#5 (n= 1376 cells) vs. normal granulocytes from a healthy individual (n=171 cells). (B) The ratio of the dimmest to brightest 10% of pixels in each cell was determined and the distribution of cells was graphed. Low dim/bright pixel ratio indicates granularity. (C) Percentage of granulocytes and AML blasts with a dim:bright pixel ratio <0.6 from 2 different AML patient samples (AML #2 and AML #5) and 1 normal granulocyte sample. Dim:bright ratio <0.6 indicates granularity.
**Figure 3.** CG1 binds HLA-A*0201 with a high affinity. (A) Schematic of CG showing the signal peptide, which is cleaved in the endoplasmic reticulum and is the source of CG1-CG3. Dashed lines demonstrate the activation dipeptide that is cleaved to generate active protease. (B) T2 cells were co-cultured for 90 minutes with CG derived peptides at increasing concentration and then stained with FITC conjugated anti-HLA-A*0201 antibody. Results presented are the mean ± SD mean fluorescence intensity (MFI) from triplicate staining groups and are representative of 3 independent experiments. (C) Peptides were incubated in HLA-coated wells at concentrations ranging from $10^{-4}$ to $10^{-8}$ molar (M) at 21°C overnight. Results represent duplicate data points and were graphed relative to the binding of the positive (POS) control peptide (FLPSDFFFPSV) at $10^{-4}$ M. The ED$_{50}$ was determined using GraphPad Prism’s nonlinear regression ‘log (agonist) versus response –variable slope (four parameter)’ curve. (D) CG1 was incubated in HLA-coated wells at a concentration of 11 mM at 21°C overnight, then washed and incubated at 37°C and read over the course of 8 hours. Results represent duplicate data points and were graphed relative to the positive (POS) control peptide as 100% binding. CG1 ED$_{50}$=1.1 μM; t$_{1/2}$≈14 hours. The t$_{1/2}$ was calculated using GraphPad Prism’s nonlinear regression, ‘dissociation – one phase exponential decay’ curve.

**Figure 4.** Killing of HLA-A*0201 leukemia by CG1-specific CTLs. (A) Calcein AM cytotoxicity assay using CG1-CTL as effector cells shows specific killing of CG-expressing HLA-A*0201 (HLA-A2⁺) primary AML, with minimal to no killing of HLA-A*0201 negative (HLA-A2⁻) or normal HLA-A2⁺ bone marrow. CG1-pulsed and PR1-pulsed T2 cells were used as positive and negative controls, respectively. (B) CG1-CTL
cytotoxicity is HLA-A*0201 dependent. HLA-A*0201 primary AML targets were cultured with CG1-CTLs +/- the anti-HLA-A*0201 antibody BB7.2. Calcein AM release was used to measure cytotoxicity. (C) Cytotoxicity assays showing that CG1-CTL specifically lyse CG-expressing HLA-A*0201 AML. The median fluorescence intensity (MFI) of intracellular CG staining of each of the AML samples was determined using flow cytometry and is shown in parenthesis (Supplementary Fig. S3). Pt. 6 is HLA-A2 negative. ND indicates not determined by flow cytometry.

**Figure 5.** Higher expression of CG is detected in leukemia stem cells (LSC). (A) Sorted Lin\(^-\) CD34\(^+\) CD38\(^-\) stem cells were permeabilized and stained with alexa-647 conjugated anti-CG antibody (red) and DAPI (blue) nuclear stain. Confocal microscopy imaging of stem cells demonstrates higher expression of CG in LSC in contrast with normal stem cells (NSC). (B) Graphical representation showing the mean\(\pm\)SD intensity of CG staining in 2 LSC samples, LSC 1 (n=234 cells) and LSC 2 (n=570 cells), in contrast with normal stem cells pooled from four different individuals (n=357 cells). Asterisks indicate statistically significant higher expression of CG in LSC: \(P<0.0001\). ANOVA followed by Tukey test was performed using Prism 5.0 software.
Figure 1

A

- 25 kDa → CG
- 29 kDa → NE
- 29 kDa → P3
- 42 kDa → Actin

B

- 25 kDa → CG
- 50 kDa → Actin
Figure 2

A

Granulocytes                         AML #2                                   AML #5

B

C

Percent of Cells with Dim:Bright ratio<0.6

Percent of Total Cells

Granulocytes AML #2 AML #5

Dim/Bright Pixel Ratio (Granularity)

Total Pixels (Cell Size)
Figure 3

A

Signal Peptide

N-terminus

C-peptide

MQPLLALLFLPTGAEAGTGEIGG-------------------------TTMRS

FKLDQMETPL

FlLPTGAE

LLLAFLLT

LLLAFLL

FlVREDFVL

MLQLSRRV

CG1

CG2

CG3

CG4

CG5

B

Peptide Binding Assay

MFI HLA-A2-FITC

Peptide Concentration [ug/mL]

CG1

CG2

CG3

CG4

CG5

PR1

C

Affinity of CG1 for HLA-A*0201

% Peptide Binding of Positive Control

-9 -8 -7 -6 -5 -4 -3

Log [Peptide] (M)

0 2 4 6 8

D

Off-Rate of CG1

% Peptide Binding of Positive Control

0 2 4 6 8

Time (hours)
**Figure 4**

A

- T2-CG1 pulsed
- T2-PR1 pulsed
- AML HLA A2+
- Normal BM HLA A2-
- Normal BM HLA A2+

Effector: Target Ratio

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<thead>
<tr>
<th>Effector: Target Ratio</th>
<th>% Specific Lysis</th>
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<tr>
<td>5:1</td>
<td>80%</td>
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<td>2.5:1</td>
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<td>1.25:1</td>
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<td>0.987:1</td>
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B

- T2 CG1
- T2 CG1 + BB7.2
- HLA-A2+ AML
- HLA-A2+ AML + BB7.2

Effector: Target Ratio

<table>
<thead>
<tr>
<th>Effector: Target Ratio</th>
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<tbody>
<tr>
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<tr>
<td>5:1</td>
<td>40%</td>
</tr>
<tr>
<td>2.5:1</td>
<td>20%</td>
</tr>
</tbody>
</table>

C

- Pt. 1 (MFI=948)
- Pt. 2 (MFI=1,488)
- Pt. 3 (MFI=3,714)
- Pt. 4 (MFI=2,008)
- Pt. 5 (MFI=ND)
- Pt. 6 (MFI=3,466)

Effector: Target Ratio

<table>
<thead>
<tr>
<th>Effector: Target Ratio</th>
<th>% Specific Killing</th>
</tr>
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<tbody>
<tr>
<td>20:1</td>
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<td>40%</td>
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<tr>
<td>2.5:1</td>
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</table>
A

Figure 5

LSC 1

LSC 2

NSC

B

Pixel Intensity

0 200 400 600 800

LSC 1  LSC 2  NSC
Clinical Cancer Research

A Novel HLA-A*0201 Restricted Peptide Derived From Cathepsin G Is An Effective Immunotherapeutic Target in Acute Myeloid Leukemia

Mao Zhang, Pariya Sukhumalchandra, Atim Enyenihi, et al.

Clin Cancer Res Published OnlineFirst November 12, 2012.