Short Report

Novel myeloperoxidase-derived HLA-A2-restricted peptides as therapeutic targets against myeloid leukemia

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ABSTRACT

Background aims: Human myeloperoxidase has been shown to be overexpressed in many types of leukemia, such as chronic myeloid leukemia, acute myeloid leukemia and myelodysplastic syndrome. The authors identified two myeloperoxidase-derived HLA-A2-restricted peptides, MY4 and MY8, as novel leukemia-associated antigens.

Methods: Ex vivo-elicited MY4- and MY8-specific cytotoxic T lymphocytes were generated, and tested for leukemia cell lysis in vitro and in NOD/SCID AML xenograft model.

Results: These MY4- and MY8-specific cytotoxic T lymphocytes killed leukemic blasts while sparing healthy donor bone marrow cells. In addition, co-injection of MY4- and MY8-specific cytotoxic T lymphocytes into nonobese diabetic/severe combined immunodeficiency mice with acute myeloid leukemia drastically reduced tumor burden in vivo. The authors also found that MY4- and MY8-specific T cells could be detected in the peripheral blood mononuclear cells of allogeneic stem cell transplant recipients.

Conclusions: These antigen-specific T cells were significantly increased in blood samples from patients compared with healthy donors, suggesting that both MY4 and MY8 are immunogenic and that MY4- and MY8-specific cytotoxic T lymphocytes may play a role in reducing leukemia in vivo. Thus, the discovery of MY4 and MY8 as novel leukemia-associated antigens paves the way for targeting these antigens in immunotherapy against myeloid leukemia.

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Introduction

Allogeneic stem cell transplantation (allo-SCT) is effective in the treatment of hematologic malignancies but is often accompanied by detrimental graft-versus-host disease [1,2]. T-cell receptor recognition of the peptide–HLA complex on the cell surface plays a critical role in the activation of cytotoxic T lymphocytes (CTLs) and the anti-leukemic function known as the graft-versus-leukemia (GVL) effect. To enhance the GVL effect while tempering graft-versus-host disease, we must identify peptides that are derived from the antigens preferentially or exclusively expressed by leukemia cells, known as leukemia-associated antigens (LAAs).

The authors have previously shown that neutrophil elastase, proteinase 3 and cathepsin G—serine proteases localized within azurophilic granules of myeloid cells, including myeloid leukemia cells—are

promising immunotherapeutic targets in myeloid malignancies [3,4]. Myeloperoxidase (MPO), which has an expression pattern and function similar to those of neutrophil elastase, proteinase 3 and cathepsin G, is synthesized at the pro-myelocyte stage and later localizes to mature neutrophils. The MPO gene encodes an 83-kD precursor polypeptide that is post-translationally processed into 13-kD and 59-kD subunits [5]. In the presence of hydrogen peroxide and chloride, MPO catalyzes the formation of hydrochloric acid in the microbicidal function of neutrophils [3]. MPO is involved in the pathogenesis of autoimmune diseases such as Wegener granulomatosis, in which auto-antibodies against MPO have been detected [6]. MPO is a distinct marker for the classification of myeloid leukemias and is overexpressed in subtypes of acute myeloid leukemia (AML) [7,8].

Previous studies of proteinase 3 and neutrophil elastase led to the identification of PR1, an HLA-A*0201-restricted non-amer peptide and an LAA [3]. Likewise, CG1, an HLA-A*0201-restricted peptide derived from cathepsin G, has been demonstrated to be an effective immunotherapeutic target against AML [4]. However, MPO as a potential LAA has not been extensively studied. Because of the heterogeneity of AML, targeting a single peptide epitope from one antigen might not be enough to achieve an optimal clinical outcome. A multi-epitope,

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multi-antigen immunotherapy regimen would conceivably reduce the possibility of antigen escape and increase the likelihood of clinical benefit. Given the high MPO expression in AML and the natural immunogenicity of this protein, the authors investigated whether MPO-derived peptides might be targets for anti-leukemia T-cell recognition.

In the current study, the authors identified two HLA-A*0201-restricted peptides, MY4 (RLFEQVMRI) and MY8 (LLREHNRL), as novel LAAs. Both MY4- and MY8-specific CTLs killed primary leukemia cells *in vitro* and reduced primary leukemia engraftment when co-injected into immunodeficient (nonobese diabetic/severe combined immunodeficiency [NOD/SCID]) mice. Moreover, MY4- and MY8-specific CTLs were detected at significantly higher frequencies in AML patients who had undergone allo-SCT compared with healthy donors. Taken together, the authors' data show that the MPO-derived peptides MY4 and MY8 are potential immunotherapeutic targets against AML.

Methods

Cell lines and patient samples

The T2 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Patient samples were obtained after appropriate informed consent through an institutional review board-approved protocol at The University of Texas MD Anderson Cancer Center. Informed consent was obtained for experimentation with human subjects and the privacy rights of human subjects always be observed. The samples were stored along with the HLA status of the patients, as determined by the HLA typing laboratory. Healthy donor peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat preparations derived from the whole blood of healthy volunteer donors (Gulf Coast Regional Blood Center, Houston, TX, USA). HLA-A2*0201 (HLA-A2+) status was identified by staining with the anti-HLA-A2 antibody (clone BB7.2; BD Pharmingen, San Jose, CA, USA).

Peptide–T2 binding assay

Stable HLA-A2.1 expression on T2 cells is observed only when peptides that are capable of binding to and stabilizing the cell surface expression of the HLA-A2.1 molecule are added exogenously. One million T2 cells were pulsed with MY1–MY10 peptides individually and then washed and stained with the fluorescein isothiocyanate-conjugated HLA-A2 antibody BB7.2. The samples were analyzed using a BD LSRFortessa X-20 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Unpulsed T2 cells were used to determine background fluorescence.

Peptide-specific CTL lines

MY-specific CTL lines were expanded by stimulating PBMCs from HLA-A*0201 healthy donors *in vitro* as previously described [3]. Briefly, T2 cells were incubated with individual MY peptides (MY1 to MY10) first and then irradiated and co-cultured with freshly isolated PMBCs at a 1:1 ratio. Cultures were restimulated with peptide-pulsed T2 cells on day 7, day 14 and day 21 with the addition of recombinant human IL-2 (Invitrogen, Carlsbad, CA, USA) on each following day. At the end of the culture period, tetramer staining and phenotypical analysis were used to evaluate the quality of the CTLs (see supplementary Figure 1).

Cell-mediated cytotoxicity assay

A standard calcein AM cytotoxicity assay was used to determine specific lysis as described previously [3]. Briefly, 1000 peptide-loaded T2 target cells and T2 alone were stained with calcein AM (BD Biosciences, San Jose, CA, USA) and then washed and co-incubated with peptide-specific CTLs at varying effector:target ratios. After a 4-h incubation period,

Trypan Blue was added to each well and fluorescence was measured using an automated CytoFluor II plate reader and an FLx800 fluorescence reader (BioTek Instruments, Inc, Winooski, VT, USA). Cytotoxicity assay results using AML blasts as targets were similar to those using T2 targets except that the number of blasts was 3000. The percentage of specific cytotoxicity was calculated using the following formula: $[1 - (\text{Fluorescence}_{\text{Target+Effector}} - \text{Fluorescence}_{\text{media}}) / (\text{Fluorescence}_{\text{Target alone}} - \text{Fluorescence}_{\text{media}})] \times 100$.

NOD/SCID AML xenograft model

NOD/SCID-HLA-A2.1 mice were provided by Dr Leonard Shultz of The Jackson Laboratory (Bar Harbor, ME, USA) and housed at MD Anderson according to the protocol approved by the Institutional Animal Care and Use Committee. The authors used a human AML sample (unique patient number 1) from an HLA-A02:01 patient with refractory disease and French–American–British classification of M2. A total of 1×10^6 AML blast cells were transferred intravenously into irradiated (200 cGy) NOD/SCID mice either alone or mixed with MY4-, MY8- or MY9-specific CTLs. Animals were killed 2 weeks after transfer and tissues were harvested. Differential cell count was determined on smears prepared from bone marrow.

MY4 and MY8 tetramer staining

To detect MY4- and MY8-specific CTLs, the authors used PE-MY4/HLA-A02:01 tetramer (Baylor) and APC-MY8/HLA-A02:01 tetramer (Baylor) to stain HLA-A02:01⁺ PBMC samples from 14 patients with AML who had undergone allo-SCT as well as from 10 healthy donors. The samples were analyzed using the LSRFortessa X-20 flow cytometer. Live cells that were negative for CD4, CD14, CD16, CD19, CD33, CD34 and CD56 and positive for CD3, CD8 and tetramers were expressed as a percentage of the total CD8⁺T-cell population.

Statistical analysis

For the *in vitro* and *in vivo* studies, *t*-tests were used to analyze the differences between treatment groups and controls. *P* < 0.05 was considered statistically significant.

Results

HLA-A*0201 binding peptides can be identified from MPO

To determine the potential HLA-A*0201-binding peptides from MPO, the authors screened the MPO sequence *in silico* against the binding motif of the HLA-A2.1 molecule (www.biomass.ncbi.nlm.nih.org). Ten peptides (MY1–MY10) were predicted to bind the HLA-A2.1 molecule (see supplementary Table 1). Individual peptides were synthesized and incubated with T2 cells in the presence of β 2 microglobulin. Stable HLA–peptide complexes on the cell surface were recognized by the anti-HLA-A2.1 antibody BB7.2. As shown in Figure 1A, the mean fluorescence intensity, representing the relative binding ability of each peptide to HLA-A2.1, was substantially above the background control, which was T2 cells incubated in the absence of exogenous peptides. Compared with the positive control PR1, which is a known HLA-A2-restricted LAA, these MPO-derived peptides showed a similar binding capacity. Thus, the authors' findings indicated that MY1–MY10 peptides are capable of binding to HLA-A2.1 molecules *in vitro*.

MY4- and MY8-specific CTLs kill leukemia cells but not healthy donor marrow cells

The authors used MY1–MY10 peptides to stimulate PBMCs from HLA-A2⁺ healthy donors to test whether each peptide was able to generate peptide-specific CTLs *in vitro*. All 10 tested peptides were

Figure 1. HLA-A2-restricted peptides derived from MPO lyse human myeloid leukemia cells *in vitro*. (A) Binding of MPO-derived peptides to T2 cells. A representative histogram of MY1, MY2, MY3, MY4 and MY5 (left) and MY6, MY7, MY8, MY9 and MY10 (right) is shown. Unstained and unpulsed T2 cells were used to determine the background. PR1 peptide was used as a positive control. (B,C) Peptide-specific cytotoxicity of MY4-, MY8- and MY9-specific CTLs. CTLs elicited from the same PBMCs were incubated with either T2 target cells loaded with the respective peptides (B) or HLA-A2⁺ AML cells from UPN1 and HLA-A2⁺ HD bone marrow cells as a control (C). The percentage of specific lysis was determined at the indicated E:T ratio. Results are shown as the mean and standard error of the mean of three independent experiments. (D) Statistical analysis of AML blast counts in NOD/SCID bone marrow. Irradiated NOD/SCID mice received AML (UPN1) cells alone or AML plus MY4-, MY8- or MY9-specific CTLs. On day 14, mice were killed and analyzed for leukemia burden. Human CD45 antibody and mouse CD45.2 antibody were used to identify cells of human origin. Results are expressed as the percentage of AML blast counts in NOD/SCID bone marrow. Comparisons between mice treated with MY4- and MY8-specific CTLs ($n = 4$ and $n = 4$, respectively) and those treated with MY9-specific CTLs or AML alone were performed using a two-tailed *t*-test. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$. E:T, effector:target; HD, healthy donor; UPN1, unique patient number 1.

capable of eliciting peptide-specific CTL lines (see supplementary Table 2), as demonstrated by the killing of respective peptide-loaded T2 targets in cytotoxicity assays (Figure 1B). Next, the authors tested

whether MY-specific CTLs could kill primary leukemic blasts from an HLA-A2⁺ AML patient (unique patient number 1, French–American–British classification M2 refractory relapsed disease, 98%

blasts, MPO⁺). Both MY4- and MY8-specific CTLs showed a similar capacity to kill the same leukemic blasts while sparing the healthy donor's bone marrow cells (Figure 1C). However, the MY9-specific CTLs failed to lyse either the leukemia target or the bone marrow cells from the healthy donor, suggesting that the MY9 peptide may not be present on the cell surface of either target. Among all tested MY-specific CTLs against primary leukemia, only the MY4- and MY8-specific lines could preferentially kill the leukemic target (see supplementary Table 2). These results suggested that the MY4 and MY8 peptides may be processed and presented preferentially on AML cells of HLA-A2⁺ patients. Thus, MY4 and MY8 are potential LAAs.

MY4- and MY8-specific CTLs kill leukemia ex vivo in NOD/SCID mice

Using NOD/SCID-HLA-A2.1 mice, the authors next examined whether MY4- and MY8-specific CTLs could eliminate primary AML

cells *ex vivo*. The authors used an HLA-A2.1⁺ AML patient sample that could be successfully engrafted in bone marrow 2 weeks after transfer, at a dose of 1×10^6 AML cells, in the xenograft model [9]. Four sets of experiments were performed using MY4- or MY8-specific CTLs elicited from the same healthy donor in each set of experiments (see supplementary Table 3). A total of 1.75×10^6 to 4.5×10^6 purified CTLs were collected and used in each single mouse experiment. As shown in Figure 1D, mice that received MY4- or MY8-specific CTLs had reduced blast counts in the bone marrow compared with mice that received AML cells alone as a control (MY4-specific CTLs 25% compared with 65% for control, MY8-specific CTLs 19% compared with 65% for control).

To determine whether alloreactivity of the healthy donor against the AML patient contributed to the reduced blast count, the authors elicited MY9-specific CTLs from the same donor as a control. Mice that received MY9-specific CTLs had 84–85% blasts in the bone marrow,

Figure 2. High frequency of MY4- and MY8-specific CTLs in AML patients who had undergone allogeneic stem cell transplantation. MY4-HLA tetramer (A) and MY8-HLA tetramer (B) were used to stain the PBMCs of 14 patient (AML) and 10 HD samples. Percentages of tetramer-specific CTLs among total CD8⁺ T cells are shown on the left. Representative flow cytometry plots demonstrating tetramer staining of AML patient and HD samples were used in the experiments. Significantly higher frequencies of MY4-specific CTLs ($P = 0.0008$) and MY8-specific CTLs ($P = 0.0116$) were detected in the AML group compared with the HD control group. HD, healthy donor.

suggesting that MY4- and MY8-specific CTLs had specific activity against AML. Compared with the untreated AML control, mice treated with MY4- and MY8-specific CTLs ($P = 0.015$ and $P = 0.03$, respectively), but not those treated with MY9-specific CTLs ($P = 0.108$), showed a significant reduction in blast counts. Significant differences were seen in mice treated with MY4- and MY8-specific CTLs ($P = 0.002$ and $P = 0.008$, respectively) compared with MY9-specific CTLs as a control. Thus, MY4- and MY8-specific CTLs were able to eliminate primary AML cells *ex vivo*. This was consistent with the cytotoxicity assay results (Figure 1C), which showed specific lysis of AML cells by MY4- and MY8-specific CTLs, but not MY9-specific CTLs, *in vitro*.

MY4- and MY8-specific CTLs are detected at increased frequency in AML patients after allo-SCT

To validate the role of MY4 and MY8 peptides as potential immunotherapeutic targets in patients with AML, the authors examined PBMC samples from 14 patients diagnosed with MPO⁺ AML who had undergone allo-SCT following non-myeloablative conditioning (see supplementary Table 4) and 10 healthy donors for MY4- and MY8-specific CTLs. The authors detected higher frequencies of MY4- and MY8-specific CTLs in PBMC samples from AML patients compared with those of healthy donors ($P = 0.0008$ and $P = 0.0116$, respectively) (Figure 2; also see supplementary Table 5). These results confirmed that MY4 and MY8 peptides could trigger immune responses in patients with AML after allo-SCT.

Discussion

In the current study, the authors demonstrated that the MPO-derived HLA-A*0201-restricted peptides MY4 and MY8 were able to elicit CTLs from HLA-A2⁺ healthy donor cells that specifically kill T2 targets loaded with the respective peptides. More importantly, the MY4- and MY8-specific CTL lines were able to lyse MPO⁺HLA-A2⁺ primary leukemic blasts while sparing bone marrow cells from HLA-A2⁺ healthy donors, suggesting that aberrantly expressed MPO in leukemia is likely the source of the peptide antigens recognized by MY4- and MY8-specific CTLs.

Kumari *et al.* [10] demonstrated that when T cells from HLA-A2⁺ donors were primed with A2⁺ antigen-presenting cells co-expressing HLA-A2 and MPO proteins, the resulting allo-T cells could recognize 23 of 30 predicted MPO-derived peptide–HLA-A2 complexes. Among the 10 peptides the authors of the current study tested, nine (excluding MY8) overlapped with the 30 predicted epitopes. MY4 was not in the 23 peptides that were recognized by primed allo-T cells, suggesting that MY4 was not overtly represented on non-malignant cell surfaces. This was consistent with the authors' cytotoxicity results, which showed that MY4-specific CTLs failed to kill bone marrow cells from healthy donors. The selective lysis of MPO⁺ HLA-A2⁺ leukemic blasts by MY4-specific CTLs further indicated that MY4 presentation was likely elevated owing to the overexpression of MPO in leukemia cells.

In comparison to healthy donors, higher frequencies of MY4- and MY8-specific CTLs were found in samples from AML patients who underwent non-myeloablative allo-SCT (Figure 2; also see supplementary Table 5). Moreover, the majority of these patients did not have detectable AML blasts after allogeneic SCT. Non-myeloablative conditioning regimens allow exploitation of the T-cell-mediated GVL effect without the toxicity of myeloablative therapy to achieve complete response. The authors' data suggest that the immune responses elicited by MY4 and MY8 peptides in patients with AML after allo-SCT could potentially contribute to the GVL effect to eradicate AML blasts.

The recent clinical success of anti-CD19 chimeric antigen receptor cells in the treatment of acute lymphoblastic leukemia and diffuse large B-cell lymphoma ushered in the era of refined adoptive T-cell therapy for cancer [11,12]. However, unlike CD19—a surface

protein—many tumor antigens arise from intracellular proteins that must be processed and presented by the HLA molecules, leading to recognition, activation and ultimately elimination by CTLs [13]. MPO is a unique diagnostic marker for myeloid leukemia, and overexpression of MPO is observed in certain subsets of AML, suggesting that MPO may be associated with high-risk disease [6].

Although an MPO-derived HLA-B7-specific peptide has been previously identified as an LAA [14], MY4 and MY8 are the first HLA-A2-restricted peptides identified as putative AML-associated antigens. At present, only a very small number of AML-associated peptide targets have been identified, including PR1 [3], CG1 [4], CCNE1/2 [15] and WT1 [16]. The discovery of MY4 and MY8 as novel HLA-A2⁺ LAAs certainly adds to the repertoire of potential targets against AML. The authors have previously generated a T-cell receptor-like monoclonal antibody against the PR1–HLA-A2 complex (8F4) as well as an 8F4-derived chimeric antigen receptor. Each has shown an anti-leukemic effect as a single agent in pre-clinical settings [17,18]. The authors envision a similar approach to utilizing MY4 and MY8 for future development of anti-leukemic agents, which could be used alone or in combination with other agents to treat AML.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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Author Contributions

Conception and design of the study: SL, ET, QM and JJM. Acquisition of data: SL, ET, XD, DL, KC and QM. Analysis and interpretation of data: SL, ET, XD, DL, KC, QM and MJY. Drafting or revising the manuscript: LS, GA, SL, ET, QM and JJM. All authors have approved the final article.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2021.05.003.

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