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Preferentially expressed antigen in melanoma (PRAME) is a cancer-testis antigen that is expressed in many cancers and leukemias. In healthy tissue, PRAME expression is limited to the testes and ovaries, making it a highly attractive cancer target. PRAME is an intracellular protein that cannot currently be drugged. After proteasomal processing, the PRAME$^{300–309}$ peptide ALYVDSLFFL (ALY) is presented in the context of human leukocyte antigen HLA-A*02:01 molecules for recognition by the T cell receptor (TCR) of cytotoxic T cells. Here, we have described Pr20, a TCR mimic (TCRm) human IgG1 antibody that recognizes the cell-surface ALY peptide/HLA-A2 complex. Pr20 is an immunological tool and potential therapeutic agent. Pr20 bound to PRAME$^+$HLA-A2$^+$ cancers. An afucosylated Fc form (Pr20M) directed antibody-dependent cellular cytotoxicity against PRAME$^+$HLA-A2$^+$ leukemia cells and was therapeutically effective against mouse xenograft models of human leukemia. In some tumors, Pr20 binding markedly increased upon IFN-γ treatment, mediated by induction of the immunoproteasome catalytic subunit β5i. The immunoproteasome reduced internal destructive cleavages within the ALY epitope compared with the constitutive proteasome. The data provide rationale for developing TCRm antibodies as therapeutic agents for cancer, offer mechanistic insight on proteasomal regulation of tumor-associated […]

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A therapeutic T cell receptor mimic antibody targets tumor-associated PRAME peptide/HLA-I antigens

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Preferentially expressed antigen in melanoma (PRAME) is a cancer-testis antigen that is expressed in many cancers and leukemias. In healthy tissue, PRAME expression is limited to the testes and ovaries, making it a highly attractive cancer target. PRAME is an intracellular protein that cannot currently be drugged. After proteasomal processing, the PRAME peptide ALYVDSLFFL (ALY) is presented in the context of human leukocyte antigen HLA-A*02:01 molecules for recognition by the T cell receptor (TCR) of cytotoxic T cells. Here, we have described Pr20, a TCR mimic (TCRm) human IgG1 antibody that recognizes the cell-surface ALY peptide/HLA-A2 complex. Pr20 is an immunological tool and potential therapeutic agent. Pr20 bound to PRAME-HLA-A2+ cancers. An afucosylated Fc form (Pr20M) directed antibody-dependent cellular cytotoxicity against PRAME-HLA-A2+ leukemia cells and was therapeutically effective against mouse xenograft models of human leukemia. In some tumors, Pr20 binding markedly increased upon IFN-γ treatment, mediated by induction of the immunoproteasome catalytic subunit PSi. The immunoproteasome reduced internal destructive cleavages within the ALY epitope compared with the constitutive proteasome. The data provide rationale for developing TCRm antibodies as therapeutic agents for cancer, offer mechanistic insight on proteasomal regulation of tumor-associated peptide/HLA antigen complexes, and yield possible therapeutic solutions to target antigens with ultra-low surface presentation.

Introduction

Effective and safe cancer therapy is premised on the idea that neoplastic cells can be specifically identified and eliminated while healthy cells remain unharmed. Although a large number of cancer-specific changes in the cell have been identified, including tumor-specific mutations, glycosylation patterns, and gene expression signatures, the vast majority of these cancer-specific markers and tumor-associated antigens cannot currently be targeted with either small molecule inhibitors or traditional Abs. Recently, a strategy to target these heretofore-untargetable epitopes has been developed by use of T cell receptor (TCR) mimic mAbs (TCRm). TCRm have specificities similar to those of T cell receptors and are directed to peptides presented in complex with MHC or HLA-I. In contrast with TCR-based therapies, TCRm can be delivered to patients as off-the-shelf pharmaceutical agents in a variety of formats ranging from IgG to bispecific T cell engagers (BiTEs), which allows for exquisite control over handling and pharmacology. In the present study, we designed a TCRm against the cancer-testis antigen preferentially expressed antigen in melanoma (PRAME) and studied the regulation of the epitope expression.

Cancer-testis antigens are a group of tumor antigens that are overexpressed in many cancers, but exhibit limited expression in healthy adult tissue except for in the testes, ovaries, and endometrium (1). The protein PRAME is a cancer-testis antigen that is overexpressed in a broad range of cancer types, including primary and metastatic melanoma (80%-90% of cases) (1, 2), breast cancer (27% of cases) (3), and neuroblastoma (>90% of cases) (3, 4). PRAME is also highly expressed in hematopoietic malignancies including acute myeloid leukemia (AML) (40%-60% of cases) (5, 6), acute lymphoblastic leukemia (ALL) (20%-40% of cases) (1, 6), myeloma (20%-50% of cases) (1), and chronic myeloid leukemia (CML) (30%-40% of cases) (1, 7). PRAME expression has been linked to poor prognosis in breast cancer (8) and neuroblastoma (4). PRAME is also expressed in the stem cells of CML (9), suggesting that targeting PRAME could preferentially deplete the leukemia-initiating cell population. PRAME is a retinoic acid receptor–binding protein that functions to block retinoic acid–mediated proliferation arrest, differentiation, and apoptosis (1, 10). This tumor-selective expression profile makes PRAME a highly attractive therapeutic target.

PRAME is an intracellular protein (1, 11, 12), making it impossible to target using traditional Abs directed at cell-surface proteins, and it cannot currently be inhibited using small molecules. Its function in tumor progression is complex, and in some contexts, PRAME overexpression can reduce malignancy...
of leukemia in vivo (11). Due to its context-dependent role of both promoting and inhibiting tumorigenesis, direct functional inhibition of the protein may not prove to be therapeutically effective as compared to cytotoxicity against PRAME-expressing cells. After proteasomal processing, PRAME-derived peptides, including the PRAME<sup>300–309</sup> peptide ALYVDSLFFL (ALY), are presented on the cell surface in the context of HLA-A*02:01 (HLA-A2) molecules (13, 14). HLA-A2 is the most common HLA-I subtype, found in approximately 40% of the United States population (15), and generating cancer immunotherapies against antigens presented by HLA-A2 would benefit a substantial population. Several groups have demonstrated the ability to generate ALY/HLA-A2-specific CD8 cytotoxic T lymphocytes (CTLs) that can specifically lyse PRAME-HLA-A2<sup>+</sup> tumors and are reactive against primary leukemia (16–18), providing proof that this epitope is presented and can be targeted by immunotherapy. Clinical trials have also demonstrated that patients vaccinated against PRAME can develop PRAME-specific CTLs (19) and helper T cells (20). There are several major constraints to cellular and vaccine-based strategies. CTL-based therapies are patient specific and often require laborious manipulation before reinfusion, while vaccines may be less potent and responses are difficult to predict or control, depending on the patient’s immune repertoire and immunological status (21).

mAbs have demonstrated potent antitumor efficacy in the clinic. Despite promising results, a major limitation of currently marketed mAbs is that they bind exclusively to cell-surface and extracellular antigens, whereas the majority of aberrantly expressed proteins in cancer, including PRAME, are intracellular (1, 11, 12, 15). We hypothesized that a TCRm Ab directed against the peptide-HLA complex formed by ALY and HLA-A2 would be capable of specifically binding to PRAME-expressing tumors and would be a cancer therapeutic against a formerly untargetable protein.

Presentation of tumor-associated antigens requires appropriate protein degradation, typically through the proteasome, additional processing steps, and loading onto HLA-I in the endoplasmic reticulum. Peptides presented by HLA-A2 are typically 9–11 residues in length and require hydrophobic anchor residues at the second and last position (22). The proteasome is a multisubunit complex that can exist in 2 major forms: the constitutive proteasome and the immunoproteasome, which have altered cleavage specificities and thus generate unique repertoires of peptides. They differ in 3 catalytic subunits: β1, β2, and β5 are found in the constitutive proteasome, while β1i, β2i, and β5i are part of the immunoproteasome (23–26). The immunoproteasome generally favors cleavage after hydrophobic residues, which enhances generation of peptides that can fit into the groove of HLA-I (23, 27). Several antigens are restricted to a specific proteasome form, and such knowledge can help dictate immunotherapy strategies against these targets (28).

We report the discovery and characterization of Pr20, which we believe is the first TCRm against PRAME, which we have generated to recognize the ALY peptide in complex with cell-surface HLA-A2. We characterized the ability of Pr20 to bind PRAME-HLA-A2<sup>+</sup> cancers and mediate cytotoxicity against PRAME-expressing malignancies in vitro and in vivo. In addition, we studied the role of the constitutive proteasome and immunoproteasone and their pharmacologic manipulation in generating the ALY peptide epitope, which may be important in the use of this and other TCR-based agents therapeutically.

Results

**Pr20 binds to ALY/HLA-A2 complexes in PRAME/HLA-A2-expressing leukemias.** TCRm clones reactive with ALY/HLA-A2 complexes were identified through a phage-display library screen as described previously (15). We aimed to identify a TCRm Ab that recognized ALY/HLA-A2, but not HLA-A2 alone or in complex with irrelevant HLA-A2-binding peptides. Briefly, single phage clones selective for the ALY/HLA-A2 complex were picked by a positive panning strategy on in vitro-folded ALY/HLA-A2 monomers and a negative panning strategy against RHAMM-R3/HLA-A2 irrelevant peptide control monomers. Specificity of phage clones was further screened on live cells using transporter-associated with antigen processing-deficient (TAP-deficient) HLA-A<sup>+</sup>-T2 cells, which have low levels of endogenously presented HLA-A2 peptides) pulsed with or without ALY or an irrelevant peptide. A more detailed description of phage-display library panning, positive clone screening, and single-chain variable fragment (scFv) characterization can be found in the Supplemental Methods (supplemental material available online with this article; https://doi.org/10.1172/JCI92335DS1). Four phage clones that selectively bound ALY peptide–pulsed T2 cells were engineered into full-length human IgG1. Pr20 IgG1 was selected as the lead clone after it was determined to have a low nM affinity (approximately 4–5 nM K<sub>d</sub>), as measured by a binding assay with HLA-A2/ALY monomers using ForteBio and by Scatchard analysis of binding PRAME-HLA-A2<sup>+</sup> AML14 cells (Supplemental Figure 1). mAb clones Pr8, Pr17, and Pr29 were not pursued due to nonspecific binding to HLA-A2<sup>+</sup> healthy donor peripheral blood mononuclear cells (PBMCs), lower estimated affinity, or inability to bind to target cells, possibly due to subtle structural differences between in vitro-folded ALY/HLA-A2 and endogenously presented ALY/HLA-A2.

To determine the specificity of Pr20, T2 cells were pulsed with ALY peptide or with the irrelevant control EW peptide (Figure 1A). Pr20 did not bind T2 cells pulsed with the EW peptide, but readily bound T2 cells pulsed with the ALY peptide, as measured by flow cytometry, demonstrating that Pr20 bound to the ALY/HLA-A2 complex and not to HLA-A2 alone or an irrelevant peptide/HLA-A2 complex. To more carefully map the TCRm epitope, each residue on the ALY peptide was replaced with alanine (except the canonical anchor residues on positions 2 and 10, which are important for binding to HLA-A2) and Pr20 binding was assessed on peptide-pulsed T2 cells (Figure 1B). Single alanine residue substitutions on positions 5, 7, 8, and 9 reduced or abrogated Pr20 binding at a saturating concentration of Pr20, suggesting that Pr20 primarily contacted the ALY peptide’s C-terminal half (Figure 1B). Decrease in Pr20 binding was not due to instability of peptide/HLA-A2 complexes, as each peptide increased surface HLA-A2 over unpulsed T2 cells in the assay, indicating that the peptides complexed with and stabilized HLA-A2 (Figure 1B). The data demonstrated that specific changes to the native peptide sequence can abrogate Pr20 binding, consistent with other reported TCRm (15, 29).
The preliminary biochemical and specificity characterization, we sought to determine whether Pr20 could recognize cancer cells expressing endogenous PRAME protein. PRAME mRNA expression was assessed by quantitative PCR (qPCR), and surface HLA-A2 expression and Pr20 binding were assessed by flow cytometry across a panel of HLA-A2+ hematopoietic and solid tumor cell lines, several of which have been reported to express PRAME by other groups (10, 12, 16, 30, 31) (Table 1 and Figure 1C). Pr20 binding was readily detected in PRAME+HLA-A2+ leukemia AML14, SET2, BV173, and the T cell lymphoma MAC2A, demonstrating that Pr20 can detectably bind endogenously processed and presented peptides (Figure 1D). Pr20 did not bind the PRAME+HLA-A2+ AML cell line HL60, indicating that the epitope was restricted by HLA-A2. In addition, Pr20 did not bind PRAME+HLA-A2+ tumors of various histological types, including SKLY16 lymphoma, MDA-MB231 breast adenocarcinoma, and NCI-H2228 lung carcinoma. (Figure 1D and Table 1). We detected minimal or no Pr20 binding on T, B, myeloid, monocyte, or neutrophil populations in whole blood taken from HLA-A2+ healthy donors (Figure 1E), demonstrating that Pr20 binds specifically to PRAME-positive tumors. To determine whether Pr20 bound primary human AML cells, we stained 9 frozen samples from HLA-A2+ AML patients and assayed for binding by flow cytometry. Only minimal positive shifts in median fluorescence intensity (MFI)

Figure 1. Pr20 binds ALY/HLA-A2 complexes and PRAME+HLA-A2+ leukemia. Pr20 was directly labeled by conjugation to the fluorophore APC. (A) TAP-deficient T2 cells were pulsed overnight with 50 μg/ml of ALY peptide or irrelevant control EW peptide or left unpulsed. Flow cytometry was used to determine Pr20 binding. (B) Each nonanchor residue in the ALY peptide was substituted for alanine, and peptides were pulsed onto T2. Pr20 binding was determined by flow cytometry relative to native ALY peptide–pulsed T2. Cell-surface HLA-A2 was also measured by flow cytometry to ensure altered peptides maintained the ability to bind and stabilize HLA-A2 compared with unpulsed T2. (C) PRAME mRNA expression was determined by qPCR, and samples that did not amplify after 40 cycles were considered negative. (D) The indicated cell lines were stained with Pr20 or an isotype control Ab, and binding was determined by flow cytometry. Surface HLA-A2 was also assessed compared with an isotype control. All data from A–D are representative of at least 3 experiments. (E) Whole blood populations from HLA-A2+ healthy donors were stained with Pr20 to determine possible crossreactivity. A representative gating strategy and Pr20 histogram compared with isotype control are shown, and data from all HLA-A2+ healthy donors (n = 5) are summarized. Staining was performed once independently for each healthy donor and an AML14 PRAME+HLA-A2+ leukemia–positive control was included in each assay to ensure assay reliability. SSC, side scatter; FSC, forward scatter.
Table 1. PRAME expression, Pr20 binding, and surface HLA-A2 expression on cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor origin</th>
<th>PRAME mRNA</th>
<th>Pr20 binding</th>
<th>Surface HLA-A2</th>
</tr>
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<tr>
<td>BV173</td>
<td>B-ALL</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>AML14</td>
<td>AML</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>SET2</td>
<td>AML</td>
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<td>AML</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>U266</td>
<td>Myeloma</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>MAC1</td>
<td>T lymphoma</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
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<td>+++</td>
<td>++</td>
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<td>+</td>
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<td>++</td>
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<td>–</td>
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</tr>
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</tr>
<tr>
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<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>SW480</td>
<td>Colon adenocarcinoma</td>
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<td>–</td>
<td>++</td>
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<tr>
<td>B-JAB</td>
<td>Burkitt lymphoma</td>
<td>+</td>
<td>–</td>
<td>++</td>
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<tr>
<td>SUDHL4</td>
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</tr>
<tr>
<td>PC9</td>
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<td>–</td>
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</tr>
<tr>
<td>NC-H228</td>
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<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>SKLY16</td>
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<tr>
<td>HL60</td>
<td>AML</td>
<td>+</td>
<td>–</td>
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</table>

PRAME expression in a panel of cell lines was determined by qPCR, and expression was binned into the follow groups based on relative expression to the constitutive gene TBP as calculated by standard 2−ΔΔCt method based on a standard 40-cycle qPCR: negative (−) = no amplification; low (+) = <0.01; medium (+++) = <5; and high (++++) = >5. Surface HLA-A2 expression and Pr20 binding were determined by flow cytometry with the following bins determined by MFI relative to an isotype control. Pr20 binding was binned based on Pr20 MFI/isotype MFI: negative (−) = <2; low (+) = <5; medium (+++) = <10; and high (++++) = >10. Surface HLA-A2 was binned based on HLA-A2 MFI/isotype MFI: negative (−) = <2; low (+) = <10; medium (+++) = <50; and high (++++) = >50.

were detected compared with an isotype control in 3 samples, and there was no relationship to PRAME mRNA levels as measured by qPCR. Several primary AMLs that had high expression of PRAME by mRNA did not bind Pr20, suggesting that mRNA expression alone was insufficient for Pr20 binding and that additional regulatory mechanisms are required for cell-surface presentation of the ALY peptide. While mRNA expression may not always equate to sufficient protein expression, which is required for generation of the ALY peptide, we pursued a detailed investigation of the ALY presentation process as described below.

Pr20M mediates Ab-dependent cellular cytotoxicity against PRAME+ leukemia. Therapeutic mAbs can mediate cytotoxicity by various mechanisms, including direct cytotoxicity and Ab-dependent cellular cytotoxicity (ADCC), but low expression of peptide/HLA-I epitopes can reduce activity of the TCRm. To study whether Pr20 could be cytotoxic against leukemia, we engineered an afucosylated Fc form of the Ab (designated Pr20M) that provides enhanced effector recruitment properties via increased FcR affin-
Pr20M-treated mice at day 13 had reduced leukemia burden compared with day 6 (Figure 3A). In the AML14 model, 3 out of 4 mice in the isotype-treated group succumbed to severe hind-leg paralysis by day 29, whereas none of the Pr20M-treated mice displayed such clinical signs. On day 29, recurrent AML14 leukemia was examined in the bone marrow. Bone marrow leukemia burden was significantly reduced in mice treated with Pr20M, as measured by flow cytometry (Figure 3D). No downmodulation of HLA-A2 or the Pr20 epitope was detected in AML14 cells harvested from Pr20M-treated mice compared with isotype-treated mice (Figure 3E). Target downregulation was therefore not a major mechanism of resistance to Pr20M in these models, confirming previously described observations with other TCRm therapies (38). Our data demonstrate that Pr20M has broad therapeutic activity against several human leukemias.

PRAME protein expression alone is not sufficient for Pr20 binding, but IFN-γ can enhance Pr20 binding in PRAME+ solid tumors and enhance ADCC. Interestingly, neither PRAME mRNA levels nor PRAME protein levels correlate with Pr20 cell-surface binding. Several HLA-A2+ cancers that expressed high levels of PRAME, such as the melanoma cell lines SK-Mel5, UACC257, and A375, did not readily bind Pr20 (Table 1). Therefore, we hypothesized that PRAME and HLA-A2 expression alone are necessary but not sufficient to generate the ALY/HLA-A2 complex. Hematopoietic cells are well known to express an alternative form of the proteasome called the immunoproteasome (24), and indeed, most PRAME-positive leukemias bound Pr20. We hypothesized that the immunoproteasome is important for processing the ALY peptide. Although not highly expressed in most tissues, the immunoproteasome can be upregulated by proinflammatory cytokines such as IFN-γ and TNF-α (26).
Figure 3. Pr20M is therapeutically active against ALL and AML in vivo, and target epitope downregulation is not a mechanism of Pr20M resistance. BV173 (ALL), SET2 (AML), and AML14 (AML) were transduced to express luciferase and GFP. NSG mice were engrafted though tail-vein injection, and on day 6 or 7, mice were randomized into groups and treated with 50 μg of Pr20M twice a week, left untreated (control for BV173 and SET2), or treated with an afucosylated isotype control Ab (AML14). Tumor burden was determined by BLI for BV173 (n = 5 mice) (A), SET2 (n = 5 mice) (B), and AML14 (n = 4 mice) (C) once a week throughout the experiment, and the BLI data are summarized below the images. The scales for days 7 and 14 for AML14 are lowered to indicate engraftment and early tumor growth. Total flux (photos/s) was normalized to each mouse’s total flux on day 6 or 7 immediately before initiation of Pr20M therapy and summarized with mean ± SEM. (D) Mice from the AML14 experiment were sacrificed on day 29, and bone marrow was harvested to determine tumor burden by flow cytometry for GFP+HLA-A2+ AML14 cells. Representative plots (n = 4 mice per group) are shown, and data are summarized. (E) MFI of AML14 for HLA-A2 and Pr20 was determined by flow cytometry. Because Pr20M-treated mice presumably had Pr20M already bound on tumor cells, staining was performed by an additional Pr20 stain on all samples followed by a secondary PE-conjugated anti-human Ab (n = 4 mice per group). Experiments were performed once per model. Differences were evaluated using the unpaired t tests on indicated times and samples. AML14 BLI data are representative of 3 similar experiments, while SET2 and BV173 BLI data are from 1 experiment. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
However, Pr20 binding increased far more (up to 10-fold) than HLA-A2 (2- to 6-fold) (Figure 4A), suggesting that increases in HLA-A2 staining were not the dominant cause of the increased Pr20 binding. Importantly, pretreatment of the tumor cells with IFN-γ led to enhanced Pr20M-mediated ADCC in vitro, indicating that upregulation of the target epitope might enhance therapeutic utility (Figure 4B). Increased Pr20 binding was not observed in several samples of HLA-A2+ healthy donor PBMC populations after IFN-γ treatment (Supplemental Figure 4). PRAME mRNA and protein expression did not increase after IFN-γ treatment and, indeed, decreased slightly (Figure 4C), suggesting that IFN-γ-mediated regulation of PRAME protein expression was not the cause of increased ALY peptide presentation. Protein expression of the immunoproteasome subunits β1i, β2i, and β5i increased after IFN-γ treatment (Figure 4D), possibly leading to enhanced generation of the ALY peptide.

The immunoproteasome catalytic subunit β5i is important for IFN-γ-mediated regulation of Pr20 binding. We hypothesized that IFN-γ could enhance generation of the ALY peptide by altering the proteasome components. To determine whether increased Pr20 binding was due to immunoproteasome upregulation, we generated CRISPR knockouts of each immunoproteasome subunit in the SK-Mel5 melanoma. After knockout by Cas9, β1i, β2i, and β5i were not measurable by Western blot analysis compared with a vector control (Figure 5A). The immunoproteasome subunit knockouts were treated with IFN-γ for 72 hours, and Pr20 binding was assessed by flow cytometry (Figure 5B). β5i knockout led to substantially less Pr20 binding, demonstrating that β5i plays an important role in IFN-γ-mediated processing of the ALY peptide epitope. CRISPR knockout of β5i yielded the same effect in UACC257, another PRAME+HLA-A2+ melanoma (Figure 5B), and SW480, an PRAME+HLA-A2+ colon adenocarcinoma (Supplemen-
The constitutive proteasome mediates internal destructive cleavage of the ALY peptide. Proteasomal degradation can regulate the generation of a specific HLA-I–associated peptide through enhancing the required N- or C-terminal cleavages or through reducing destructive internal cleavages. Several tumor-associated antigens exhibit restriction to the immunoproteasome because the peptide is largely destroyed by the constitutive proteasome and thus intact peptide cannot be presented (28, 40). To elucidate the differing proteolytic mechanisms between the constitutive and immunoproteasome involved in generating increased ALY peptide epitope on the surface, we examined the possible involvement of the immunoproteasome catalytic subunit β5i.

Figure 5. Immunoproteasome catalytic subunit β5i is important for IFN-γ–mediated Pr20 binding in melanomas and other solid tumors. βi, β2i, and β5i were knocked out in the SK-Mel5 melanoma line using a CRISPR approach. A CRISPR construct against GFP was used as a vector control. (A, left panel) Cells were treated with 10 ng/ml IFN-γ for 72 hours, and Western blot analysis was used to demonstrate successful knockouts. Blots were derived from replicate samples run on parallel gels with the GAPDH loading control shown from the β2i blot. (B) Flow cytometry was used to determine Pr20 binding and surface HLA-A2 on the indicated knockouts (sgRNA against βi, β2i, and β5i) untreated or treated with IFN-γ for 72 hours. (B, top panels). Data are normalized to MFI of untreated GFP sgRNA CRISPR control. (B, lower panels) β5i CRISPR knockout experiments were performed in the same manner on the UACC257 melanoma line. Successful knockdown was determined by Western blot (A, right panel), and Pr20 binding and surface HLA-A2 were determined by flow cytometry (B, lower panels). (C) SK-Mel5 and UACC257 cells were left untreated or treated with 10 ng/ml IFN-γ for 72 hours in the presence or absence of 200 nM of the β5i inhibitor ONX-0914. Flow cytometry was used to determine MFI relative to untreated cells. All data are representative of 3 experiments with 3 technical replicates per experiment and mean ± SEM plotted. Statistical significance was determined by unpaired t test compared with control. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

tal Figure 5). Surface HLA-A2 expression was not affected by β5i knockout in the SK-Mel5 model and only minimally decreased in the UACC257 model (Figure 5B). ONX-0914, a selective inhibitor of β5i, was used to provide orthogonal validation that the immunoproteasome is important for generation of ALY/HLA-A2. SK-Mel5 and UACC257 were treated with IFN-γ for 72 hours with or without the presence of ONX-0914. ONX-0914 was used at 200 nM, a concentration reported to have potent biochemical inhibition of β5i, but minimal inhibition of other proteasome catalytic subunits (25). As expected, cells treated with ONX-0914 had reduced Pr20 binding compared with cells treated with IFN-γ alone (Figure 5C).

Taken together, our data suggest the shift from the constitutive proteasome to the immunoproteasome is an important mechanism for increased epitope presentation and Pr20 binding. Furthermore, SK-Mel5 cells treated with bortezomib alone, a potent inhibitor of the constitutive proteasome β5 subunit and, to a lesser extent, the β1 subunit (39), did not substantially alter binding to Pr20 at doses that were not cytotoxic. We also explored the use of demethylating agents in an attempt to increase the level of PRAME protein expression and thereby possibly peptide epitope on the surface. We observed only modest increases in Pr20 binding after decitabine treatment (Supplemental Figure 6).
face, an elongated 20-mer ALY-precursor peptide was synthesized with 5 residues extending from each terminus (PRAME295-314). The ALY-precursor peptide was incubated with either purified constitutive proteasome or immunoproteasome in vitro, and digest fragments were analyzed by mass spectrometry. The major detectable fragments were then mapped to specific cleavage sites. Of the detected major digest fragments, the immunoproteasome had increased ratio of nondestructive/destructive cleavages in the ALY sequence (Figure 6A), while the immunoproteasome maintained the intact ALY 10-mer. In addition, the immunoproteasome catalyzed a major cleavage site after Q296 and L298 (LQ/CLQ ALYVDSLFFL RGRLD and LQCL/Q ALYVDSLFFL RGRLD, where bold letters indicate the ALY 10-mer) 1 and 3 residues N-terminal to the ALY 10-mer (Figure 6B). Such cleavage may prime the peptide for amino-peptidase trimming. In contrast, the constitutive proteasome mediated a major destructive cleavage site after A300 (LQCLQA/LYVDSLFFL RGRLD) and after V303 (LQCLQA/LYVDSLFFL RGRLD). Our analysis demonstrates that the relative cleavage after the C-terminal L309 (LQCLQA/LYVDSLFFL RGRLD) was comparable between constitutive and immunoproteasome, suggesting that C-terminal cleavage of the ALY peptide was not a major mechanism of immunoproteasome restriction (Figure 6B). An N-terminal cleavage after the Q296 (LQCLQA/LYVDSLFFL RGRLD) was not a major cleavage site for either proteasome form. The major differences in cleavage specificities between the constitutive and immunoproteasome on the precursor peptide are schematized (Figure 6C). Taken together, the biochemical data show that the immunoproteasome enhances generation of the ALY 10-mer peptide through decreased internal destructive cleavage and increased N-terminal upstream cleavage, relative to the constitutive proteasome.

Discussion
Immunotherapy has demonstrated potent clinical utility for several cancers. However, successful therapy would be improved by use of targets that are cancer selective to minimize toxicity to
essential healthy tissue. Although highly selective onco-fetal or cancer-testis tumor-associated antigens have been described, most are intracellular proteins that cannot be targeted by small molecule inhibitors or by using Abs directed at cell-surface targets. Furthermore, most of these tumor antigens, such as PRAME, have context-dependent function and are not necessarily oncogenic. Thus, functional inhibition may not offer therapeutic benefit. Several groups have studied TCR-transgenic T cells specific against intracellular tumor-associated antigens, but this strategy has been limited due to challenges of large-sale manufacturing and safety concerns of the transgenic TCR recombining with the native TCR, generating unknown specificity and possible autoimmune reactivity (41). Recently described “ImmTAC” molecules use a TCR-based recognition domain offering similar reactivity to TCRm Abs, but they are typically 100-fold lower affinity (42). Instead, TCRm Abs such as Pr20 can target these “undruggable” proteins with high affinity for redirected immune-mediated cytolysis. Pr20 specificity analyses were consistent with binding to the ALY peptide primarily on the C-terminal half amino acids at positions 5–9. However, because minimal contact residues were predicted in the N terminus, we cannot exclude the possibility of crossreactive peptide/HLA-A2 epitopes in the exome that share high C-terminal homology to ALY. Whether other theoretical peptide epitopes in the exome are expressed and processed appropriately in normal tissues is difficult to explore. The data shown here suggest that if potential crossreactive peptides in the human exome are expressed, properly processed, and sufficiently presented, they are infrequent. Peptides have specific processing requirements, including not only proteasomal degradation, but also aminopeptidase and oligopeptidase processing (43), which likely limit generation of crossreactive epitopes. In addition, potential crossreactive peptides may lack high affinity to HLA-A2 and thus will not form stable peptide/HLA-A2 complexes at the cell surface. Pr20 did not bind PRAME HLA-A2+ cells and did not bind more than a dozen other HLA-A2+ tumor lines, suggesting there were no broadly presented crossreactive peptides. In addition, Pr20 did not bind to or accumulate in any major organ in HLA-A2 transgenic mice, nor bind to normal human blood cell populations in healthy HLA-A2+ donors. Taken together, the data suggest that crossreactive epitopes presented on HLA-A2 are non-abundant. Importantly, such off-targets are not increased in normal cells after IFN-γ treatment. The afucosylated Pr20M demonstrated therapeutic efficacy in vivo. Interestingly, at the experimental end point, Pr20 binding to AML14 extracted from Pr20M-treated mice was slightly higher than in AML14 extracted from isotype-treated mice. This is intriguing because, first, it shows that target downregulation is not a mechanism of tumor escape in this model, and second, it also suggests that cellular interactions during Pr20M therapy may increase epitope expression on target cells. This may be due to cytokines released by immune effectors during ADCC, as observed with other therapeutic Abs (44, 45). It is important to note that the NSG mouse xenograft poorly models the human effector populations and the cytokine milieu that would be produced in a patient. In addition, because HLA-A1 is well characterized as regulated by inflammatory cytokines and TCRm targets are presented in complex with HLA-A1, it is possible that TCRm targets are regulated in a “feed-forward” system where TCRm-mediated cytolysis leads to local cytokine release and increased target expression on neighboring tumor cells. Human IgG1 also has different affinities to mouse FcR and human FcR, and NSG mice lack functional NK cells and have defective macrophages and dendritic cells. Therefore, lack of and poorly functional effector cell populations in this model may limit efficacy of a TCRm that requires immune effectors for ADCC (38), which may in part explain the lack of tumor eradication and relapse within weeks. It is reasonable to hypothesize that TCRm therapy in an immunocompetent patient or model would demonstrate more potent therapy. To better understand the incomplete responses to the therapy in vivo, we tested whether combination of Pr20M Ab therapy with a second therapeutic TCRm Ab directed to an unrelated epitope also found on the target cells would increase therapeutic effects (Supplemental Figure 7). This would yield more than double the target epitope numbers on each cell and also rule out the issue of leukemia escape by downregulation or loss of the PRAME epitopes from the leukemia cells. No significant improvement in leukemia control was demonstrated in these experiments, further bolstering the argument that lack of effector cell function and effector cell numbers were the critical deficiencies, not lack of (or loss of) target PRAME epitopes on the leukemia.

Generating an immunocompetent syngeneic mouse model in which to test these agents is difficult because murine PRAME does not comprise the human ALY peptide, which would have to be introduced genetically along with the use of mice with transgenic human HLA-A2. It is also unknown whether the ALY peptide can be properly cleaved and processed by the murine antigen presentation machinery and presented on transgenic HLA-A2 molecules. Coinfusion of human immune cell populations into NSG mice may provide an alternative model for effector cells and cytokines in these mice, but also leads rapidly to graft-versus-host disease and graft-versus-leukemia activity, complicating the analysis, as seen in previous studies (15).

Binding studies demonstrated that Pr20 robustly bound to several PRAME+HLA-A2+ leukemias and lymphoma cell lines, but did not bind well to a small sample size (n = 9) of previously frozen PRAME+HLA-A2+ primary AML. This is consistent with the lack of binding to several PRAME+HLA-A2+ cancer cell lines. Our data demonstrate that PRAME and HLA-A2 expression alone is necessary, but insufficient for Pr20 binding. It is also important to note that the number of ALY peptide epitopes presented on HLA-A2 is highly limited (estimated at less than 0.1% of the HLA molecules on the surface based on Scatchard analyses) and may be below the detection limit of the flow cytometry assay with Pr20 in some cells. It is also possible that low epitope presentation is undetectable with our assays, yet sufficient to initiate redirected lysis; however, this could not be reliably studied with frozen primary AML samples.

Pr20 did not initially bind several PRAME+HLA-A2+ melanomas and other solid tumors despite high levels of PRAME expression, but Pr20 binding dramatically increased upon treatment with IFN-γ, which was partially mediated by increases in immunoproteasome β5i expression. β5i is well characterized as having chymotrypsin-like enzymatic activity, cleaving after hydrophobic amino acids (23). However, the specificity is complex and not fully understood. For instance, β5i cleavage can be inhibited by
the presence of an additional hydrophobic residue directly C-termi-
nal of a site as demonstrated in vitro using the enolase-1 pro-
tein as a model substrate (46). IFN-γ decreased PRAME protein
expression, which may be caused by decreased mRNA expression
but may also be due to differing kinetics of the immunoprotea-
some. Using a biochemical digestion assay in vitro, we dem-
onstrated that the immunoproteasome cleaves and yields a present-
able ALY-precursor peptide more efficiently than the constitutive
proteasome. ALY peptide precursors generated through proteaso-
mal digestion in vitro have been described (18); however, direct
comparison between constitutive proteasome and immunoprotea-
some on digestion of the ALY peptide has not been studied.
The constitutive proteasome catalyzed a major destructive cleav-
age site after the first A300 of ALY (LQCLQA/LYVDSLFFL-
GRLD), whereas the immunoproteasome did not, possibly due to
inhibition by the adjacent hydrophobic leucine. In addition, the
immunoproteasome better catalyzed cleavages slightly N-termi-
nal to the ALY peptide. Minor N-terminal elongated intermediate
peptide may prime the peptide for aminopeptidase trimming into
the ALY 10-mer; however, this was not studied. The knowledge
of target presentation has broad implications when designing
peptide vaccines, TCR, and TCRm Abs for determining which
tumors may respond best to these therapies. In addition, check-
point blockade therapy, which has demonstrated effective clinical
utility, relies on tumor-antigen presentation and CTL recognition
to direct tumor cell lysis. Therefore, understanding the biochemi-
cal mechanisms of immunogenic peptide generation and presen-
tation is critical for designing checkpoint blockade strategies and
determining ideal tumor targets. Our data suggest that tumors
expressing the immunoproteasome such as leukemia and lymph-
omas would better respond to immunotherapies against ALY/
HLA-A2 and that other cancer types may need pharmacologic
upregulation of the immunoproteasome in conjunction to make
the immunotherapy effective.

Pr20 binding requires peptide presentation in the context of
HLA-A2, and thus strategies to enhance HLA-A2 expression may
also augment Pr20M-mediated therapy. It will be important to
discover pharmacological modulators of HLA-I that can be used
for combination therapy with TCRm Abs or other HLA-I-based
immunotherapies. For example, recent reports demonstrate that
inhibition of MEK can increase cell-surface HLA-I, which may
enhance TCRm Ab therapy (47). Additionally, several pharma-
cochemical agents that target histone-modifying enzymes, such as
methyltransferase inhibitors and histone deacetylase inhibitors,
can induce expression of tumor-associated antigens, including
PRAME, and lead to enhanced cytolyis by effector T cells (48–
50). It would be important to understand whether these agents
could enhance antigen expression and synergize with TCRm ther-
apy. However, these epigenetic drugs can also have context-de-
pendent effects on immune cell function and therefore must be
evaluated carefully to ensure they do not also inhibit the effector
cells required for TCRm-mediated cytotoxicity (49, 51).

Our data demonstrate the ability to target PRAME with a
TCRm Ab. This approach enables us to target intracellular pro-
teins that cannot be modulated with small molecule inhibitors.
TCRm allow access to a new universe of Ab protein targets,
far larger and more tumor specific than the currently available
cell-surface protein targets. They also bypass the patient-specific
limitations of CTL-based therapies. Only a few TCRm have been
studied in preclinical models as agents for cancer therapy (15, 29,
36, 52–55). Therefore, the present study on Pr20 adds additional
proof-of-concept that TCRm can be potent and selective thera-
peutic agents. Finally, due to the well-characterized mAb format
of TCRm, they can be readily engineered into alternative formats
such as Fe-enhanced forms, as shown in this study, and BiTE (56,
57) forms, as done with a TCRm to WT1, or transduced as chime-
ric antigen constructs (CARs) in T cells (58, 59). These additional
formats may be required for effective targeting of these ultra–low
density targets. Radioimmunoconjugates (60) and Ab-drug conju-
gates may also be explored in the context of TCRm in an effort to
enhance potency against cancer cells.

Methods

Peptides. All peptides were purchased and synthesized by and pur-
chased from Genemed Synthesis Inc. The peptides were dissolved
in dimethyl sulfoxide and frozen at −80°C. Peptide-pulsing exper-
iments were performed by incubating TAP-deficient T2 cells
overnight with 50 μg/ml of peptide with 20 μg/ml B2M in either
serum-free media or in the presence of 5% dialyzed FBS overnight.
Control peptides used were established HLA-A2–binding peptides
RHAMM-R3 (ILSLELMKL) and EW (QLQNPSYDK). Experimental
peptides included the ALY peptide (ALYVDSLFFL) and the elongat-
ed 20-mer ALY-precursor peptide (LQCLQALYVDSLFFLGRLD).

Cells. PBMCs from HLA-typed healthy donors were obtained by
Ficoll density centrifugation. Cell lines were maintained at MSKCC
and were originally obtained from ATCC and frozen as aliquots in liq-
uid nitrogen. BV173 was provided by H. J. Stauss (University College
London, London, United Kingdom). The following cell lines were gifts
from the listed labs at MSKCC: AML14 was a gift from Ross Levine;
SET2 was a gift from Richard J. O’Reilly; SK-Me5 and SK-Me37 were
gifts from Jedd D. Wolchok; and SUDHL4 was a gift from Anas Younes.
SKLY16, FC9, SK-Me30, and SK-Me2 were from cell
line banks at MSKCC. THP1, U266, A375, SW480, BJAB, NCI-H2228,
MDA-MB231, T2, and HL60 were obtained from ATCC. UACC257
and UACC62 were obtained from the National Cancer Institute.
MAC1 and MAC2A were gifts from Mads H. Andersen (University of
Copenhagen, Copenhagen, Denmark). Cell lines of unknown HLA
were HLA typed by the Department of Cellular Immunology at MSK-
CC. All cell lines were cultured in RPMI 1640 supplemented with 10%
FCS, 1% penicillin, 1% streptomycin, 2 mM l-glutamine, and 10 mM
HEPES at 37°C and 5% CO₂.

ScFv clones specific for ALY peptide/HLA-A2 complexes. A human-de-
erived scFv Ab phage display library (7 × 10⁹ clones) was used for the
selection of mAb clones. Methods for selection and characteriza-
tion of the scFv as well as engineering of full-length Pr20 are described
in Supplemental Methods.

Flow cytometry. For cell-surface staining, cells were blocked using
FceR blocking reagent (Miltenyi Biotec, 130-059-901) at the manufac-
turer’s recommended dilution for 15 minutes on ice, then incubated
with appropriate fluorophore-conjugated mAbs for 30 minutes on ice
and washed twice before resuspension in a viability dye (either DAPI
or propidium iodide at 1 μg/ml). Abs used include anti-HLA-A2 clone
BB7.2-APC (eBioscience, 17-9876-42), BB7.2-FITC (MBL, K0186-4),
anti-CD3-PerCP clone 7D6 (Invitrogen, MHCDO331), anti-CD19-

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PE-Cy7 clone 1D3 (eBioscience, 25-0193-81), anti-CD33-BV711 clone WM53 (BioLegend, 303423), and CD14-PE clone 61D3 (eBioscience, 12-0149-42). Pr20 or its human IgG1 isotype control (Eureka Therapeutics, ET901) was conjugated to APC using the linking-light kit (Innova Bioscience, 705-0010), and staining was performed at 3 μg/ml, which was determined to be a saturating concentration. Flow cytometry data were collected on a LSRII (BD) or an Accuri C6 (BD) and analyzed with FlowJo V10 software.

ADCC. Cancer cell lines used as ADCC target cells were incubated with 50 μCi of 111In for 1 hour at 37°C and washed 3 times to remove free 111In. Indicated concentrations of Pr20M or matched isotype control hlgG1 (afucosylated Eureka Therapeutics ET901) were incubated with target cells and fresh PBMCs at effector/target ratios of 50:1 for 6 hours at 37°C. The assay was performed in 96-well format with 5,000 target cells per well and 250,000 PBMCs. The supernatant was harvested, and the cytotoxicity was measured by scintillation counting. For flow-based ADCC assays, PBMC and GFP+ tumor target cells were incubated at effector/target ratios of 30:1 overnight with 1 μg/ml of Pr20M and flow cytometry was used to determine depletion of GFP+ tumor percentage. PBMCs were also incubated alone with 1 μg/ml Pr20M to measure potential autologous toxicity to PBMC populations.

Western blot and qPCR analysis. Total cell lysate was extracted using RIPA buffer and quantified using the DC protein assay (Bio-Rad). 15–30 μg of protein was loaded and run on 4%–12% SDS PAGE gels. After 1 hour block with 5% milk at room temperature, immunoblotting was performed using the following Abs: anti-β2 (Enzo Life Science, BML-PW8845-0025), anti-β1 (Enzo Life Science, BML-PW8350-0025), anti-β1 (Enzo Life Science, BML-PW8840-0025), and anti-PRAME (Sagma-Aldrich, HPA045153). Abs were probed at the manufacturer’s recommended dilution overnight at 4°C before a secondary Ab conjugated to HRP was used for imaging. Replicate samples were probed using the indicated Abs when noted, or blots were stripped with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, 21063), reblocked with 5% milk, and reprobed with an anti-GAPDH-HRP direct conjugated Ab (Cell Signaling Technology, 3683) as a loading control. qPCR was performed using the TaqMan Real-Time PCR system. RNA was extracted using QIAGEN RNaseasy, and 1 μg of RNA was reverse transcribed into cDNA using qScript cDNA SuperMix (Quanta Biosciences). TaqMan probes and primers were designed from "assay-on-demand" gene expression products (Applied Biosystems). Primers and probes were PRA2 (assay ID number: Hs01022301_m1), and the endogenous reference gene control was TATA-box binding protein (TBP) (assay ID number: HS999999910). The results are presented as relative differences in expression versus the endogenous reference control gene (2^(-ΔΔCt)) or fold changes based on the differences of normalized Ct values compared with control samples, assuming optimal primer efficiency (2^(-ΔΔCt)). Samples that did not amplify after 40 cycles or amplified at an equal or later Ct value compared with a water sample were considered negative and are not plotted with a value.

CRISPR knockout studies. LentiCRISPRv2 (Addgene plasmid 52961) (61) was a gift from Feng Zhang (Broad Institute, Cambridge, Massachusetts, USA). Guide RNA sequences targeting the immunoproteasome subunits were as follows: β2i (PSMB10): GTCCCTTCAGGCACGCAAGAC; β5i (PSMB8): GTGGACAGACTGTCAGTAC; β1i (PSMB9): GGTGCTTTGACGGATGCCT. Cells were transduced with LentiCRISPRv2, and transduced cells were selected using 1–4 μg/ml puromycin for 48 hours. Successful knockout was confirmed by Western blot analysis.

Animals. Eight- to ten-week-old NOD.Cg-Prkd^+/+ IL2rgtm1Wjl/Szj mice (NSG) were purchased from The Jackson Laboratory or obtained from the MSKCC animal breeding facility. Female mice were used for the BV173 and SET2 models, while male mice were used for the AML14 model. C57BL/6 and B6.Cg-Tg(HLA-A/H2-D) Enge/J mice (HLA-A2 transgenic mice) (6 to 8 weeks old) for biodistribution experiments were also purchased from The Jackson Laboratory and bred at MSKCC.

Clinical trials of Pr20M. GFP/luciferase-transduced AML14 cells were passaged once in NSG mice, and bone marrow was harvested to generate a subculture line that engrafted more consistently in vivo. Using this AML14 subculture line, 3 million cells were injected intravenously into 2 groups of NSG mice. On day 7, tumor engraftment between the 2 groups was confirmed by luciferase imaging to have minimal intergroup variation. Groups were blindly assigned to either treatment group (Pr20M or Isotype-treated). 50 μg of Pr20M or an afucosylated isotype control human IgG1 (afucosylated Eureka Therapeutics, ET901) was injected intravenously twice weekly (every 3 or 4 days) starting on day 7 until the experiment end point on day 29. Tumor growth was assessed by weekly BLI, and bone marrow was harvested on day 29 for flow cytometric analysis. For BV173 and SET2 therapy experiments, 0.5 × 10^6 SET2 cells and 3 × 10^6 BV173 were engrafted into NSG mice through tail-vein injection. Mice were treated with 50 μg of Pr20M on days 6, 10, 13, and 17 after engraftment or left untreated (control), and tumor burden was assessed by BLI on the indicated days.

Pharmacokinetic and biodistribution studies. Pr20 Ab was labeled with 125I (PerkinElmer) using the chloramine-T method. 100 μg of Ab was reacted with 1 μCi 125I and 20 μg chloramine-T, quenched with 200 μg Na metabisulfite, then separated from free 125I using a 10-DG column equilibrated with 2% bovine serum albumin in PBS. Specific activities of products were in the range of 4 to 8 mCi/μg. 2.5 μg of radiolabeled mAb was administered intravenously into each mouse through retroorbital injection, and blood and/or organs were collected at indicated time points, weighed, and measured on a γ counter.

In vitro proteasome digestion and liquid chromatography–mass spectrometry/mass spectrometry analysis. A 20-mer PRAME sequence peptide (LCQLQALYVDLSFLLRGRLD) termed the precursor peptide, encompassing the ALY epitope, and elongated by 5 residues on each end was synthesized by Genemed and ensured to be over 95% pure by HPLC. The precursor peptide was dissolved in DMSO and stored at −80°C. Purified constitutive proteasome and immunoproteasome were purchased from Boston Biochem (E-360 and E-370, respectively). 10 μg of precursor peptide was mixed with either 5 μg of constitutive or immunoproteasome in 100 μl of assay buffer per replicate. Assay buffer consisted of 2 mM MgAc2, 1 mM DTT, and 20 mM HEPES/KOH at a pH of 7.8. The reaction was incubated at 37°C, and at each time point, a 20 μl aliquot was removed and quenched with 2 μl of 10% TFA in water. Samples were stored frozen at −80°C until mass spectrometry (MS) analysis. Each sample was analyzed separately by microcapillary liquid chromatography (LC) with electrospray ionization coupled with tandem MS. We used a NanoAcquity LC System (Waters) with a 100-μm inner diameter × 10-cm length C18 analytical column (1.7 μm BEH130; Waters) configured with a 180-μm × 2-cm trap column coupled to a Q-Exactive Plus Mass
Spectrometer (Thermo Fisher Scientific). A nanoelectrospray source (Proxeon, Thermo Scientific) set at 1800 V and a 25-micron (with 10-micron orifice) fused silica nanoelectrospray needle (New Objective) were used to complete the nanoelectrospray interface. For each time point, the sample was diluted 1:20 in HPLC grade water with 0.1% (v/v) formic acid and 1 μl was loaded onto the trap column and washed with 3× loop volume of buffer A (water with 0.1% [v/v] formic acid); the flow was reversed through the trap column and the peptides eluted with a 90-minute linear gradient from 1%-50% buffer B (acetonitrile with 0.1% [v/v] formic acid) at 300 nL/min. The QE Plus was operated in automatic, data-dependent MS/MS acquisition mode with 1 MS full scan (400–1800 m/z) at 70,000 mass resolution and up to 10 concurrent MS/MS scans for the 10 most intense peaks selected from each survey scan. Survey scans were acquired in profile mode, and MS/MS scans were acquired in centroid mode at 17,500 resolution, an isolation window of 1.5 amu, and normalized collision energy of 27. AGC was set to 1 × 10^6 for MS1 and 5 × 10^6 and 100 ms IT for MS2. Charge exclusion of unassigned and greater than 6 enabled with dynamic exclusion of 15 seconds. Degradation products were identified and quantified by in silico analysis of MS data. Briefly, all HPLC peaks were identified using the findpeaks method in the pracma R package. For each retention time during which a HPLC peak appeared, the ms1 spectra was analyzed to identify the identified peak series were matched to a database of all possible precursor peptide degradation products. Total intensity of each degradation product was quantified by adding up the intensities of each production. If 2 degradation products yielded the same peak series, identified peak series were matched to a database of all possible precursor peptide degradation products. Total intensity of each degradation product was quantified by adding up the intensities of each production. If 2 degradation products yielded the same peak series (e.g., FFL and LFF), intensity was assigned to each product ion.

Statistics. Values reported represent mean ± SEM unless otherwise noted. P values were calculated with GraphPad Prism 6 (GraphPad Software Inc.) using the paired Wilcoxon signed-rank test where appropriate or Student’s t test (unpaired, 2 tailed) with P < 0.05 considered significant. Binding affinity of Pr20 was determined on AML14 cells using Scatchard analysis after linear transformation of [bound] and [bound]/[free] Pr20. The 2-phase exponential decay model was used for analyzing Pr20M pharmacokinetics. Experiments were performed at least 3 times unless otherwise noted.

Study approval. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Memorial Sloan Kettering Cancer Center (New York, NY) under protocol number 96-11-044. After informed consent on and approval by MSKCC Institutional Review Board (IRB) #95-054 (New York, NY), PBMCs from HLA-typed healthy donors were obtained by Ficoll density centrifugation. Frozen cells from AML patients were obtained under specific biospecimen banking protocols at MSKCC after informed consent and research authorization.

Author contributions

AYC, TD, and DAS designed experiments, interpreted the data, and wrote the manuscript. AYC and TD did biochemical characterization of Pr20 and immunological studies. TD designed experiments for immune clone selection and characterization of Pr20. RSG performed molecular biology experiments and MS analysis. CAJ, AS, TK, and VZ performed technical cell biology, immunochemistry. MC performed mAb radiolabeling and pharmacology experiments. LD and MDM performed animal therapy experiments. RCH performed MS experiments and analysis. CL performed phage display selection, generation of mAbs, and manufacturing of mAbs. FAS was the principle investigator.

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