Generation of a new therapeutic peptide that depletes myeloid-derived suppressor cells in tumor-bearing mice

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Immune evasion is an emerging hallmark of cancer progression. However, functional studies to understand the role of myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment are limited by the lack of available specific cell surface markers. We adapted a competitive peptide phage display platform to identify candidate peptides binding MDSCs specifically and generated peptide-Fc fusion proteins (peptibodies). In multiple tumor models, intravenous peptibody injection completely depleted blood, splenic and intratumoral MDSCs in tumor-bearing mice without affecting proinflammatory immune cell types, such as dendritic cells. Whereas control Gr−1−specific antibody primarily depleted granulocytic MDSCs, peptibodies depleted both granulocytic and monocytes MDSC subsets. Peptibody treatment was associated with inhibition of tumor growth in vivo, which was superior to that achieved with Gr−1−specific antibody. Immunoprecipitation of MDSC membrane proteins identified S100 family proteins as candidate targets. Our strategy may be useful to identify new diagnostic and therapeutic surface targets on rare cell subtypes, including human MDSCs.

Activating the immune system has emerged as a promising way to treat cancer. Recent successful phase 3 clinical trials of therapeutic cancer vaccines include the US Food and Drug Administration–approved Sipuleucel-T prostate cancer vaccine, melanoma peptide vaccines and personalized lymphoma vaccines1–3. However, tumor-induced immune suppression limits their potency. MDSCs are heterogeneous cells that coexpress Gr−1− and CD11b+ myeloid lineage differentiation markers4–6. Functional studies have shown that MDSCs are potent inhibitors of T cells in mice7–10, but more specific surface markers that would allow isolation of viable MDSCs would facilitate additional studies to precisely understand tumor-MDSC interactions in the microenvironment. New specific markers are also needed for targeting MDSCs in vivo to test the hypothesis that MDSC inhibition enhances antitumor immunity4,10.

RESULTS
Identification of mouse MDSC-binding peptides
MDSC frequency is low in naive C57BL/6 mice; however, after transplantation of syngeneic EL4 thymomas MDSC numbers are increased, accounting for approximately 10% of total splenocytes5. Splenic MDSCs from EL4-bearing mice consist of two distinct subpopulations characterized by Gr−1−CD11b+ granulocytic (P7) and Gr−1intCD11b+ monocytic (P10) staining (Fig. 1a). With the goal of selecting peptide ligands that specifically bound MDSCs by phage display, we separated Gr−1−CD11b+ MDSCs from non-MDSCs by cell sorting after incubation with a Ph.D.-12 peptide phage library. Phages eluted from granulocytic or monocytic MDSCs were then expanded through rounds of competitive biopanning. We analyzed enrichment by the number of phage eluted from 1 × 10^6 MDSCs (Fig. 1b) and by phage output normalized to the initial input of 2 × 10^10 phages (Fig. 1c).

Sequencing of enriched phages revealed overrepresented peptide sequences, and two predominant peptides (H6 and G3) were selected for further study (Table 1). Each clone that was expanded and tested for binding to MDSCs revealed the specificity of both H6 and G3 phage for MDSCs, without significant binding to non-MDSCs (data not shown). We isolated additional candidate MDSC-binding phages but did not give them further consideration because their binding was not specific. Corresponding synthetic FITC-conjugated H6 and G3 peptides bound specifically to Gr−1−CD11b+ MDSCs but not Gr−1−CD11b− non-MDSC splenocytes from EL4-bearing mice (Fig. 1d).

Generation of MDSC-specific peptibodies
We genetically fused sequences encoding H6 and G3 peptides with a sequence encoding the Fc portion of mouse IgG2b to generate peptibodies (Pep-H6 and Pep-G3, respectively) (Fig. 2a). We also made control peptibodies (Pep-irrel) using nonspecific sequences. Then we produced recombinant peptibodies from 293T human embryonic kidney cells, followed by purification of the peptibodies

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Figure 1 Identification and characterization of MDSC-binding peptides. (a) Identification of Gr-1+/CD11b+ MDSCs in spleens of C57BL/6 mice (n = 5) challenged subcutaneously with EL4 mouse lymphoma cells for 3 weeks. Double-positive cells contain two distinct populations, including Gr-1intCD11b+ granulocytic (P7) and Gr-1+CD11b+ monocytic (P10) MDSC subsets. (b,c) Biopanning with Ph-D21-12 peptide phage display library on Gr-1+CD11b+ splenocytes showed enriched phage eluted from sorted MDSC subsets. Biopanning enrichment was expressed in either number of plaques per 1 × 106 cells (b) or phage output/input ratio (×10−8) (c). (d) Binding of synthetic FITC-conjugated G3 and H6 peptides on Gr-1+CD11b+ gated MDSCs from EL4-bearing C57BL/6 mice (n = 4), compared with Gr-1+CD11b−-gated non-MDSC splenocytes. A nonspecific peptide (irrel peptide) was used as a negative control to exclude nonspecific binding. The data are representative of three identical experiments.

Peptide treatment on monocytic MDSCs was especially apparent when we first subjected the blood to Ficoll sedimentation to remove granulocytes and granulocytic MDSCs (Fig. 3a–d). Notably, we observed that peptide treatment depleted intratumoral MDSCs in both EG.7 (Fig. 3b–d) and EL4 (Supplementary Fig. 3) models. Peptibodies also depleted blood and splenic MDSCs in mice bearing A20 lymphomas (Fig. 3e).

Peptibodies specifically depleted MDSCs in tumor-bearing mice without affecting other proinflammatory cells, including DCs and lymphocytes (T, B and NK cells), which is consistent with their lack of binding to these cells, although Pep-G3 treatment was associated with a minor reduction in NK cell numbers (Fig. 3f). Although we observed staining of Gr-1+CD11b− immature myeloid cells from bone marrow with peptibodies, peptide treatment of tumor-bearing mice did not deplete myeloid precursor cells in this compartment, either (Fig. 3f). Analysis of other blood cells subsets showed peptibody-associated depletion of elevated numbers of neutrophils in tumor-bearing compared with naive mice, but peptibodies had no effect on numbers of red blood cells or platelets (Supplementary Table 1).

Finally, to test our hypothesis that MDSC depletion can augment antitumor immunity, we administered peptibodies to EL4-bearing mice every other day to achieve a continuous depletion of systemic MDSCs. Mice treated with Pep-H6 or Pep-G3 alone showed a significant delay in tumor development, measured by tumor size (Fig. 3g) and tumor mass (Fig. 3h), compared with untreated or Pep-irrel-treated control mice. Whereas both peptibodies clearly inhibited tumor growth in vivo, Gr-1–specific mAb treatment was associated with less consistent inhibition of tumor growth (Fig. 3h). This effect of peptibody treatment correlated with MDSC depletion at the end of the 2-week treatment (Supplementary Fig. 4).

Cell surface–bound alarmin is a candidate target
To explore the target of our peptibody, we developed a strategy to identify peptibody-bound proteins on the surface of MDSCs (Fig. 4a). We biotinylated surface proteins on sorted Gr-1+CD11b+ MDSCs and subsequently captured them with monomeric avidin after cell lysis. Then, we used Pep-H6 that was immobilized on protein A to immunoprecipitate the surface protein of interest. Proteomic analysis of eluted proteins suggested that S100A9 was the source protein, with sequence coverage above 40% (Fig. 4b). Consistent with these results,
immunoprecipitation studies (without prior biotinylation) showed that eluted proteins from Pep-H6–bound, sorted MDSCs co-migrated with recombinant S100A9 (6× His tagged) and were recognized by S100A9–specific antibodies (Fig. 4c), but we did not detect any signal when using lysates from MDSCs without peptibody, excluding contamination by intracellular S100A9 (Fig. 4c). Furthermore, such direct immunoprecipitation of MDSC cell surface proteins with Pep-H6 revealed a protein band that more closely co-migrated with native S100A9 identified by immunoblotting of MDSC total cell lysates (Fig. 4d). Immunoprecipitation experiments suggested that the peptibody also recognizes S100A8, which is consistent with the formation of S100A9-S100A8 heterodimers (Fig. 4d). Immunoprecipitation with Pep-G3 also suggested binding to S100A9 and S100A8 proteins (Supplementary Fig. 5). These results suggested either cross-reactivity with S100A8 or perhaps recognition of a combinatorial determinant on the S100A9 and S100A8 complex.

We then tested our peptibodies in S100A9-deficient mice and observed that peptibodies could bind MDSCs (Fig. 4c) and also partially deplete MDSCs in vivo (Fig. 4f). Taking these results together, the most likely explanation is that peptibodies are cross-reactive for S100A9 and S100A8. This explanation is consistent with the fact that S100A9-deficient mice express S100A8 (ref. 11). Unfortunately, S100A8-deficient mice show an early embryonic lethal phenotype, precluding their analysis for our study. Thus, further characterization of the specificity of our peptibodies for S100 family proteins must await the availability of S100A9 and conditional S100A8 double-knockout mice.

**DISCUSSION**

We adapted a competitive peptide phage display platform to generate new diagnostic and therapeutic MDSC-specific peptide-Fc fusion (peptibody) reagents. Peptibodies successfully depleted blood and splenic MDSCs, as well as intratumoral MDSCs, in multiple syngeneic tumor models. The superiority of the peptibody therapeutic effects over an available Gr-1–specific mAb was suggested by their ability to deplete both granulocytic and monocytic MDSC subsets (Gr-1–specific
Figure 3 Peptibodies specifically depleted tumor-induced MDSCs in multiple tumor models and inhibited tumor growth in vivo. (a,b) Depletion of Gr-1+CD11b+ MDSCs in the blood and spleens in EL4-challenged C57BL/6 mice (n=5 per group) after treatment with 50 µg peptibody intravenously for three consecutive days. Control mice received Gr-1–depleting mAb (Gr-1 mAb; positive control), irrelevant control peptibody (Pep-irrel) or PBS. Plots are shown for individual representative mice (a) and composite results (mean ± s.e.m.) (b) in a representative experiment out of five, with percentages of MDSC subsets indicated (granulocytic, m, monocytes). (c,d) Depletion of MDSCs in the blood and subcutaneous tumors of EG.7-challenged C57BL/6 mice (n=5 per group) by peptibody treatment. Percentage of Gr-1+CD11b+ MDSCs from Ficolled blood and single-cell suspensions prepared from harvested tumors is shown for individual representative mice (c) and composite results (mean ± s.e.m.) (d) in a representative experiment out of two. (e) Peptibody treatment depleted MDSCs in vivo from the blood and spleens of A20 lymphoma-challenged BALB/c mice (data pooled from 2 experiments) (mean ± s.e.m.). (f) Frequencies of Gr-1+CD11b+ MDSCs, Ly6G+CD11c+ DCs, CD3+ T cells, CD19+ B cells and CD3−CD49b+ NK cells in spleens and Gr-1+CD11b+ immature myeloid cells in the bone marrow from peptibody-treated, EL4-bearing C57BL/6 mice. Data are shown as the mean ± s.e.m. of 5 mice per group. (g,h) Inhibition of EL4 tumor growth in C57BL/6 mice following peptibody treatment every other day. Tumor size is shown as the mean ± s.e.m. of 5 mice per group in a representative experiment out of four. (h) Tumor mass data are pooled results from 4 independent experiments (mean ± s.d.). *P < 0.05 and **P < 0.01 compared with tumor-challenged mice without peptibody treatment (PBS) by two-tailed Student’s t-test.

mAb depleted primarily granulocytic MDSCs) and more consistent tumor inhibition in vivo.

To evaluate potential off-target activity of our specific peptibodies, we also assessed potential depletion of normal cell types, including those of myeloid origin. With the exception of transient reduction of blood neutrophils, and possibly monocytes, we did not observe any depletion of DCs, T, B or NK cells or Gr1+CD11b+ immature myeloid precursor cells in the bone marrow in tumor-bearing mice. We also observed that in naive mice, peptibody treatment reduced circulating Gr-1+CD11b+ splenocyte numbers (Supplementary Fig. 6a) but did not affect immature myeloid cells in the bone marrow (Supplementary Fig. 6b), suggesting that peptibody treatment may not have an effect on myeloid-lineage cells other than systemic (or intratumoral) MDSCs. Consistent with this possibility, systemic MDSC numbers recovered 3 d after single-dose treatment (data not shown). The precise explanation for the lack of depletion of myeloid cells in the bone marrow,
Peptibodies recognize extracellular S100 family proteins on the surface of MDSCs. (a) Schematic representation of a strategy for peptibody-based isolation of candidate cell type–specific surface markers. (b) Proteomic analysis from sorted Gr-1+CD11b+ splenic MDSCs pooled from 5 EL4-bearing C57BL/6 mice revealing predominant peptides with homology to S100A9. The data are representative of 2 independent experiments. (c) Identification of S100A9 protein in protein A eluates of Pep-H6–bound, sorted MDSC lysate (without biotinylation) by western blotting. Recombinant mouse S100A9 protein served as a positive control (left), and lysates from unbound MDSCs were negative controls (right). Input lysates were blotted with actin as an internal control. S100A9 Ab, S100A9–specific antibody. (d) Detection of both S100A9 and S100A8 proteins in protein A eluates of Pep-H6–bound, sorted MDSC lysate by western blotting. All western blot data in c and d are representative of 3 independent experiments. The MDSC lysates were pooled from 5 EL4-bearing C57BL/6 mice. (e) Binding of Pep-H6 and Pep-G3 peptibodies with CD11b+Gr-1+–gated splenic MDSCs from EL4-bearing, S100A9–deficient C57BL/6 mice (n = 3). The data are representative of 2 independent experiments. (f) Frequencies of CD11b+Gr-1+–gated splenic MDSCs from EL4-bearing, S100A9–deficient C57BL/6 mice (n = 3) after peptibody treatment as in Figure 3a. This experiment was performed once. Despite binding by peptibody, is unclear. However, our peptibodies were engineered to express mouse IgG2b Fc portion with the goal of enhancing antibody-dependent cell-mediated cytotoxicity (ADCC). One possible explanation is that the effector cells (mature NK cells and macrophages) required for ADCC are present in insufficient numbers in the bone marrow. Finally, peptibody-treated mice suffered no visible adverse effects or constitutional signs of cachexia or ruffled appearance, compared with control naive or tumor-bearing mice, and had otherwise normal blood counts. Altogether, the data suggest limited off-target effects of peptibody treatment.

The identification of surface S100A9 and S100A8 as potential peptibody targets validates our protocol as a strategy for cell type–specific surface marker discovery. S100 family calcium-binding proteins are intracellular molecules released to the extracellular milieu by myeloid cells in response to inflammation and function as proinflammatory danger signals (alarmins). Though there is limited evidence showing cells in response to inflammation and function as proinflammatory intracellular molecules released to the extracellular milieu by myeloid surface marker discovery. S100 family calcium-binding proteins are limited off-target effects of peptibody treatment.

Peptibodies recognize extracellular S100 family proteins on the surface of MDSCs. (a) Schematic representation of a strategy for peptibody-based isolation of candidate cell type–specific surface markers. (b) Proteomic analysis from sorted Gr-1+CD11b+ splenic MDSCs pooled from 5 EL4-bearing C57BL/6 mice revealing predominant peptides with homology to S100A9. The data are representative of 2 independent experiments. (c) Identification of S100A9 protein in protein A eluates of Pep-H6–bound, sorted MDSC lysate (without biotinylation) by western blotting. Recombinant mouse S100A9 protein served as a positive control (left), and lysates from unbound MDSCs were negative controls (right). Input lysates were blotted with actin as an internal control. S100A9 Ab, S100A9–specific antibody. (d) Detection of both S100A9 and S100A8 proteins in protein A eluates of Pep-H6–bound, sorted MDSC lysate by western blotting. All western blot data in c and d are representative of 3 independent experiments. The MDSC lysates were pooled from 5 EL4-bearing C57BL/6 mice. (e) Binding of Pep-H6 and Pep-G3 peptibodies with CD11b+Gr-1+–gated splenic MDSCs from EL4-bearing, S100A9–deficient C57BL/6 mice (n = 3). The data are representative of 2 independent experiments. (f) Frequencies of CD11b+Gr-1+–gated splenic MDSCs from EL4-bearing, S100A9–deficient C57BL/6 mice (n = 3) after peptibody treatment as in Figure 3a. This experiment was performed once. Despite binding by peptibody, is unclear. However, our peptibodies were engineered to express mouse IgG2b Fc portion with the goal of enhancing antibody-dependent cell-mediated cytotoxicity (ADCC). One possible explanation is that the effector cells (mature NK cells and macrophages) required for ADCC are present in insufficient numbers in the bone marrow. Finally, peptibody-treated mice suffered no visible adverse effects or constitutional signs of cachexia or ruffled appearance, compared with control naive or tumor-bearing mice, and had otherwise normal blood counts. Altogether, the data suggest limited off-target effects of peptibody treatment.

The identification of surface S100A9 and S100A8 as potential peptibody targets validates our protocol as a strategy for cell type–specific surface marker discovery. S100 family calcium-binding proteins are intracellular molecules released to the extracellular milieu by myeloid cells in response to inflammation and function as proinflammatory danger signals (alarmins). Though there is limited evidence showing that soluble S100A9 binds MDSCs in vitro; it is likely that MDSCs secrete S100A9 in an autocrine feedback mechanism through receptors. The precise nature of the peptibody target remains to be elucidated. Although the epitopes recognized may be derived from S100 proteins, because our protocol used viable cells to screen the peptide library, combinatorial native conformational epitopes composed of the S100–receptor complex would have also been preserved. Given that our peptibody did not bind or deplete DCS, the cell surface receptor for S100A9 and S100A8 on MDSCs may be an as yet unidentified or different from the receptors on DCS, such as RAGE and Toll-like receptor 4. We also acknowledge that it is formally possible that other MDSC-specific targets exist that were not detected by our methods, as biotinylation might mask some epitopes.

Hypothetically, an MDSC-depleting peptibody could cause tumor regression by depleting MDSCs by complement-dependent cytotoxicity or ADCC, inducing apoptosis by blocking the binding of the S100 family protein with its receptor on MDSCs or on tumor cells14,15 and interfering with S100–induced survival signals, and inducing direct cytotoxicity against tumor cells14,15. In order to rationally combine peptibody treatment to reverse immune suppression with other immunotherapy strategies, it will also be important to formally demonstrate that inhibition of tumor growth after MDSC depletion is immune mediated. Future studies are needed to investigate these possibilities. Finally, human MDSC targets are needed. Indeed, S100A9 was recently reported on MDSCs isolated from patients with colon cancer16. Additional studies are in progress applying the technology platform described de novo to identify new targets on human MDSCs.

**Methods**

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.
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AUTHOR CONTRIBUTIONS
H.Q. designed the project and experiments, analyzed data and wrote the manuscript. B.L. and I.S. performed most of the experiments. G.W., S.S.R., S.C.C., J.Q., Y.H., R.N. and K.C.D. assisted with mouse studies, flow cytometry experiments and cell sorting. J.R., Q.Y. and W.W.O. provided S100A9-knockout mice, the EL4 tumor model and Gr-1–specific mAb, respectively, and also reviewed the manuscript. L.W.K. supervised the project, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Phage display. Total splenocytes from EL4 tumor (ATCC)-bearing C57BL/6 mice were blocked with formaldehyde-inactivated M13 phage and 2.4G2 antibody (BD Biosciences, San Jose, CA), followed by staining with anti-CD11b-APC and anti-Gr-1-FITC (BD Biosciences) (Supplementary Table 2). Gr-1 and CD11b labeled splenocytes were then incubated with 2 × 10^5 Ph.D.-12 peptide phage display library (New England BioLabs Inc., Ipswich, MA) for 1 h at 4°C. Gr-1<sup>hi</sup>/CD11b<sup>granulocytic</sup> and Gr-1<sup>lo</sup>/CD11b<sup>monocyte</sup> MDSC subsets were sorted separately, and their bound phage was eluted, titrated and amplified for the next round of biopanning. After the third round of biopanning, predominant MDSC-binding peptides were identified by PCR analysis of 66 individual phage, generated using specific primers spanning encoded peptides, was sequenced.

MDSC specificity of synthetic peptide. FITC-conjugated H6 or G3 synthetic peptides were made by Pl Proteomiks, LLC (Huntsville, AL). After blocking with 2.4G2 antibody, splenocytes from EL4-bearing C57BL/6 mice were co-stained with anti-CD11b-APC, anti-Gr-1-PE and FITC-conjugated peptides. H6 and G3 peptides were analyzed for their binding to Gr-1<sup>−</sup>/CD11b<sup>+</sup> gated MDSCs, compared with Gr-1<sup>+</sup>/CD11b<sup>−</sup> gated non-MDSC splenocytes. A nonspecific peptide (irrelevant peptide) was used as a negative control to exclude nonspecific binding. FACS analysis was performed using LSRFortessa cell analyzer (BD Biosciences), and results were analyzed using FlowJo software.

Generation and production of MDSC-specific peptibody. Synthetic, complementary double-stranded oligonucleotides encoding H6 or G3 peptide were fused with a human IL-2 signal peptide and 6× His tag and then cloned into EcoRI and BglII sites of pTINFUSE-mlgG2b-Fc vector (InvivoGen, San Diego, CA). For initial characterization, we produced recombinant peptibodies by transfecting 293T human embryonic kidney cells with the plasmid constructs using Lipofectamine 2000 kit (Life Technologies, Carlsbad, CA). Peptibodies excreted into growth medium were purified using protein A chromatography (GE Healthcare Life Sciences, Pittsburgh, PA). The identity of peptibodies was verified by western blotting using anti-His-HRP (BD Biosciences), anti-mouse IgG-HRP antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) (Supplementary Table 2). Recombinant peptibodies used in all in vivo studies were produced by Aldevron (Madison, WI).

Animals used for in vivo studies. Six-week-old C57BL/6 and BALB/c female mice were purchased from US National Cancer Institute. St100A9-deficient female mice were a generous gift from D. Kusewitt (M.D. Anderson Cancer Center, Smithville, TX) by permission of J. Roth (University of Muenster, Germany), who originally generated the model. Mice were maintained in a pathogen-free mouse facility according to institutional guidelines. All animal studies were approved by the Institutional Animal Care and Use Committee at MD Anderson Cancer Center. The experimental sample sizes were chosen to ensure adequate statistical power. No animals were excluded. Randomization and blinding were not used in animal studies.

FACS analysis of binding specificity of peptibodies. Recombinant peptibodies were conjugated with FITC or APC using FluorReporter Protein Labeling kits (Life Technologies). All other fluorophore-labeled monoclonal antibodies used for immune staining of cell type–specific markers including CD11b, Gr-1, Ly6C, Ly6G, CD3, CD19, CD49b and CD11c were purchased from BioLegend (San Diego, CA) (Supplementary Table 2). To determine the binding pattern of Pep-H6 and Pep-G3, splenocytes from EL4-bearing C57BL/6 mice, EG.7-bearing C57BL/6 mice, B16-bearing C57BL/6 mice and A2O-bearing BALB/c mice were co-stained with anti-CD11b-APC, anti-Ly6G-PE, anti-Ly6C-PerCP and FITC-conjugated peptides. Peptibody binding was analyzed on CD11b<sup>−</sup>/Ly6G<sup>+</sup> and CD11b<sup>+</sup>/Ly6G<sup>−</sup> gated granulocytic MDSCs and CD11b<sup>−</sup>/Ly6G<sup>+</sup> and CD11b<sup>+</sup>/Ly6G<sup>−</sup> gated monocyte MDSCs, respectively. To further characterize the binding specificity of peptibodies, splenocytes from EL4-bearing C57BL/6 mice were co-stained with anti-CD11c-PerCP, anti-Ly6G-PE and peptibody-FITC. CD11c<sup>+</sup>Ly6G<sup>−</sup> DCs were analyzed for peptibody binding. In a separate experiment, splenocytes from EL4-bearing C57BL/6 mice were co-stained with anti-CD19-APC, anti-CD3-PerCP, anti-CD49b-PE and peptibody-FITC. CD3<sup>+</sup> T cells, CD19<sup>+</sup> B cells and CD3<sup>−</sup>CD49b<sup>+</sup> NK cells were gated and analyzed for peptibody binding. Likewise, bone marrow cells from EL4-bearing C57BL/6 mice were isolated and co-stained with anti-CD11b-APC, anti-Gr-1-PE and FITC-conjugated peptibodies. CD11b<sup>+</sup>/Gr-1<sup>−</sup> immature myeloid cells were analyzed for their binding to peptibodies. Finally, to determine whether Pep-H6-bound population would overlapped with Pep-G3-bound populations, splenocytes from EL4-bearing C57BL/6 mice were co-stained with anti-Gr-1-PE, anti-CD11b-PerCP and either APC-labeled Pep-G3 or FITC-labeled Pep-H6, or both. CD11b<sup>+</sup>/Gr-1<sup>−</sup> gated MDSCs were analyzed for peptibody binding.

In vivo MDSC depletion. Groups of 5 C57BL/6 mice were challenged subcutaneously with 1 × 10<sup>6</sup> EL4 or EG.7 mouse thymoma tumor cells on day 0. Tumor-bearing mice were treated intravenously with 50 µg of peptibodies per day for 3 consecutive days (days 17, 18 and 19), and then killed on day 20 to harvest blood and spleens. Control mice received Gr-1<sup>−</sup> single monoclonal antibodies (clone 1A8, BioXcell, West Lebanon, NH), irrelevant control peptibody (Pep-irrel) or PBS. Ficolled blood and splenocytes were stained with anti-Gr-1-PE and anti-CD11b-APC to identify MDSCs. To prepare single-cell suspensions from harvested tumors, EL4 or EG.7 subcutaneous tumors were cut into small pieces of 2–4 mm and digested with the enzyme mix (40 min at 37°C) provided in the Tumor Dissociation Kit (Miltenyi Biotec Inc., Auburn, CA). Tumors were dissociated into single-cell suspension with a gentleMACS Dissociator (Miltenyi Biotec Inc.). The tumor cells were then stained with anti-Gr-1-PE and anti-CD11b-APC to identify intratumoral MDSCs. To determine whether the in vivo depletion effect of peptibodies is MDSC specific, splenocytes from peptibody-treated, EL4-bearing C57BL/6 mice were co-stained with anti-CD11b-APC, anti-CD11c-PerCP and anti-Ly6G-PE and myeloid cells were gated based on forward and side scatter profile. Ly6G<sup>−</sup>CD11c<sup>+</sup> DCs were enumerated and their representative frequencies were calculated by multiplying the myeloid cell frequency in total splenocytes. Splenocytes were also stained with anti-CD19-APC, anti-CD3-PerCP and anti-CD49b-PE, and frequencies of CD3<sup>+</sup> T cells, CD19<sup>−</sup> B cells and CD3<sup>−</sup>CD49b<sup>+</sup> NK cells in total splenocytes were analyzed. Bone marrow cells were stained with anti-CD11b-APC and anti-Gr-1-PE. Double-positive immature myeloid cells were enumerated.

In vivo therapeutic studies. Groups of 5 C57BL/6 mice were challenged with 1 × 10<sup>6</sup> EL4 tumor cells on day 0. Twenty-four hours later, mice started to receive 50 µg of peptibodies every other day for 2 weeks. Control mice received Gr-1 monoclonal antibodies (BioXcell), Pep-irrel or PBS. Tumor dimensions were measured daily with caliper to monitor growth. On day 14, mice were killed, and spleens and subcutaneous tumors were isolated. Tumor mass was measured on analytical balances. Splenocytes were stained for MDSCs using anti-Gr-1-PE and anti-CD11b-APC antibodies.

Identification of targets for peptibodies on the surface of MDSCs. Splenocytes from day 21 EL4-bearing C57BL/6 mice were immunolabeled with anti-Gr-1-PE and anti-CD11b-APC, and double-positive MDSCs were sorted. Cell surface proteins on sorted cells were biotinylated with EZ-Link Amine-PEG-Biotin kit (Thermo Scientific, Rockford, IL) and precipitated by monomeric avidin after cell lysis. A sequential second immunoprecipitation was performed using Pep-H6 immobilized on protein A–agarose. After washing away unbound proteins, the eluate was analyzed by proteomic sequencing in the Proteomic core at MD Anderson Cancer Center.

Immunoprecipitation and western blot. To confirm the results of proteomic analysis, total cell lysates prepared from Pep-H6–bound, sorted MDSCs (without biotinylation) were loaded onto a protein A column. The eluate was separated by SDS-PAGE followed by immunoblotting with S100A9 antibodies (Abcam, Cambridge, MA) (Supplementary Table 2). Recombinant mouse S100A9 protein (protein) (Fitzgerald, Acton, MA) served as a positive control, and lysates from unbound MDSCs were negative controls. Input lysates were blocked with actin as an internal control. Western blot was also performed on
the protein A eluates of Pep-H-bound MDSC lysates or total cell lysates alone using anti-S100A8 antibodies (Abcam).

**S100A9-deficient mouse studies.** Groups of 3 S100A9-deficient C57BL/6 mice were challenged subcutaneously with $1 \times 10^6$ EL4 tumors on day 0. Three weeks later, splenocytes were harvested and co-stained with anti-CD11b-APC, anti-Gr-1-PE and peptibody-FITC. CD11b$^+$Gr-1$^+$ MDSCs were analyzed for peptibody binding. In a separate experiment, EL4-bearing S100A9-deficient C57BL/6 mice were treated i.p. with 50 µg of peptibodies for 3 consecutive days (days 17, 18 and 19). Splenocytes were harvested on day 20 and stained for MDSCs, as above.

**Statistical analyses.** Statistical analyses were performed using two-tailed Student’s $t$-test to determine statistical significance that is expressed as $^*P < 0.05$ and $^{**}P < 0.01$ compared with tumor-challenged mice without peptibody treatment (PBS).