A Synthetic CD8α:MyD88 Coreceptor Enhances CD8+ T-cell Responses to Weakly Immunogenic and Lowly Expressed Tumor Antigens

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Abstract

T-cell–based immunotherapies are a promising approach for patients with advanced cancers. However, various obstacles limit T-cell efficacy, including suboptimal T-cell receptor (TCR) activation and an immunosuppressive tumor environment. Here, we developed a fusion protein by linking CD8α and MyD88 (CD8α:MyD88) to enhance CD8+ T-cell responses to weakly immunogenic and poorly expressed tumor antigens. CD8α:MyD88-engineered T cells exhibited increased proliferation and expression of effector and costimulatory molecules in a tumor antigen–dependent manner. These effects were accompanied by elevated activation of TCR and Toll-like receptor signaling-related proteins. CD8α:MyD88–expressing T cells improved antitumor responses in mice. Enhanced antitumor activity was associated with a unique tumor cytokine/chemokine signature, improved T-cell infiltration, reduced markers of T-cell exhaustion, elevated levels of proteins associated with antigen presentation, and fewer macrophages with an immunosuppressive phenotype in tumors. Given these observations, CD8α:MyD88 represents a unique and versatile approach to help overcome immunosuppression and enhance T-cell responses to tumor antigens.

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Introduction

T-cell–based immunotherapies are one of the most promising treatments for patients with advanced cancers, including melanoma. Several approaches have been developed to harness the anti-tumor activity of cytotoxic T cells, including vaccine-based therapies, adoptive cell transfer of tumor-infiltrating lymphocytes (1, 2) or T cells engineered to express a tumor-reactive T-cell receptor (TCR; refs. 3, 4), and antibody-mediated checkpoint blockade (5). Despite encouraging clinical results demonstrating tumor regression and prolonged survival, durable antitumor responses are observed only in a subset of patients, highlighting the need to improve these therapies. Limitations include low tumor antigen–specific T-cell precursor frequencies, suboptimal TCR responses due to low TCR affinity (or avidity) to weakly immunogenic tumor antigens, and low antigen expression and/or presentation on tumor cells (1, 2, 5). Furthermore, various suppressive mechanisms within the tumor microenvironment (TME), such as tumor-associated macrophage (6) and Th2 cytokine accumulation, hamper antitumor T-cell responses. Moreover, chronic exposure to factors in the TME induces the expression of receptors that foster T-cell exhaustion, such as Tim-3, Lag-3, and PD-1 (7).

Various studies have demonstrated that activating MyD88 signaling in T cells via Toll-like receptor (TLR) engagement enhances cytokine production, cytotoxicity, and proliferation (8–10). Yet, applying TLR costimulation specifically to T cells is challenging due to their low and transient expression of TLRs, insufficient intratumoral TLR agonist localization for T-cell costimulation, and the potential for TLR engagement on tumor cells to promote tumor growth (10).

Here, we present a novel strategy to activate MyD88 signaling within CD8+ T cells in a TLR-independent but TCR-dependent fashion to overcome weak tumor antigenicity and an immunosuppressive TME. This strategy utilizes the CD8α chain, an endogenous TCR coreceptor that interacts with MHC I. By fusing CD8α to MyD88 (CD8α:MyD88), we developed a novel platform that enhances various parameters of T-cell function. CD8α:MyD88–expressing T cells respond to suboptimal levels of tumor antigen and display reduced levels of exhaustion markers and increased production of effector molecules. The enhanced antitumor activity of CD8α:MyD88–expressing T cells is associated with their ability to reshape various facets of the TME, including the cytokine milieu as well as the phenotype of other immune cells, resulting in a TME that favors T-cell infiltration and antitumor activity.

Materials and Methods

Plasmids, retroviral production, and T-cell transduction

The CD8α:MyD88 coreceptor was designed by fusing the murine CD8α extracellular and transmembrane domain sequences...
to the human MYD88 death domain and intermediate domain sequences (Supplementary Fig. S1A). The CD8αΔIC construct lacked any intracellular domains. Genes were cloned into the pMIG-w vector containing a GIP reporter gene. Retroviral vector supernatants were produced from Phoenix Amphot and Eco packaging cell lines we have described previously (11). For generating engineered mouse T cells, pmel or OT-I T cells were activated for 48 hours using 1 mg/mL hgp10025-33 or SIINFEKL peptide, respectively, and 50 U/mL of IL2 prior to transduction. Efficiency was determined by assessing the frequency of GFP+ cells by flow cytometry. For generating DMF5-CD8α:MyD88 cells, human peripheral blood mononuclear cells (PBMC) were engineered to express DMF5 as described previously (11), expanded human peripheral blood mononuclear cells (PBMC) were engineered to express DMF5 as described previously (11), expanded

Mice, tumor model, and ex vivo analysis

Studies were approved by the UMB Institutional Animal Care and Use Committee. C57BL/6 and pmel (B6.Cg-Thy1/Cy Tg (TcrαTcrβ)Rest/J) mice were purchased from The Jackson Laboratory. T cells from IRAK-4 kinase dead mice were kindly provided by Dr. Stefanie Vogel at the University of Maryland (Baltimore, MD). C57BL/6 mice were injected with 2 x 10^6 B16-F1 melanoma cells subcutaneously on the right flank. Mice were irradiated with 350 rads on day 9 after tumor inoculation and intravenously injected with engineered pmel T cells on day 10. Mouse body weight and tumor size were monitored every 2 to 3 days. Tumor volume was calculated by the ellipsoid formula: length x width x height x (4/3)π. Specific tissues were harvested one week after T-cell transfer for ex vivo analyses including flow cytometry and cytokine/chemokine Lumimex. The following antibodies were used: CD8α, Lag3, Tim3, I-A/E, CD8ε, CD11b, CD11c, F4/80, CD206, Gr-1, NK1.1 (BioLegend), CD45.2, CD8α, CD19 (BD Pharmingen), MHC class I H2 Kb + Db (Abcam).

T-cell proliferation assays, cytokine measurements, and intracellular staining

Splenocytes from C57BL/6 mice were irradiated (3,000 rads) and pulsed with varying concentrations of hgp10025-33 or SIINFEKL peptide for 2 hours at 37°C. Pmel or OT-I T cells were cocultured with peptide-pulsed splenocytes at a 1:1 ratio, and supernatant was collected after 24 hours. Alternatively, B16-F1 cells (ATTC CRL-6323, obtained within three years of using them) were irradiated (20,000 rads) and plated at various cell numbers together with 1 x 10^5 transduced T cells for 48 hours. Cytokine concentrations were determined by ELISA (eBioscience) or the Milliplex Cytokine/Chemokine Kit (Millipore). IFNγ and TNFα production by DMF5 T cells was evaluated at 2:1 and 1:2 T to PBMC ratios after 4 days of stimulation. We assessed T-cell proliferation by adding 1 μCi/well of tritiated thymidine (methyl-3H, PerkinElmer) and measured thymidine incorporation 24 hours later. Intracellular levels of signaling proteins were evaluated by flow cytometry. Briefly, cells were permeabilized in BD Pharmingen Phosflow Perm Buffer III (BD Bioscience) and stained with anti-p-p65 and anti-rabbit IgG F(ab)2 Fragment-PE (Cell Signaling Technology), or anti-p-ERK1/2/Pacific Blue and anti-p-PNK-PE, or anti-p-p38/Pacific Blue and anti-p-Zap70-PE (BD Biosciences). For flow cytometry–based proliferation assays, transduced T cells were pulsed with cell proliferation dye eFluor 450 (eBioscience), washed, and cocultured with hgp10025-33-pulsed splenocytes at a 1:1 ratio for 72 hours. In other experiments, T cells were coincubated with tumor antigen-pulsed splenocytes (mouse pmel or OT-I T cells) or autologous PBMCs (human DMF5 T cells) for 48 or 96 hours with brefeldin A added the last 6 hours of incubation prior to staining. For evaluating human T-cell proliferation, transduced T cells were cocultured with Malme-3M melanoma cell (HLA-A2* MART-1+) or with A375 melanoma cells (HLA-A2+) pulsed or unpulsed with 10 μg of MART-1 27-35 peptide at a ratio of 1:1 T cell to tumor cell ratio. For the phenotyping screen, transduced T cells were cocultured with 0.12 μg/mL of hgp10025-33-pulsed splenocytes at a 1:2 splenocyte to T-cell ratio for 48 hours, stained with the Zombie Aqua viability dye (BioLegend), anti-CD45.2 and anti-CD8α antibodies, and stained with the LegendScreen Mouse Cell Screening (PE) Kit (BioLegend). Malme-3M and A375 cell lines were obtained from ATCC, used, and were tested for mycoplasma within 3 years of purchasing them. All flow cytometry experiments were performed on the BD LSRII at the Greenebaum Comprehensive Cancer Center Flow Cytometry Shared Service Lab and analyzed by FlowJo (TreeStar).

Statistical analysis

Proliferation and ELISA experiments were performed in triplicate in at least two independent experiments and analyzed by one-way ANOVA. Animal studies contained 8 to 10 animals per group for growth and survival, and 5 per group for ex vivo flow cytometry analysis. For flow cytometry and the cytokine arrays, the values and error bars represent mean ± SEM (”, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; one-way ANOVA with Tukey multiple comparison test; n = 3 experimental replicates and are representative of at least two independent experiments). Tumor sizes were analyzed by a mixed model repeated measures with AR(1) covariance structure (yielding smallest BIC), followed by Sidak-adjusted comparisons at each time point.

Results

CD8α:MyD88 lowers the threshold of T-cell activation, resulting in increased proliferation and cytokine production

The extracellular and transmembrane domain of CD8α were fused to the human MYD88 death and intermediate domain to generate a CD8α:MyD88 coreceptor (Supplementary Fig. S1A). As controls, we generated CD8α lacking the intracellular domain (CD8αΔIC), or an empty vector control. Each vector also contained a GFP reporter to distinguish transduced T cells (Supplementary Fig. S1B). CD8α:MyD88 expression was confirmed by Western blot and imaging flow cytometry (Supplementary Fig. S1C and S1D).

Gp10025-33-specific TCR transgenic pmel CD8+ T cells were engineered to express CD8α:MyD88, CD8αΔIC, or only GFP using retroviral vectors and stimulated with gp10025-33-pulsed splenocytes. Pmel T cells expressing CD8α:MyD88 exhibited a lower activation threshold as demonstrated by increased proliferation in response to lower tumor antigen (TAg) levels compared with control T-cell groups (Fig. 1A). Notably, although only half of the CD8+ T cells expressed CD8α:MyD88, proliferation was doubled at most antigen concentrations (Supplementary Fig. S1B, Fig. 1A). The ability for T cells to
recognize exceedingly low TAg levels is relevant as tumor cells can downregulate TAg or MHC I expression to evade T-cell detection. In the absence of TAg, CD8α:MyD88 T cells displayed little to no baseline proliferation, indicating that the enhancement of TCR responses by MyD88 is TCR dependent. To determine whether the increased proliferation observed in CD8α:MyD88 T cells was due to their ability to promote the division of nontransduced T cells, we compared cell division of CD8α:MyD88 (GFP+) and nontransduced (GFP−) T cells. CD8α:MyD88 T cells loaded with a proliferation dye...
expanded to a greater degree than nontransduced T cells, as indicated by a lower median fluorescence intensity in GFP cells (Fig. 1B, right). The increased proliferation by CD8α:MyD88+ T cells was more pronounced at suboptimal Tag concentrations that were too low to activate control T cells (Supplementary Fig. S1E). In addition, the degree of cell division between the GFP–T cells from each of the groups was comparable. These data indicate that the costimulatory effects of CD8α:MyD88 occur within engineered T cells and do not impact the proliferation of neighboring T cells.

One of the potential advantages to using CD8α:MyD88 is the ability to boost responses with any given TCR. This was confirmed using OT-I TCR transgenic CD8+ T cells specific for an ovalbumin (OVA) peptide presented on MHC I, which also showed increased proliferation to antigen stimulation in a dose-dependent manner (Fig. 1C). Likewise, human T cells engineered to express fully human CD8α:MyD88 and the DMF5 TCR, which recognizes the MART-127-35 Tag (12), demonstrated greater proliferation than control T cells following stimulation with MART-1+ Malme-3M melanoma cells (Fig. 1D). In addition, DMF5 CD8α:MyD88–expressing T cells proliferated in response to MART-127-35 peptide-pulsed but not unpulsed A375 (HLA-A2+ MART-1+) melanoma cells (Fig. 1E). Further highlighting that the enhancing effects of CD8α:MyD88 are strictly regulated by tumor antigen expression and are effective in multiple tumor and antigen models.

CD8α:MyD88 transduces signals by recruiting IL1 receptor associated kinase-4 (IRAK-4). In the absence of functional IRAK-4, CD8α:MyD88 did not enhance T-cell proliferation (Supplementary Fig. S1F), demonstrating the dependence of CD8α:MyD88 costimulation on the canonical MyD88 pathway. CD8α: MyD88 led to the enhanced and more rapid activation (phosphorylation) of downstream signaling proteins ERK1/2, JNK, and p38 (Fig. 1F). Increased ERK1/2 activation is advantageous as ERK2 drives CD8+ T-cell proliferation and survival (13). CD8α:MyD88 also enhanced the activation of the proximal TCR protein ZAP-70, a CD3 receptor–associated protein tyrosine kinase (Fig. 1F).

CD8α:MyD88 expression in pmel and OT-I T cells also increased IFNγ and TNFα production over CD8α:IC and empty vector T cells (Fig. 2A and B) and occurred in an antigen concentration-dependent fashion (Fig. 2C). Increased cytokine levels were in part due to the ability of CD8α:MyD88 to increase the frequency of T cells capable of producing both IFNγ and TNFα or TNFα alone (Fig. 2D). In addition, increases in IFNγ-producing CD8α:MyD88 T cells were more apparent at lower Tag levels (Supplementary Fig. S2A). MART-127-35–specific human DMF5 CD8+ T cells also showed enhanced IFNγ and TNFα production in response to stimulation with Tag (Fig. 2E).

CD8α:MyD88 upregulates cytokine receptors and costimulatory molecules while reducing molecules associated with T-cell exhaustion

To better understand changes induced by CD8α:MyD88, we conducted a flow cytometry–based protein expression array evaluating the levels of 252 surface proteins. Changes in the expression of 89 surface proteins were identified comparing the fold change between vector control and CD8α:MyD88 or CD8α:ΔIC T cells (Fig. 2F). CD8α:MyD88 increased expression of IL2 receptor chains CD25 and CD132, costimulatory molecules involved in T-cell proliferation and cytolytic function, such as CD71, CD26, and CD137 (4-1BB; ref. 14), and various activation-associated adhesion molecules, including CD44 and CD69. CD8α:MyD88 also decreased the expression of coinhibitory molecules including CD160, CD73, Tim-3, and CD39 (Fig. 2F). Notably, CD39 has been reported to be a marker of terminally exhausted CD8+ T cells and was considerably reduced in CD8α: MyD88 T cells (15). The most differentially expressed proteins fell into a wide variety of classes, but changes by CD8α:MyD88 universally favored improved T-cell function (Fig. 2F, bottom; Supplementary Fig. S2B).

CD8α:MyD88 increases T-cell responses to weakly immunogenic B16-F1 melanoma cells

Cancer cells, including the melanoma cell line B16-F1, often carry alterations in antigen presentation machinery (16, 17) that make them poor T-cell targets. Despite having nearly undetectable MHC I levels (Supplementary Fig. S3A), CD8α:MyD88 substantially enhanced T-cell proliferation following coculture with B16-F1 as compared with control T cells (Fig. 3A). Proliferation occurred in a tumor cell number–dependent manner, and T cells did not proliferate in the absence of tumor cells. Notably, CD8α:MyD88–expressing T cells required nearly 10-fold fewer melanoma cells to achieve the same level of proliferation as control T cells (Fig. 3A, hashed lines). CD8α: MyD88 T cells produced higher levels of IFNγ and granzyme B than control T cells in response to tumor cells (Fig. 3B). Furthermore, CD8α:MyD88 T cells produced IFNγ at tumor cell numbers at which control T cells could not, emphasizing the propensity of CD8α:MyD88 to lower the TCR activation threshold, a beneficial feature for destroying weakly immunogenic tumor cells. CD8α:MyD88 also induced multiple chemokines important for immune cell recruitment, including RANTES, CXCL9, and CCL3 (Fig. 3C; Supplementary Fig. S3B). In addition, we evaluated changes in the levels of other cytokines/chemokines following stimulation with B16-F1 melanoma cells and found that CD8α:MyD88 increased CXCL1, CXCL2, CCL4, CXCL10, GM-CSF, IL3, and LIF levels in tumors and serum (Supplementary Fig. S4A and S4B).

CD8α:MyD88 expression increases the infiltration of T cells into the tumor and reduces T-cell exhaustion phenotype

On the basis that MyD88 activation in T cells enhanced proliferation and production of various cytokines/chemokines, we evaluated T cells in the tumor and spleen 7 days after adoptive transfer into tumor-bearing mice. CD8α:MyD88–expressing T cells comprised a greater proportion of the CD8+ T-cell population within the tumor as compared with vector control T cells (Fig. 3D). However, the numbers of control and CD8α: MyD88–expressing T cells were similar in the spleen (Fig. 3D). These data suggest that the costimulatory effect of CD8α: MyD88 signaling occurs in a manner that depends strictly on TCR engagement in the tumor and does not occur in a nontumor-specific manner in other tissues. Furthermore, in agreement with data demonstrating a reduction of exhaustion markers on CD8α:MyD88 T cells (Fig. 2F), there were nearly four times fewer Lag3+Tim3+ CD8α:MyD88–expressing T cells in tumors as compared with control T cells, but similar proportions in spleens (Fig. 3E). Lag3+Tim3+ expression on T cells is associated with a reduced ability to produce effector molecules, including IFNγ. Fittingly, CD8α:MyD88 T cells induced higher levels of IFNγ in tumors, whereas IFNγ was nearly undetectable.
Figure 2.
CD8xMyD88 T cells exhibit enhanced cytokine production and alter the expression of costimulatory and coinhibitory molecules in response to tumor antigen. A and B, IFNγ and TNFα levels measured by ELISA in supernatants from pmel T cells cocultured with gp10025-33-pulsed splenocytes (A) and OT-I T cells with OVA (SIINFEKL)-pulsed splenocytes at 24 hours (B). C, IFNγ production by engineered pmel T cells cocultured with gp10025-33-pulsed splenocytes. D, Intracellular cytokine staining of pmel T cells stimulated with splenocyte pulsed with 5 μg/mL gp10025-33. E, Cytokine production by DMF5 empty vector, DMF5 CD8xΔIC, or DMF5 CD8xMyD88 human T cells in response to stimulation with MART-127-35–pulsed PBMCs as measured by ELISA 4 days later. Data from three independent experiments with three replicates per reaction are shown. Values, mean ± SEM. **, P ≤ 0.01; one-way ANOVA. F, Flow cytometry screen of pmel T cells cocultured for 48 hours with splenocytes pulsed with 0.12 μg/mL gp100. The fold change of the median fluorescence intensity of CD8xΔIC and CD8xMyD88 T cells over control vector T cells from three independent experiments is displayed in a heatmap. Gray boxes represent data not available (na). Representative histograms are shown of the expression of most upregulated and downregulated molecules. Values and error bars represent mean ± SEM. ***, P ≤ 0.001; one-way ANOVA with Tukey multiple comparison test at each concentration; n = 3 experimental replicates; representative of at least two independent experiments.
Figure 3. CD8α:MyD88 T cells display improved responses to melanoma in vitro and in vivo. A, Proliferation of engineered pmel T cells stimulated with irradiated B16-F1 tumor cells for 72 hours. B and C, ELISA and Luminex analysis of cytokines and chemokines in the supernatant of T cells cocultured with B16-F1 tumor cells for 48 hours. D–I, Mice bearing established B16-F1 tumors were exposed to a sublethal dose of irradiation (550 rads), followed by transfer of 3 × 10^6 T cells by intravenous injection one day later. Tissues were harvested and analyzed by flow cytometry one week after T-cell transfer. D, The frequency of GFP^+ cells in the tumor and spleen. E, The percentage of CD8^+ GFP^+ cells expressing exhaustion markers Tim-3 and Lag-3. F, IFNγ levels in tumor tissue measured by ELISA of tumor homogenate. G, Protein levels of CXCL9 in the tumor and serum. H, Frequency of CD8^+ T cells in the tumor and spleen. I, Frequency of CD45^+ cells, CD19^+ cells, and NK1.1^+ cells in the tumor and spleen. Values and error bars represent mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***; P ≤ 0.001 (A–C), one-way ANOVA with Tukey multiple comparison test at each concentration; n = 3 experimental replicates or n = 5 for D–I.
in tumors of control-treated or untreated mice (Fig. 3F). IFNγ is known to induce CXCL9, a potent chemoattractant that recruits T cells to the tumor and skews responses toward a Th1 phenotype (18, 19). Indeed, CXCL9 was elevated in tumors and serum of CD8α:MyD88 T cell–treated mice (Fig. 3C). Accordingly, the percentage of CD8+ T cells was higher in the tumors, but not the spleens, of mice treated with CD8α:MyD88 T cells (Fig. 3H). Differences were not detected in the number of CD45− hematopoietic cells, B cells, or NK cells in the tumors or spleens of mice (Fig. 3I).

CD8α:MyD88 T cells alter the TME and induce tumor regression

In accordance with increased IFNγ levels, MHC I expression on nonlymphoid (CD45−) cells was increased in mice treated with CD8α:MyD88 T cells (Fig. 4A and B). Treatment with CD8α:MyD88 T cells also increased the expression of MHC II and CD86 on dendritic cells (DC; Fig. 4A and B). The percentage of activated DCs coexpressing MHC II and CD86 was also elevated in the tumors, but not spleens, of CD8α:MyD88 T cell–treated mice (Fig. 4C).

The percentage of intratumoral CD45−CD11c−CD11b−F4/80+ macrophages was decreased in mice treated with CD8α:MyD88 T cells as compared with control-treated or untreated mice (Fig. 4D). Further analysis of the macrophage population revealed an increase in the percentage of macrophages with an M1-like phenotype (CD38−CD206+), typically associated with antitumor responses, following administration of CD8α:MyD88 T cells (Fig. 4E, left). The rise of M1-like macrophages in the tumor was associated with reduced M2-like protumor macrophages (CD38−CD206−) (Fig. 4E, right). In contrast, the percentage of M1-like and M2-like macrophages remained

![Figure 4](https://www.aacrjournals.org/CancerRes/77/24/December15/2017/7055.png)

**Figure 4.**

CD8α:MyD88 T cells alter the tumor microenvironment. **A**–**G.** Mice bearing an established B16-F1 tumor (~50 mm2) were exposed to a sublethal dose of irradiation (550 rads), followed by transfer of the CD8α:MyD88 or vector control pmel T cells (3 × 10^6) by intravenous injection one day later. Tissues were harvested and analyzed by flow cytometry one week after T-cell transfer. Transduction efficiency of CD8α:MyD88 T cells was 50%. Each data point represented one mouse. A, Median fluorescence intensity (MFI) of MHC I on CD45− cells and MHC II and CD86 on CD11c+ DCs. B and C, Frequency of CD11c+ DCs coexpressing activation markers CD86 and MHC class II in the tumor and spleen. D, Frequency of macrophages in the tumor and spleen as defined by CD11b+ F4/80+ cells. E, Frequency of M1 (CD8α:MyD88−CD38−CD206+) macrophages in the tumor and spleen. F, Frequency of myeloid-derived suppressor cells (MDSC) in the tumor and spleen as defined by coexpression of Gr1 and CD11b. G, LumineX analysis of CCL2, IL-4, and IL-5 in tumor homogenate. ns, nonsignificant.
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similar in the spleens of all groups. We did not detect any
differences in the number of CD45<sup>+</sup>G1<sup>+</sup>CD11b<sup>+</sup> myeloid-
derived suppressor cells (Fig. 4F).

We characterize the levels of cytokines and chemokines asso-
ciated with myeloid cell migration and differentiation. CCL2,
which is involved in monocyte recruitment, Th2 polarization, and
tumor progression (20, 21), were decreased nearly 3-fold in the
tumors of CD8<sup>a</sup>:MyD88 T cell–treated mice (Fig. 4G, left).
Likewise, IL5, which has been associated with Th2 responses and
protumor effects (22), was decreased following treatment with
CD8<sup>a</sup>:MyD88 T cells (Fig. 4G, middle). IL4 levels remained
similar in all groups (Fig. 4G, middle). These studies highlight
the propensity for CD8<sup>a</sup>:MyD88 to alter different facets of the
TME, including the cellular composition, cell surface protein
expression, and cytokine profile toward one that can support
CD8<sup>a</sup> T-cell expansion and antitumor activity.

Given these findings, we assessed the antitumor activity of
CD8<sup>a</sup>:MyD88–expressing T cells in tumor-bearing mice. Mouse
hharbing established B16-F1 tumors (~30 mm<sup>3</sup>) were treated
with control pmel or CD8<sup>a</sup>:MyD88–expressing pmel T cells.
Two injections of CD8<sup>a</sup>:MyD88 pmel T cells were sufficient to
delay tumor growth as compared with mice treated with control
T cells or untreated mice. Importantly, although control T cells
temporarily delayed or reversed tumor growth, CD8<sup>a</sup>:MyD88 T cells
treated and maintained tumor regression of large established
tumors (Fig. 5A and B). All mice treated with CD8<sup>a</sup>:
MyD88<sup>T</sup> cells exhibited tumor regression and 50% (4/8) of
mice cleared tumor. Notably, these antitumor responses were
accomplished in the absence of additional T-cell support such as
TAG vaccination, cytokines, or checkpoint blockade, emphasize-
ing the potent antitumor effects of activating MyD88 signal-
ing in T cells. A single injection of CD8<sup>a</sup>:MyD88 T cells
moderately delayed tumor growth as compared with mice
mixed with vector control T cells or untreated mice (Supple-
mentary Fig. S5A). Furthermore, we observed tumor control,
and tumor regression in some animals lasting up to one week
after cell transfer (Supplementary Fig. S5B). In sharp contrast,
tumor growth rates and mouse survival of the control T-cell-
treated group were similar with those of the untreated group
(Supplementary Fig. S5A–S5C), indicating that control tumor-
specific T cells were ineffective at the given dose, exemplifying
the potent antitumor effects of CD8<sup>a</sup>:MyD88 T cells. We also
analyzed whether checkpoint blockade using anti–PD-L1 anti-
odies or antibodies to stimulate 4-1BB signaling would potent-
able antitumor T-cell responses. We observed that although
blocking PD-L1 had a negligible effect, stimulating 4-1BB
increased the antitumor activity of control T cells, but it did
not improve CD8<sup>a</sup>:MyD88 T-cell responses (Supplementary
Fig. S6A and S6B). These data suggest that the costimulatory
signals activated by 4-1BB signaling might be redundant to
those activated by CD8<sup>a</sup>:MyD88.

**Discussion**

Through these studies, we present a novel strategy in which
expressing the fusion protein CD8<sup>a</sup>:MyD88 activated MyD88
signaling in cytotoxic CD8<sup>a</sup> T cells in a TLR-independent but
TCR-dependent fashion, resulting in enhanced responses to sub-
optimal TAgs levels and in an ability to reshape the TME toward
one favoring antitumor immune responses. Notably, the
enhancement of TCR responses by MyD88 was strictly dependent
on TCR stimulation with the tumor antigen. To the best of our
knowledge, this is the first strategy designed to boost TCR
responses against weakly immunogenic or lowly expressed anti-
gens without the need to alter the TCR sequence or identify
stronger MHC I epitopes.

The use of CD8<sup>a</sup>:MyD88 offers several unique strategies to
control tumor growth. First, the costimulatory effects of CD8<sup>a</sup>:
MyD88 are not limited to a single tumor antigen nor are they
MHC restricted, as CD8<sup>a</sup> interacts with the α<sub>1</sub> domain on all
human MHC I complexes. Therefore, immunotherapy using
CD8<sup>a</sup>:MyD88–expressing T cells could be utilized as a method
to enhance TCR responses against a variety of tumor antigens and
thus could be applied to patients with various malignancies
regardless of HLA haplotype or tumor antigen specificity. Fur-
thermore, ongoing studies in our laboratory suggest that CD4:
MyD88 extends CD4<sup>T</sup> cells a similar costimulatory effect and
could be used as a strategy to potentiate responses against tumor
antigens expressed on MHC II molecules.

One limitation to T-cell–based therapies is low TCR affinity
and/or avidity (3, 4). T cells with high affinity toward self-
antigens, such as tumor antigens, are eliminated in the thymus
during development, leaving behind low-affinity or low-avidity
tumor-reactive T cells that recognize subdominant epitopes and
weakly immunogenic antigens including neoantigens. There-
fore, a second benefit of expressing CD8<sup>a</sup>:MyD88 is that it
lowers the TCR activation threshold, providing the potential
to expand both high and low affinity TCR T cells while preserving
endogenous TCR signaling. The ability to enhance weak TCR
responses is especially relevant when redirecting T cells toward
neoantigens that can be expressed at low levels and/or have
lower affinity than nonmutated self-antigens. Amplifying T-cell

**Figure 5.**

CD8<sup>a</sup>:MyD88 T cells induce tumor regression of established melanoma
tumors. A and B, Mice bearing an established B16-F1 tumors were
exposed to a sublethal dose of irradiation (550 rad), followed by transfer
of 6 × 10<sup>5</sup> pmel T cells engineered to express CD8<sup>a</sup>:MyD88 or vector
control-GFP by intravenous injection one day and 8 days later. Average
tumor volume as well as tumor volumes for individual mice are shown.
Values represent mean ± SEM. *P < 0.05 starting on day 28 and
onward. Tumor growth was analyzed by a mixed model repeated
measures with AR(1) covariance structure, followed by Sidak-adjusted
comparisons at each time.
responses toward neoantigens is important for developing effective cancer therapies, as they offer the ability to target bona fide tumor-specific antigens. Furthermore, it is estimated that up to 20% of cancers are associated with oncogenic viruses. Therefore, another attractive strategy to specifically target cancer while eliminating off-target effects is to develop CD8α:MyD88 T cells reactive toward viral antigens expressed by tumor cells.

Another important and potentially clinically valuable use of CD8α:MyD88 is its ability to enhance the function of T cells engineered to express tumor-reactive TCRs, such as those targeting MART-1 or Ny-Eso (3, 4). For example, our data demonstrating engineered T cells reactive toward viral antigens expressed by tumor cells.

Figure 6. Proposed cellular processes impacted by CD8α:MyD88-expressing T cells in the tumor microenvironment. A, We observed an increased frequency of CD8α:MyD88 T cells in the tumor, indicating enhanced infiltration, expansion, and/or survival of CD8α:MyD88 T cells and increased MHC I expression or antigen expression to evade detection by T cells. A third advantage offered by CD8α:MyD88 is the ability to activate MyD88 signaling specifically in transduced T cells. Tumor-specific cells can be isolated and activated against the tumor prior to introduction of the CD8α:MyD88 to limit immune-related adverse events. This is an important quality as it indicates that the boosting effects of CD8α:MyD88 occur only in gene-modified T cells and do not nonspecifically costimulate nontransduced T cells, such as auto-reactive T cells. Importantly, the ability to activate MyD88 signaling in engineered T cells would eliminate the need to provide systemic TLR agonists, which could have the undesirable effect of promoting tumor growth by stimulating TLRs on cancer cells (10).

Fourth, CD8α:MyD88-expressing T cells exhibited a reduced ability to become exhausted in vitro and in vivo. This was exemplified by reduced frequencies of Lag3+Tim3+CD8+ T cells and an ability for CD8α:MyD88-expressing T cells to produce higher amounts of IFNγ (Fig. 3B and F) and/or granzyme B (Fig. 3B) than control T cells, which did not produce detectable IFNγ levels under those stimulation conditions. At present, we are unsure as to the molecular mechanisms underpinning this important observation, which is an important part of our ongoing investigations. In addition to demonstrating a reduced tendency to become exhausted, CD8α:MyD88-expressing T cells altered various facets of the TME toward one that favors antitumor immune responses.
These changes included increased T-cell infiltrates, an altered cytokine/chemokine milieu, increased expression of stimulatory molecules on DCs, and a skewing of the macrophage population from a protumor to an antitumor population. The proposed mechanisms by which CD8α:MyD88 T cells enhance antitumor responses are provided in Fig. 6.

In summary, the use of CD8α:MyD88 represents a universal approach to potentiate T-cell responses in cancer patients, regardless of the HLA type. Moreover, that the TCR-enhancing effects of CD8α:MyD88 occur strictly upon antigen recognition is innovative and highly suitable for self-regulating T-cell responses.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Kaczanowska, E. Davila
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Writing, review, and/or revision of the manuscript: S. Kaczanowska, Y. Zhang, E. Davila
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Younger

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