Cancer Cell
Article

Relapse or Eradication of Cancer Is Predicted by Peptide-Major Histocompatibility Complex Affinity

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http://dx.doi.org/10.1016/j.ccr.2013.03.018

SUMMARY

Cancers often relapse after adoptive therapy, even though specific T cells kill cells from the same cancer efficiently in vitro. We found that tumor eradication by T cells required high affinities of the targeted peptides for major histocompatibility complex (MHC) class I. Affinities of at least 10 nM were required for relapse-free regression. Only high-affinity peptide-MHC interactions led to efficient cross-presentation of antigen, thereby stimulating cognate T cells to secrete cytokines. These findings highlight the importance of targeting peptides with high affinity for MHC class I when designing T cell-based immunotherapy.

INTRODUCTION

Relapse of cancers is very common, even following combinatorial therapy of surgery, chemotherapy, radiation, and/or immunotherapy. For maximal efficacy, drugs depend on reaching the necessary concentration in the tumor microenvironment (Skipper, 1986). This critical concentration concept also applies to cellular effectors, such as neutrophils and T cells (Budhu et al., 2010; Li et al., 2002, 2004). While cellular effectors or drugs at optimal concentrations can eradicate all sensitive cancer cells, relapse may still occur because of the outgrowth of variants. Cancer cells show extremely high genetic instability, and cancers always contain variants that are resistant to destruction by a particular drug or T cell (Anders et al., 2011; Hanson et al., 2000), very similar to what is found for viruses (Hensley et al., 2009).

For complete eradication, it is important to eliminate every residual cancer cell, including heritable variants (Singh et al., 1992; Spiotto et al., 2004; Zhang et al., 2007). However, factors responsible for T cell elimination of variants have not been determined. In experiments designed to explore the reason for failed T cell treatment, we took a reductionist approach, ultimately directing our focus to the target peptides and, in particular, to their affinities for major histocompatibility complex (MHC) class I. We selected several peptides that, when targeted, caused tumor eradication and others that caused relapse. To reduce the influence of differences between cancers, we used two cancer cell lines that were both transduced to express the different peptides. To reduce differences due to expression levels, we used the same design of triple peptides fused to fluorescent proteins. Proteasomal cleavage of proteins may not generate (Chapiro et al., 2006; Popovic et al., 2011) or destroy immunogenic peptides (Schultz et al., 2002). To minimize differences in proteasomal cleavage of the fusion proteins, we designed peptide triplets separated by "Ala-Ala-Tyr" cleavage sites. We targeted antigens with no known oncogenic activity to reduce the possibility that the nature of a particular targeted antigen prevented the cancer from escaping. To exclude the influence of other T cells helping or regulating the relevant $CD8^+$ T cells, T cell receptor (TCR)-transgenic T cells with a single specificity were adoptively transferred into hosts, which were TCR-transgenic for an irrelevant target. Finally, a single adoptive T cell transfer regimen was used without providing any additional stimulation, such as vaccination or administration of cytokine.

RESULTS

Cancer Cells Expressing Different Peptides Are Killed by T Cells with Similar Efficacy In Vitro

EGFP was fused to minigenes encoding the peptides hen egg ovalbumin$_{257–264}$ (OVA$_{257}$), model peptide SIYRYYGL

Significance

Cancer relapse remains the greatest obstacle to virtually any cancer therapy. Our data show that high affinity of the targeted peptides for MHC is required for strong stimulation of T cells to secrete cytokines and cause relapse-free tumor eradication. Adoptive T cell transfer therapies should, therefore, target peptides that have high affinities for the presenting MHC class I.

Table 1. Abbreviations, Conditions, and Summary of Results for Key Experiments

Target Peptide on Cancer Cells				Hosts		T Cells		
Designation	Sequence	MHC	Affinity of Peptide for MHC (IC_{50} [nM])[a]	Designation	Relationship of Antigen to Recipient	Designation	Relationship of Antigen to Donor	Tumor Rejection
SIY	SIYRYYGL	K^b	1.1	OT-I	nonself	2C	nonself	5/5[b,c,d,e]
						none		0/6[b]
$OVA_{257–264}$	SIINFEKL		0.9	2C	nonself	OT-I	nonself	4/4[f]
						none		0/4[f]
$Tyr_{369–377}$	FMDGTMSQV	A2	4.2[g]	OTA	self	FH	self	6/7[h]
						none		0/5[h]
$hgp100_{25–33}$	KVPRNQDWL[i]	D^b	186	OT-I	nonself	pmel	nonself	1/8[c]
						none		0/2
EGP	EGPRNQDWL		454	OT-I	nonself	pmel	nonself	1/6[d]
						none		0/5
$mgp100_{25–33}$	EGSRNQDWL		22,975	OT-I	self	pmel	self	1/12[e]
						none		0/6

See Table S1 for details.
[a]IC_{50} values represent the geometric mean of five or more experiments.
[b]$p = 0.002$.
[c]$p < 0.005$.
[d]$p = 0.015$.
[e]$p < 0.001$.
[f]$p < 0.029$.
[g]A higher IC_{50} value of 65 nM was published for this peptide earlier (Colella et al., 2000). The differences in affinity measurements likely arose as a result of small differences in reagents, methodology, and procedures.
[h]$p = 0.015$.
[i]Only the underlined amino acids differ between the three gp100 peptide variants.

(SIY), mouse $Tyrosinase_{369–377}$ (Tyr_{369}), mouse or human $gp100_{25–33}$ ($mgp100_{25}$ and $hgp100_{25}$, respectively), and a heteroclitic $gp100_{25–33}$ (EGP); EGP differs from $mgp100_{25}$ only in the third amino acid (EGPRNQDWL versus EGSRNQDWL), while it shares the proline at position 3 with $hgp100_{25}$ (KVPRNQDWL) (Table 1). A Cerulean fusion gene was generated only for SIY (Figures 1A and 1C, top). The fibrosarcoma line MC57 of C57BL/6 origin was used to generate lines that expressed the fusion genes at high levels (Figure 1B). Furthermore, the chimeric human leukocyte antigen (HLA)-A2.1/H-$2D^b$ molecule HHD was cotransduced with the Tyr_{369}-EGFP fusion protein to generate MC57-TyrHHD (Figure 1C, bottom).

Assays in vitro demonstrated similar killing of the cancer lines by cognate peptide-activated T cells (Figure 1D). 2C T cells, whose TCR binds SIY, killed the MC57-SIY line, and pmel T cells killed MC57 cells expressing mouse $gp100_{25}$, human $gp100_{25}$ or EGP. Interestingly, Tyr_{369}-specific T cells derived from the *FH* TCR-transgenic, tyrosinase (*Tyr*)-deficient albino mouse (AFH) or *Tyr*-positive black mouse (FH) killed MC57-TyrHHD target cells similarly well. Together, the results imply there is sufficient direct presentation of all processed peptides and sufficient avidity of the T cells for efficient killing in vitro.

T Cells Targeting SIY, OVA_{257}, or Tyr_{369} Eradicate Large Tumors

SIY-expressing MC57-SIY cells were injected in TCR-transgenic mice of irrelevant specificity (OT-I). OVA_{257}-transfected cancer cells were injected in *2C* TCR-transgenic mice; MC57-TyrHHD cancer cells were grown in *OT-I* TCR- and *AAD*-transgenic mice, which did (OTA) or did not express tyrosinase (albino; AOTA). In all cases, cancer cells produced progressively growing tumors within 1 week (Figure 2A). At least 2 weeks after cancer cell injection, when tumors reached about 500 mm^3, mice were treated with T cells. As published by our laboratory, tumors expressing SIY and treated with 2C T cells were eradicated (Figure 2B, upper left; Table 1; Table S1 available online; Spiotto et al., 2004). Here, we also show that OVA_{257}-expressing tumors treated with OT-I T cells were rejected (Figure 2B, middle left) and FH T cells eradicated Tyr-positive tumors (Figure 2B, lower left). In this last experiment, FH T cells derived from a *Tyr*-positive donor were transferred into a *Tyr*-positive host and eradicated a Tyr-expressing tumor. Together, this and other experiments using *FH* TCR-transgenic T cells from *Tyr*-negative donors (AFH) and *Tyr*-negative (AOTA) or *Tyr*-positive (OTA) hosts showed that tumors could be rejected (1) whether the targeted peptide was self or nonself for the tumor-bearing host and (2) whether the targeted peptide was self or nonself for the donor T cells (Figure S2A). This may be unique to our model, since a different model showed that low ubiquitous expression of a transgene prevented the rejection of antigen-expressing tumors through the induction of tolerance (Buschow et al., 2010). Levels of antigen expression in the host and/or tumor, type of cells that express the self-antigen, and the source of T cells may likely influence the outcome. Taken together, targeting any of the three peptides, SIY, OVA_{257}, or Tyr_{369}, caused eradication of established large and solid tumors.

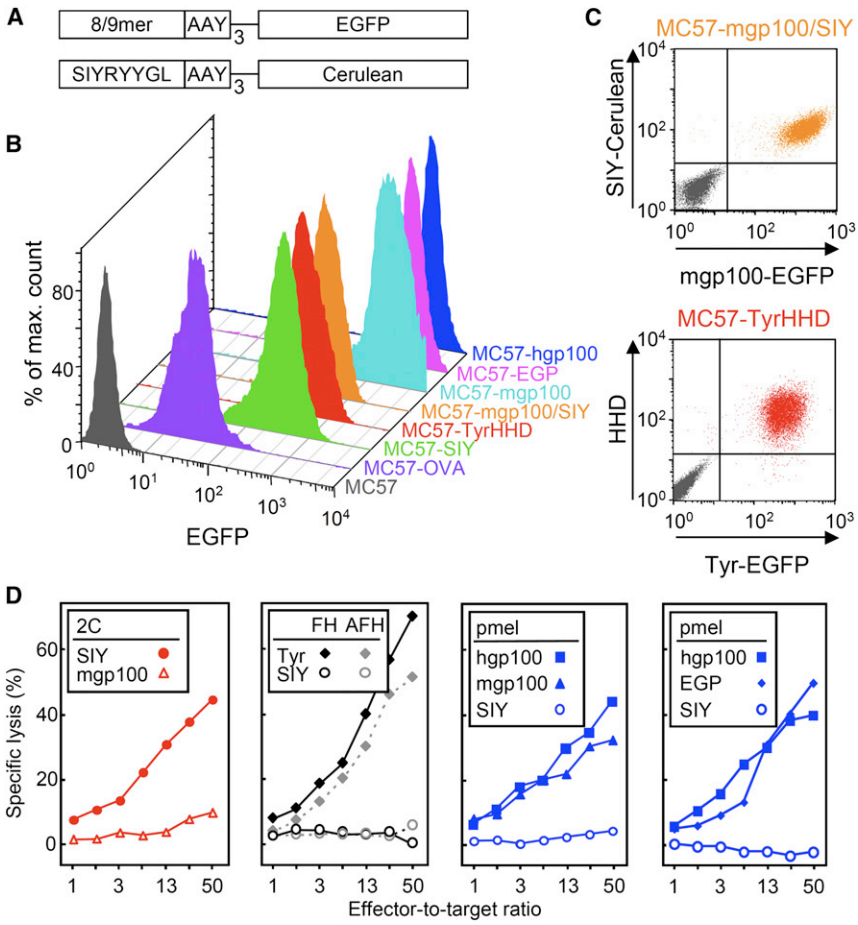

Figure 1. All Transduced Cancer Cell Lines that Express Antigens at High Levels Were Effectively Killed In Vitro

(A) Diagram of fusion proteins constructed to express antigen in MC57 cancer cells. Triple repeats of peptide and alanine-alanine-tyrosine (AAY) proteasomal cleavage sites were fused to fluorescent proteins: OVA_{257}, SIY, mouse $tyrosinase_{369\text{-}377}$ (Tyr_{369}), mouse or human $gp100_{25\text{-}33}$ ($mgp100_{25}$ and $hgp100_{25}$, respectively), and EGP; a Cerulean fusion gene was generated only for SIY.

(B) Flow cytometric analysis of peptide-EGFP fusion proteins expressed by the transduced MC57 fibrosarcoma lines.

(C) MC57-mgp100/SIY expressed $mgp100_{25}$ and SIY antigens as EGFP and Cerulean fusion proteins, respectively. The HLA-A2/D^b chimeric protein HHD was cotransduced with the Tyr_{369}-EGFP fusion protein to generate MC57-TyrHHD. Parental MC57 (gray) was analyzed for comparison.

(D) Cytolysis of MC57 target cells overexpressing SIY, $mgp100_{25}$, $hgp100_{25}$, EGP, or Tyr_{369} and HHD (Tyr) by 2C, pmel, AFH (*Tyr*-negative), or FH (*Tyr*-positive) T cells in a 4.5 hr ^{51}Cr release assay. Cancer cells expressing noncognate peptide were used as negative controls. These data are compiled of three experiments and are representative for seven independent experiments.

See Figure S1 for induction of vitiligo by FH and pmel T cells.

Large $mgp100_{25}$, $hgp100_{25}$, or EGP-Expressing Tumors Relapse after Initial Regression Caused by Transferred T Cells

In contrast, T cells targeting the self-peptide $mgp100_{25}$ and the nonself heteroclitic peptides $hgp100_{25}$ or EGP did not result in tumor eradication. MC57 lines overexpressing $mgp100_{25}$, $hgp100_{25}$, or EGP were injected into *OT-I* TCR-transgenic mice and produced progressively growing tumors within 1 week (Figure 2A). At least 2 weeks after cancer cell injection, when the tumors reached about 500 mm^3, the mice were treated with pmel T cells. The tumors regressed initially, but eventually almost all tumors relapsed (Figure 2B, right panels; Tables 1 and S1).

To exclude any nonantigenic differences in the cancer lines (caused by transduction and sorting), we used a cell line that expressed both SIY and $mgp100_{25}$ antigens (MC57-mgp100/SIY; Figure 1C). When mice bearing these tumors were treated with 2C or pmel T cells, the outcome was the same as when tumors from single antigen lines were treated (Figure 2B, upper panels, red curves). In conclusion, neither human nor mouse $gp100_{25}$ or EGP expressed by the cancer cells supported rejection by pmel T cells.

These findings were not limited to the methylcholanthrene-induced cancer line MC57 but were confirmed using the UV-induced cancer line 8101 (Figures S2B and S2D). The line was transduced to overexpress SIY, human, or mouse $gp100_{25}$. Again, we observed eradication of established tumors by adoptive T cell transfer only when SIY was targeted. Interestingly, in this model, targeting $hgp100_{25}$ was more effective than targeting $mgp100_{25}$; tumors expressing $hgp100_{25}$ regressed after pmel transfer, while tumors expressing $mgp100_{25}$ continued to grow uninhibitedly.

Treatment of Tumors Expressing Human $gp100_{25}$ but Not Murine $gp100_{25}$ or EGP Results in Outgrowth of Antigen-Loss Variants

We isolated cancer cells from tumors expressing $mgp100_{25}$, $hgp100_{25}$, or EGP that had relapsed following treatment with pmel T cells (Figure 2B) and analyzed these for antigen-loss variants (ALV). All MC57-hgp100 tumors had lost EGFP expression, which indicated loss of $hgp100_{25}$, as both were expressed as a single fusion protein (one representative tumor is shown in Figure 3). Importantly, the tumor isolated from a nontreated mouse retained EGFP expression. MC57-mgp100 and MC57-EGP tumors treated with pmel had also not lost EGFP expression. All lines expressed mgp100-EGFP or EGP-EGFP at levels similar to the isolate from a nontreated mouse (Figure 3). These data suggest that pmel T cells were capable of killing all $hgp100_{25}$-expressing MC57 cancer cells but were not capable of killing all $mgp100_{25}$- or EGP-expressing cancer cells in the respective tumors. These findings seem to be influenced also by the targeted cell, as relapsed tumors formed by 8101-hgp100 cells mostly retained expression of the antigen (only

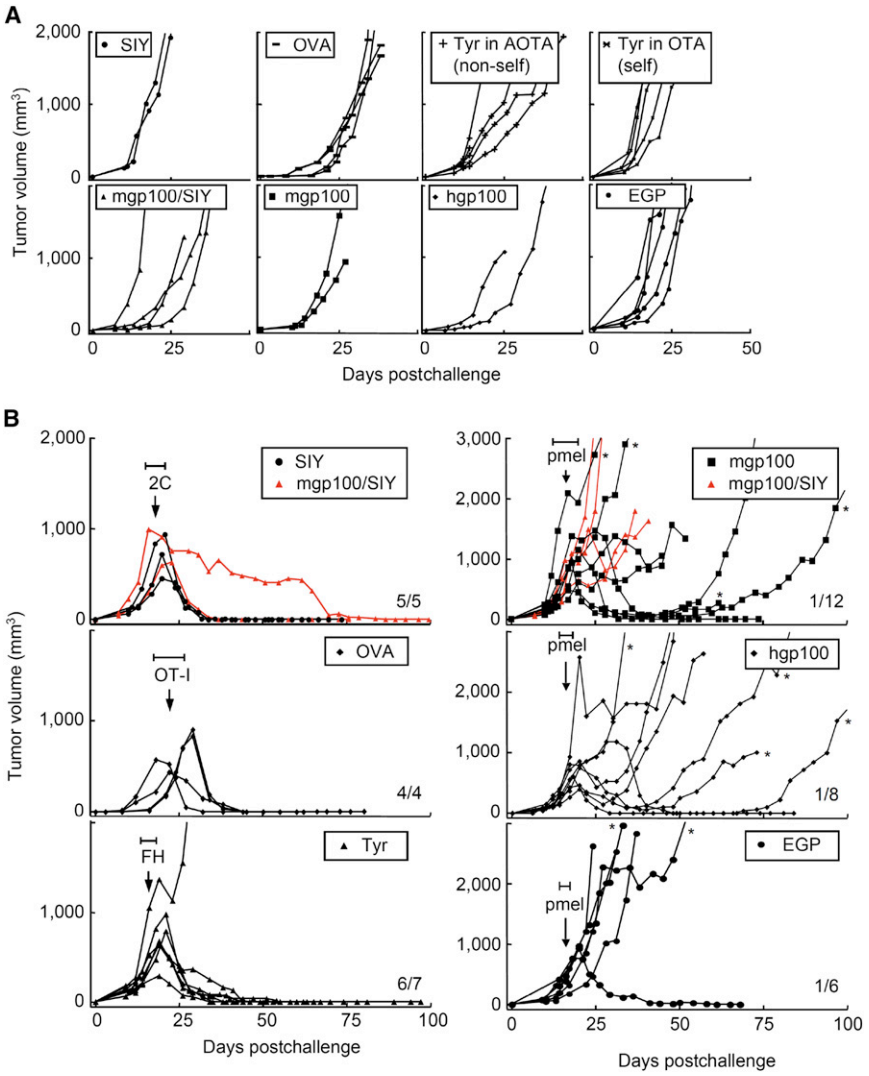

Figure 2. Targeting SIY, OVA_{257}, or Tyr_{369} Eradicated Large Tumors while Targeting $mgp100_{25}$, $hgp100_{25}$, or EGP Caused Initial Tumor Regression but Was Followed by Relapse

(A) Cancer cell lines formed progressively growing tumors within 1 week. TCR-transgenic mice with irrelevant specificity were challenged subcutaneously with 2×10^6 cancer cells. MC57-SIY, MC57-mgp100, MC57-mgp100/SIY, MC57-hgp100, and MC57-EGP were injected into OT-I mice; MC57-OVA grew in 2C mice and MC57-TyrHHD grew in AOTA (*Tyr*-deficient, nonself) and OTA (*Tyr*-positive, self). Graphs represent single mice in 11 experiments, listed as nontreated controls in Tables 1 and S1 and Figure S3C.

(B) At least 2 weeks after cancer cell injection, when tumors reached about 500 mm^3, each mouse was treated once with cognate T cells (treatment between days 13 and 26, depending on tumor size, as indicated by the horizontal bars [H]). Average size of tumors at day of treatment: 2C: 720 mm^3, ranging from 448 to 995 mm^3; OT-I: 608 mm^3, ranging from 440 to 715 mm^3; FH: 517 mm^3, ranging from 250 to 848 mm^3; pmel, targeting $mgp100_{25}$: 601 mm^3, ranging from 325 to 980 mm^3; targeting $hgp100_{25}$: 470 mm^3, ranging from 264 to 936 mm^3; and targeting EGP: 337 mm^3, ranging from 180 to 600 mm^3. MC57-TyrHHD was grown in OTA (self) and treated with FH T cells (self). The number of rejected tumors per number of tumors treated is indicated. Data are derived from 15 independent experiments, compiled in Tables 1 and S1. *Tumors were isolated and analyzed for antigen expression (see Figure 3).

See also Figure S2.

one of the isolated relapsed tumors was an ALV; data not shown).

While we did not observe significant differences when targeting either human or mouse $gp100_{25}$ in treatments of established MC57 tumors, we did see differences in protection against cancer cell inoculations. Pmel T cells prevented the outgrowth of MC57-hgp100 but not of MC57-mgp100 tumors (Figures S3A and S3C). MC57-mgp100 cells formed tumors in which a large fraction of cells still expressed the antigen (Figure S3B). Taken together, pmel T cells showed a stronger effect when targeting $hgp100_{25}$ compared to $mgp100_{25}$ and EGP.

Tumor Eradication Correlates with High Affinity of Targeted Peptides for MHC

In an effort to understand why targeting some peptides led to eradication while targeting others resulted in relapse, we first analyzed the activation status of the T cells transferred to treat the different tumors. Upon transfer, after peptide stimulation in vitro, all T cells showed the same $CD44^{hi}$ and $CD62L^{hi}$ phenotype of activated T cells (Figure 4) and demonstrated very similar killing capabilities in vitro (Figure 1). It is worth mentioning that before stimulation, splenocytes from self-reactive TCR-transgenic mice (pmel and AFH) showed an antigen-experienced phenotype ($CD44^{hi}$), while T cells from the nonself, reactive TCR-transgenic 2C mice showed a truly naive phenotype. However, this difference was overcome after peptide stimulation in vitro. Interestingly, when isolated from tumors 4 days after adoptive transfer, the T cells that led to eradication of tumors (2C and AFH) showed a more effector-like phenotype ($CD62L^{lo}$ and $CD44^{hi}$) compared to the more central memory-like phenotype ($CD62L^{hi}$ and $CD44^{hi}$) found for pmel T cells. Together, these data suggest that the differences in tumor rejection were not due to differing activation statuses of the T cells at the time of transfer.

As another variable that could influence the efficacy of tumor rejection, we analyzed the affinities of the peptides for the presenting MHC molecules. In a cell-free competition-binding assay, the concentration of inhibitor peptides needed to displace half of the probe peptide (half maximal peptide displacing concentration [IC_{50}] in nM) was determined. IC_{50} values are reasonable approximations of real K_D values (see Experimental Procedures). A wide range in binding affinities was measured (Table 1). There was a strong correlation between affinity of the

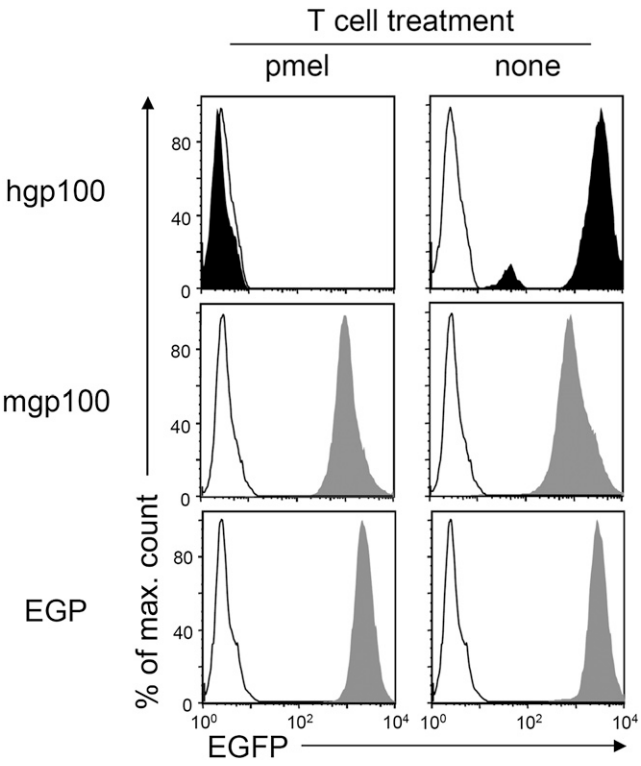

Figure 3. Outgrowth of Antigen-Loss Variants after pmel T Cell Treatment of Cancer Cells Expressing hgp100$_{25}$ but Not of Cancers Expressing mgp100$_{25}$ or EGP

Cancer cells of relapsed tumors expressing mgp100$_{25}$, EGP (both gray), or hgp100$_{25}$ (black) were isolated after pmel T cell treatment, adapted to culture, and analyzed for peptide-EGFP fusion gene expression (left panels). MC57 cells (white histogram) cultured in vitro and MC57-mgp100, MC57-EGP (both gray), or MC57-hgp100 (black) cells isolated from nontreated mice (right panels) were analyzed as controls. Isolated lines from mgp100- or hgp100-expressing tumors are representative for four lines each and the isolate from the EGP-expressing tumor is representative for two lines; all lines were isolated after relapse (respective tumors were marked with an asterisk in Figure 2B). The repeatability in independent experiments strongly suggests that loss of antigen expression from the hgp100$_{25}$ cancer cells was not an artifact caused by adaptation or postisolation culturing.

See also Figure S3.

peptide for MHC and tumor eradication. The three target peptides supporting tumor eradication, OVA$_{257}$, SIY, and Tyr$_{369}$ displayed strong binding to their cognate MHC (0.9, 1.1, and 4.2 nM, respectively). These high affinities stood in stark contrast to the affinities we measured for mgp100$_{25}$, EGP, and hgp100$_{25}$ (22,975, 454, and 186 nM, respectively). These three peptides bound the MHC poorly and, when targeted, resulted in relapse rather than tumor eradication.

Stromal Cells Isolated from Tumors Formed by Cancer Cells Expressing Peptides with High Affinity to MHC Stimulate Cognate T Cells Effectively

To analyze why high peptide-MHC affinities were required for tumor eradication, we performed assays to evaluate the level of cross-presentation in growing tumors. MC57 and 8101 lines expressing SIY, mgp100$_{25}$, or hgp100$_{25}$ were grown in *OT-I* TCR-transgenic or *Rag1*$^{-/-}$ mice, respectively; MC57-TyrHHD was grown in AOTA (nonself) mice. SIY was used as a representative peptide for the two highest binding peptides OVA$_{257}$ and SIY. Enriched populations of CD11b$^+$ stromal cells were obtained from at least 2-week-old untreated tumors and were compared in their ability to stimulate T cells in vitro to analyze the level of cross-presentation of the different peptides expressed by the tumors. For comparison, we used the transduced MC57 and 8101 cancer lines grown in vitro. As seen for the similar killing of MC57 cells presenting the different peptides in Figure 1D, direct presentation also led to comparable amounts of interferon (IFN)-γ and tumor necrosis factor (TNF)-α secretion by cognate T cells (Figure 5). However, in the 8101 model, more IFN-γ was found when targeting SIY versus hgp100$_{25}$ and mgp100$_{25}$ (Figure S2C). Even bigger differences occurred in both cancer models when T cells were stimulated with stromal cells. CD11b$^+$ stromal cells cross-presenting SIY and Tyr$_{369}$ stimulated cognate T cells even more strongly than directly presenting cancer cells (Figure 5). In contrast, both gp100$_{25}$ peptides were very poorly cross-presented. While stromal cells from hgp100$_{25}$-expressing tumors stimulated T cells to secrete low levels of both cytokines, stromal cells from mgp100$_{25}$ tumors did not stimulate T cells at all (Figures 5 and S2C). The heteroclitic peptide EGP behaved similarly to hgp100$_{25}$ when cross-presented; it stimulated pmel T cells to secrete low amounts of IFN-γ (Figure S4). Thus, the peptides with high affinities for MHC (SIY and Tyr$_{369}$) were well cross-presented, while peptides with low affinities (all three gp100$_{25}$ peptides) were so poorly cross-presented that the respective stromal cells could not efficiently stimulate cognate T cells.

Destruction of Tumor Stroma Is Stronger when Targeted Peptides Have High Affinity for MHC

We analyzed regressing MC57 tumors to help us understand how tumor eradication correlated to peptide-MHC affinities. Tumors were dissected on day 5 after adoptive T cell transfer, and we analyzed the viability of CD11b$^+$ stromal cells. Stroma from tumors expressing SIY or Tyr$_{369}$ showed a high percentage of dead cells (Figure 6), a 6- and 5.8-fold increase over background, respectively. In accordance with the relapse of tumors from gp100$_{25}$ peptide-expressing cells, death of tumor stroma was low, with only 1.9- and 2.2-fold increases over background for hgp100$_{25}$ and mgp100$_{25}$, respectively. In conclusion, tumors that were eradicated by cognate T cell therapy showed a high rate of stromal death, while relapsing tumors contained less dead CD11b$^+$ stromal cells.

DISCUSSION

Our results have a direct impact on the design of adoptive immunotherapy. First, and most importantly, the affinity of the targeted peptide for the presenting MHC was highly predictable of success or failure of T cell therapy, indicating that this is a key variable. Only high-affinity peptides that were efficiently presented by cancer cells and/or stroma induced cytokine secretion by T cells, stromal death, and relapse-free regression of tumors. Second, targeting self-antigens on tumors did not preclude eradication of large cancers, even though the treated mice developed vitiligo (data not shown). Such autoimmunity was

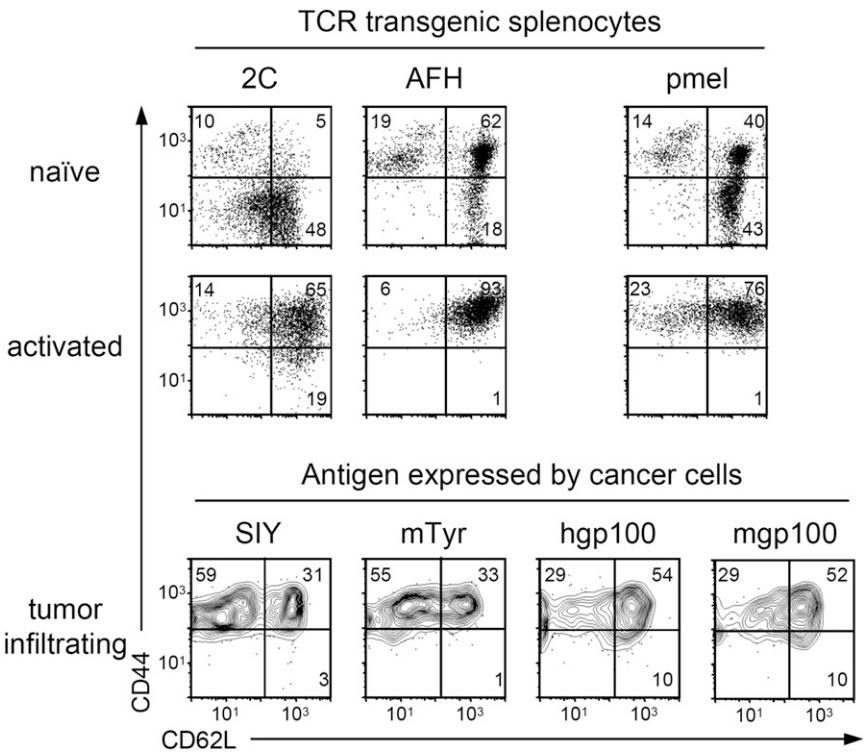

Figure 4. T Cells Transferred to Treat the Tumors Expressing the Different Peptides Showed the Same Phenotype of Activated T Cells

T cells of *2C*, *AFH*, and *pmel* TCR-transgenic mice were tested for their activation status in "naive," untreated mice (splenocytes) at day 3 after peptide activation in vitro and on day 4 after adoptive transfer (tumor infiltrating cells). Cells were analyzed by flow cytometry for expression of CD44 and CD62L and gated on $CD8^+$ T cells expressing the cognate Vβ-chain: Vβ8 for 2C, Vβ11 for AFH, and Vβ13 for pmel. Data are representative for two or three independent experiments for the data in vivo and in vitro, respectively.

also observed in patients treated with antiself T cells (Morgan et al., 2010; Palmer et al., 2008; Parkhurst et al., 2011; Yee et al., 2000). As might be expected, we found that the self-peptide with the higher affinity for the presenting MHC molecule was associated with stronger autoimmunity ($mTyr_{369}$ as opposed to $mgp100_{25}$). Vitiligo was also detected earlier in mice transgenic for the FH TCR compared to pmel (Figure S1; Gregg et al., 2010).

We have analyzed the potential of the different peptides to be cross-presented by tumor stroma. This is an effective readout to evaluate different affinities of peptides for MHC and more sensitive than direct presentation by cancer cells. All peptides were overexpressed by the cancer lines; therefore, no differences in direct presentation were detected in killing and cytokine secretion assays, but cross-presentation reflected the results obtained from the cell-free affinity measurements. Death of stroma correlated with the amount of cross-presentation and tumor relapse. Though stromal death seems to be required for tumor eradication, we do not know whether cross-presentation is essential. For example, direct T cell stimulation provided by cancer cells expressing peptides with high affinity for MHC can lead to strong cytokine production, Fas ligand upregulation, and bystander killing (Wang et al., 1996), which could destroy stroma. Stromal cross-presentation may also not be needed when an essential oncogene on the cancer is targeted (Anders et al., 2011; Listopad et al., 2013).

Targeted peptides that led to tumor eradication fell into a category of high-affinity MHC binders (IC_{50} < 10 nM), whereas affinities of peptides that led to relapse fell into a category of intermediate (IC_{50} between 50 and 500 nM) or low binders (IC_{50} > 500 nM). These data are consistent with the low nanomolar affinities needed to provide full protection against lethal *Vaccinia* virus infection (Moutaftsi et al., 2009). Low affinity peptides may allow perforin-mediated killing, which requires only two to three peptide/MHC complexes and brief T cell-target cell interactions (Purbhoo et al., 2004). However, the efficacy of adoptively transferred T cells to eradicate tumors does not depend on perforin (Garcia-Hernandez et al., 2010; Listopad et al., 2013). The high affinity of peptides for MHC is probably needed for tumor eradication, because this allows the formation of stable synapses between T cells and antigen-positive cancer cells and/or stromal cells cross-presenting the antigen. At least ten peptide/MHC complexes need to be engaged for the prolonged interactions required to stimulate T cells to secrete cytokines (Purbhoo et al., 2004), which are essential for tumor eradication (Garcia-Hernandez et al., 2010; Listopad et al., 2013; Zhang et al., 2008). It appears that targeting peptides with affinities below a certain threshold will result in a level of stimulation of effector T cells that is insufficient to eradicate the cancer, resulting in relapse of antigen-positive or negative cancer cells.

Several studies have tried to overcome relapse after adoptive T cell therapy. They show that the antitumor effects of adoptively transferred T cells can be enhanced by selecting for more effective T cell populations, multiple transfers of T cells, high-dose interleukin-2, vaccinations, and/or total body irradiation (Cheever et al., 1980; Cho et al., 2012; Dummer et al., 2002; Ho et al., 2003; Ly et al., 2010; Matsui et al., 2003; North, 1982; Overwijk et al., 2003). But even under these conditions, relapse was often observed when peptides with low affinities for MHC were targeted (Antony et al., 2005; Gattinoni et al., 2005, 2009; Overwijk et al., 2003).

TCR affinity can undoubtedly be an important factor (Gottschalk et al., 2012). However, in the study presented here, the affinity of the peptides for MHC seemed to determine if T cells could eradicate tumors or not. As reasons for this, we propose that the affinities (K_D) of the majority of natural TCRs, measured by surface plasmon resonance, are 1–100 μM (Davis et al., 1998; Williams et al., 1999). This is a very narrow range, considering the affinity range from under 1 to more than 20,000 nM measured for the different peptides binding MHC.

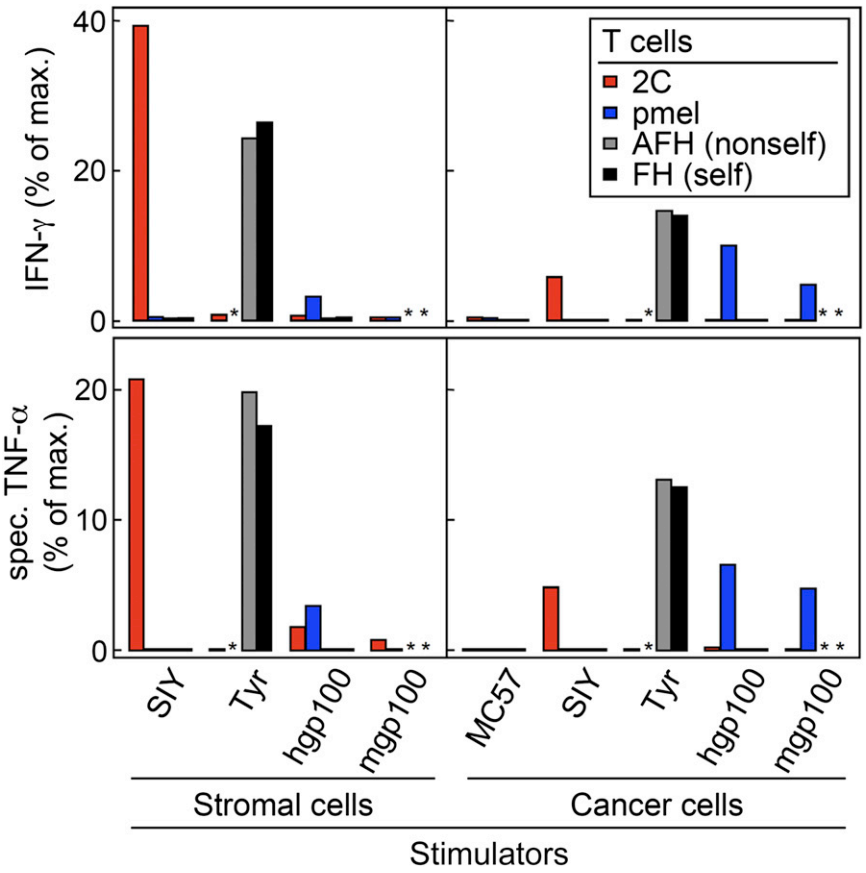

Figure 5. Only SIY and Tyr_{369} Are Cross-Presented, as Detected by Cytokine Secretion by T Cells Stimulated by Stromal Cells Isolated from Untreated Tumors

$CD11b^+$ stromal cells were isolated from established untreated tumors and were cocultured with peptide-activated T cells. Enriched stromal cells from tumors grown from MC57-SIY, MC57-hgp100, and MC57-mgp100 cells (all grown in OT-I mice) and MC57-TyrHHD (grown in AOTA mice [nonself]) were cocultured with *2C*, *pmel*, *AFH* (nonself), or *FH* (self) TCR-transgenic T cells. Stromal cells from the various tumors were compared to cultured cancer cells expressing the same antigen. Supernatants were harvested after 24 hr of coculture and amounts of IFN-γ and TNF-α measured by ELISA. Data are shown as percent of maximal cytokine secretion (anti-CD3 and anti-CD28 antibody stimulation, defined as 100%). For TNF-α, cytokine secretion by stromal cells without T cells was subtracted to obtain specific TNF-α secretion by T cells (443–975 pg/ml by 1×10^5 cells cultured in 200 μl for 24 hr, depending on experiment). Unstimulated T cells served as negative control (below 0.7% for all responders, not shown). Data shown are combined from two experiments and are representative for four independent experiments.
See also Figure S4.
The asterisk indicates experiments were not done.

In the same line of argument, a study by Bowerman and colleagues demonstrated in vitro that the magnitude of T cell activity against peptide/MHC was influenced more by peptide binding to MHC than by binding of TCR to peptide/MHC, especially for higher affinity TCRs (Bowerman et al., 2009). Finally, T cells expressing the 2C TCR, even when targeting a peptide/MHC complex with a 30-fold higher affinity, could not prevent relapse in the absence of cross-presentation. Using 2C T cells to treat MC57-SIY and MC57-L^d tumors, SIY-expressing tumors were rejected (Figure 2B; Spiotto et al., 2004; affinity of 2C TCR for SIY-K^b [K_D = 30 μM]), while MC57-L^d cancer cells grew out as ALV (Spiotto et al., 2004; affinity of 2C for QL9- and p2Ca-L^d [$K_D \approx 1$ μM]; Corr et al., 1994; Garcia et al., 1997; Holler and Kranz, 2003). In contrast to SIY, QL9- and p2Ca-L^d, recognized by 2C as alloantigens, cannot be cross-presented, as the entire peptide/MHC complex would need to be taken up by the stromal cells and then be re-presented on their surface.

While we did not study the influence of several TCRs with different affinities to one (same) peptide MHC complex, the influence of one TCR (pmel) on tumors expressing three peptides with different affinities for MHC was studied here. The affinities of the pmel TCR for the three peptide/MHC complexes studied, $mgp100_{25}$, EGP, and $hgp100_{25}$ binding D^b, are not known; however, alanine scans of murine and human $gp100_{25}$ suggest similar affinities, since the first three amino acids, which harbor the only differences between the three peptides, do not contribute to the binding of the peptide/MHC complex to the TCR (but are important for the binding of the peptides to MHC; Overwijk et al., 1998). Also, structural studies of these peptides (van Stipdonk et al., 2009) in complexes with D^b indicate that the two positions that influenced binding to D^b (p2 and p3) are both pointing down into the MHC pocket. In fact, the authors did not see significant differences in any of the exposed regions of the peptides, which would be in contact with the TCR. Nevertheless, only cancer cells expressing the peptide with the highest affinity for D^b ($hgp100_{25}$) were effectively killed in vivo, tumors expressing the other peptides relapsed being antigen positive. As none of the three peptides supported complete tumor eradication, we further analyzed their affinity for MHC in detail, which has given us insight into the importance of the stability of peptide-MHC interactions. In the original description of EGP (van Stipdonk et al., 2009), two different RMA-S cell-based assays were employed to determine the relative affinity of EGP for D^b compared to the murine gp100 peptide (murine gp100 peptide [25–33] EGSRNQDWL ["EGS"]) and human gp100 peptide "KVP"). These assays were: first, a binding assay that measured cell surface D^b levels as a function of peptide concentration; in this assay, EGP was almost 100-fold better than $hgp100_{25}$ (KVP) and 1,000-fold better than $mgp100_{25}$ (EGS). Second, a stabilization assay measured the cell surface lifetimes of the peptide/D^b complexes. In this stabilization assay, EGP and $hgp100_{25}$ (KVP) showed similar lifetimes, whereas $mgp100_{25}$ (EGS) had a considerable shorter lifetime (i.e., the complex was less stable). Consistent with their results, we observed a 50-fold increased affinity of EGP over $mgp100_{25}$, and this affinity

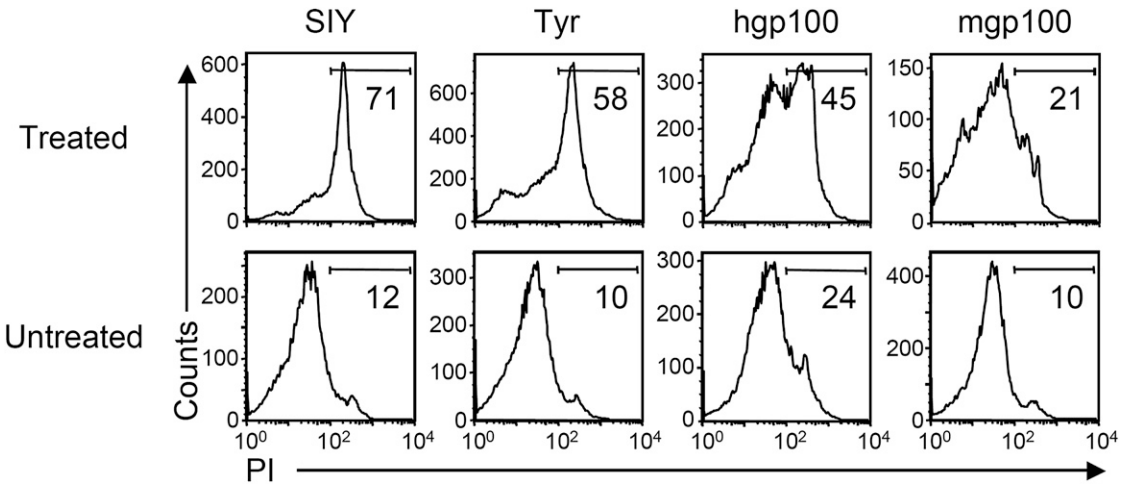

Figure 6. Death of Stromal Cells in T Cell-Treated SIY- and Tyr_{369}-Expressing Tumors

MC57-SIY, MC57-TyrHHD, MC57-hgp100, and MC57-mgp100 tumors were treated with 2C, AFH (nonself) or pmel T cells, respectively. Five days after adoptive transfer, single cell suspensions were generated from treated and untreated tumors and analyzed by flow cytometry. Histograms show $CD11b^+$ T cells stained with propidium iodide (PI) to identify cell death. Numbers indicate the percentage of dead cells among $CD11b^+$ cells. Data are representative for four independent experiments, with single mice per group.

of EGP was similar to that of $hgp100_{25}$ (454 nM and 186 nM, respectively). We used a cell-free competition-binding assay, which is influenced by on- and off-rates (giving an approximation of the dissociation constant [K_D]). Taken together, EGP demonstrated similar MHC stabilization compared to $hgp100_{25}$ (van Stipdonk et al., 2009). Indeed, like $hgp100_{25}$, the affinity of EGP for D^b was insufficient to allow for tumor eradication.

Heteroclitic peptides can induce strong T cell responses that include TCRs with high affinities (Gold et al., 2003; van Stipdonk et al., 2009). However, these T cells will not be able to eradicate tumors if the targeted tumor antigen has low affinity for its presenting MHC. An example is a recent clinical trial that showed no improvement of antimelanoma effects by addition of vaccinations with heteroclitic gp100 peptides to the immune stimulating anti-cytotoxic T-lymphocyte antigen (CTLA) 4 antibody (Hodi et al., 2010). The affinities of the corresponding natural peptides are $gp100_{209}$: 83–172 nM and $gp100_{280}$: 94–455 nM (Kawakami et al., 1995; Parkhurst et al., 1996; Tsai et al., 1997).

Since our data show that high affinity of peptide for MHC results in tumor eradication along with strong stimulation of T cells to secrete cytokines, future studies should concentrate on targeting peptides that have high affinities for presenting MHC class I. There are several algorithms that are constantly being improved to give a fairly reliable prediction of peptide affinities for MHC (e.g., Immune Epitope Database Analysis Resource). Nevertheless, the predicted affinities of the peptides show 2- to 20-fold differences when compared to the measured affinities (as analyzed here for SIY and OVA_{257}, respectively). While the affinities of peptides for MHC can be accurately measured in standardized cell-free assays, natural processing and presentation of these putative peptides needs also to be confirmed before selecting a peptide as a therapeutic target (Popovic et al., 2011). Together, it should be possible to identify optimal targets for T cell therapies when analysis of peptide-MHC affinity is included.

EXPERIMENTAL PROCEDURES

Cell Lines

Phoenix-ampho (Fujita et al., 1992) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Manassas, VA), 10% nonheat inactivated fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO) at 37°C in a 5% CO_2 humidified incubator. Cancer cells lines were cultured in DMEM, 5% FCS (Gemini Bio-Products, West Sacramento, CA) at 37°C in a 10% CO_2 dry incubator. 8101 originated in a UV-treated C57BL/6 and has been described (Dubey et al., 1997; Schreiber et al., 2001). P. Ohashi (University of Toronto, Toronto, Ontario, Canada), with permission of H. Hengartner (University Hospital Zurich, Zurich, Switzerland), provided the MC57G methylcholanthrene-induced, C57BL/6-derived fibrosarcoma (MC57). Its transfectant MC57-SIY-1 (MC57-SIY) has been described previously (Spiotto et al., 2002). The new cell lines 8101- and MC57-hgp100, 8101- and MC57-mgp100, 8101-SIY, MC57-EGP, and MC57-OVA were generated by transductions of 8101 or MC57 with the Moloney murine leukemia virus-derived retroviral vector MFG expressing peptide-EGFP fusion genes (see Supplemental Experimental Procedures for details on retroviral vectors and transductions). MC57-mgp100/SIY was derived from MC57 by subsequent transduction with MFG-$(SIY)_3$-Cerulean and MFG-mgp100-EGFP. MC57-TyrHHD was obtained by sequential transductions with MFG-Tyr-EGFP and MP71-HHD, encoding a fusion protein of a HLA-A2/D^d chimera and human β2m (Pascolo et al., 1997).

Mice

A list of the pairs of mice used as hosts of tumors and donors of T cells can be found in Table 1. OVA_{257}-K^b-specific TCR-transgenic OT-I mice were provided by M. Mescher (University of Minnesota, Twin Cities, MN), the SIY-K^b-specific TCR-transgenic 2C mice were provided by J. Chen (Massachusetts Institute of Technology, Cambridge, MA), and the human and murine $gp100_{25}$-D^b-specific pmel-1 (referred to as pmel) were provided by N. Restifo (National Cancer Institute, Bethesda, MD) (Overwijk et al., 2003). Other TCR-transgenic mice used in this study are the murine Tyr_{369}-A2-specific AFH mouse (Nichols et al., 2007), which is also *AAD*-transgenic (HLA-A2 and D^b chimera; Newberg et al., 1996) and albino (*Tyr*-deficient; Colella et al., 2000). It is important to note that the FH TCR used for targeting the self-peptide $mTyr_{369}$ has been derived in a nonself setting. The TCR was obtained from a *Tyr*-deficient albino mouse (Nichols et al., 2007), while the mouse from which pmel was obtained expressed $mgp100_{25}$ (Overwijk et al., 2003). This does not imply that the pmel TCR specific for $mgp100_{25}$ is of lower affinity but rather that TCRs with a certain

affinity for peptide/MHC can only be found naturally, if the target peptide is not expressed (FH) or of low affinity for MHC (pmel). The *Tyr*-positive, Tyr_{369}-A2-specific, *AAD*-transgenic FH mice were generated by crossing AFH to C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) and selecting black (Tyr^{+}) mice. The *OT-I*-, *Thy1.1*-, and *AAD*-transgenic strains AOTA (albino, Tyr^{-}) and OTA (Tyr^{+}) were obtained by crossing ATA (Nichols et al., 2007) to OT-I/Thy1.1 ($Thy1^{a}$; provided by T. Gajewski, The University of Chicago, Chicago, IL) and selecting for mice with white or black fur color, respectively. All colonies, including $Rag1^{-/-}$ (B6.129S7-$Rag1^{tm1Mom}$/J, The Jackson Laboratory), were maintained at the University of Chicago facilities. The Institutional Animal Care and Use Committee at the University of Chicago approved all animal experiments, and all experiments were performed to conform to the relevant regulatory standards.

Peptides

The peptides EGP (EGPRNQDWL), $hgp100_{25}$ (KVPRNQDWL), $mgp100_{25}$ (EGSRNQDWL), OVA_{257} (SIINFEKL), SIY (SIYRYYGL), and Tyr_{369} (FMDGTMSQV) were made by solid-phase peptide synthesis using standard 9-fluorenylmethoxycarbonyl chemistry (see Supplemental Experimental Procedures for details).

T Cell Cultures

NH_4Cl^{-}treated splenocytes were cultured at 4×10^6 cells/ml, 3 ml per well of a 6-well plate in Roswell Park Memorial Institute medium (RPMI), 10% FCS (Sigma-Aldrich), 2 mM glutamine, 50 μM β-mercaptoethanol, 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 1 mM sodium pyruvate, 1X nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin (all GIBCO/Invitrogen, Carlsbad, CA). T cells were activated with 1 μg/ml anti-CD3 (145-2C11) and anti-CD28 (37.51, both eBioscience, San Diego, CA) for killing assays in vitro or 1 μM cognate peptide: SIY for 2C, Tyr_{369} for AFH and FH, OVA_{257} for OT-I, and $hgp100_{25}$ for pmel. Activated T cells were used for adoptive transfer after 3 days and for assays in vitro after 4 days of culture.

Cytotoxicity Assay

Cell-mediated lysis of target cells by activated T cells was determined by standard 4.5 hr ^{51}Cr-release assay. Briefly, target cells were labeled for 1 hr with 100 μCi ^{51}Cr (Perkin Elmer, Waltham, MA) and incubated with T cells using E:T ratios from 50:1 to 1.3:1 using 5×10^3 target cells. The ^{51}Cr-released was measured using a gamma counter (Titertek, Huntsville, AL). The percentage of specific lysis was calculated as: % specific lysis = ([experimental release − spontaneous release]/[maximum release − spontaneous release]) × 100.

Tumor Challenge and Treatment

Cultured cancer cells were trypsinized and washed with PBS. Cancer cells in suspension (MC57: 2×10^6/200 μl, 8101: 5–10 $\times 10^6$/200 μl) were injected subcutaneously onto the shaved back of mice. Tumor volumes were measured along three orthogonal axes (*a*, *b*, and *c*) every 3 to 4 days and tumor volume calculated as *abc*/2. MC57 tumors were treated after at least 14 days, when tumors reached approximately 500 mm^3; 8101 tumors were treated after at least 5 weeks, when tumors reached approximately 300 mm^3. Mice were treated with 3 day activated T cells, one spleen per recipient. We injected $5.5 \pm 1.3 \times 10^7$ activated 2C T cells, $5.3 \pm 2.4 \times 10^7$ activated FH T cells, and $6.9 \pm 2.2 \times 10^7$ activated pmel T cells (numbers were derived from six independent experiments). 8101 tumors were treated with cells from half a spleen only and with naive T cells in some of the experiments (see Table S2). T cell suspensions were injected into the recipient via the retro orbital plexus in two doses of 150 μl. For tumor protection, T cells were injected on the day of tumor challenge or 3 days later, as indicated.

Isolation of Stromal and Cancer Cells from Tumors

Two-week-old, untreated tumors were used for functional analysis; tumors of mice treated with T cells 4 or 5 days prior were used for flow cytometric analysis of T cells and stromal death, respectively. Tumors were surgically excised and single cell suspensions generated by enzymatic digestion (see Supplemental Experimental Procedures). For stromal cross-presentation, $CD11b^{+}$ cells were enriched using magnetic beads (Dynabeads FlowComp Flexi [Invitrogen Dynal, Oslo, Norway] and anti-CD11b antibody [M1/70, BD Bioscience, Franklin Lakes, NJ]).

To analyze antigen loss, relapsed tumors were surgically excised under sterile conditions and placed in DMEM on ice. Tumors were minced to 1 to 2 mm pieces and seeded in DMEM, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 50 μg/ml nystatin. Cells and fragments in the flask were not moved for the initial three days and then cultured normally.

Cytokine Release Assay

T cells activated in vitro for 4 days were incubated with cancer cells cultured in vitro or tumor stromal cells obtained ex vivo. For 24 hr, 1×10^5 responders were cultured with 1×10^5 stimulators per well of a 96-well U-bottom plate. Wells coated with 1 μg/ml of anti-CD3 (145-2C11) and anti-CD28 (37.51, eBioscience) served as positive controls and maximal stimulation. All supernatants were removed and tested for IFN-γ and TNF-α using ELISA Kits ("Femto-HS" High Sensitivity, eBioscience), according to the manufacturer's protocol.

Flow Cytometry

Cells were stained using directly labeled antibodies (see Supplemental Experimental Procedures). Flow cytometry data were acquired on FACSCalibur or FACSCanto machines (BD), and data were analyzed using FlowJo (Tree Star, Ashland, OR) software. Cell sorting was performed using FACSAria (BD) or MoFlo-HTS (Beckman Coulter, Brea, CA) at the Flow Cytometry Facility of The University of Chicago.

MHC Peptide-Binding Assays

MHC purification and quantitative assays to measure the binding affinity of peptides to purified H2-K^b, H2-D^b, and HLA-A*0201 molecules were performed as previously described (Assarsson et al., 2007; Sidney et al., 2001; see Supplemental Experimental Procedures for details). Under the conditions used, where [label] < [MHC] and $IC_{50} \geq$ (MHC), the measured IC_{50} values are reasonable approximations of the true K_D values.

Statistical Analysis

Results of treatment of small groups of mice were analyzed using the two-tailed probability calculated by the Fisher's exact probability test ($p \leq 0.05$ is considered significant, $p \leq 0.01$ highly significant).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.03.018.

ACKNOWLEDGMENTS

We thank Dr. Theodore Karrison (The University of Chicago) for help with statistical analysis, Zhang Yi for generating the cancer lines MC57-hgp100 and MC57-mgp100, Andrea Schietinger for the pMFG-$(SIY)_3$-Cerulean vector, Andrew Hawk for peptide synthesis, and the University of Chicago Flow Cytometry Core Facility. We also thank Ainhoa Arina and Christian Idel for critical review of the manuscript. This work was supported by a Research Fellowship of the DFG EN 703/3-1 (to B.E.); NIH grants P01-CA97296 (to D.M.K. and H.S.), R01-CA22677, and R01-CA37156 (to H.S.); a grant from the Melanoma Research Alliance (to D.M.K.), and the Cancer Center at the University of Chicago.

Received: March 28, 2012
Revised: February 21, 2013
Accepted: March 19, 2013
Published: April 15, 2013

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