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The Effect of Adjuvanting Cancer Vaccines with Herpes Simplex Virus Glycoprotein D on Melanoma-Driven CD8+ T Cell Exhaustion

Ying Zhang* and Hildegund C. J. Ertl*†

Two vaccines expressing CD4+ and CD8+ T cell epitopes of melanoma-associated Ags (MAAs) by a chimpanzee-derived replication-defective AdC68 vector were compared in a mouse model of melanoma. In one vaccine, termed AdC68-gDMelapoly, the epitopes were expressed as a fusion protein within HSV-1 glycoprotein D (gD), which blocks immunoinhibitory signaling through the herpes virus entry mediator pathway. The other vaccine, termed AdC68-Melapoly, expressed only the MAA epitopes. AdC68-gDMelapoly induced more potent MAA-specific CD8+ T cell responses especially to the subdominant MAA epitopes. Upon prophylactic vaccination, mice that developed CD8+ T cell responses to the two vaccines that were comparable in magnitude showed equal protection against tumor challenge. When mice were first challenged with tumor cells and then vaccinated, differences in the magnitude of MAA-specific T cell responses, especially to subdominant MAA epitopes, were linked to reduced expression of 2B4, LAG-3, and CTLA-4 (10, 11), and programmed death-1 on tumor-infiltrating MAA-specific CD8+ T cells elicited by the gD-adjuvanted vaccine suggesting that CD8+ T cells induced in presence of gD are less susceptible to tumor-driven exhaustion. The Journal of Immunology, 2014, 193: 1836–1846.

Evven cancer vaccines that are highly immunogenic in animal models commonly fail to provide benefits to patients with advanced cancers (1, 2). This has partially been linked to the highly immunosuppressive tumor microenvironment, which expresses immunoinhibitory ligands (3), recruits suppressive cell subsets such as regulatory T cells (4) and myeloid suppressor cells (5) and provides a metabolically stressed milieu (6). Biologicals that block immunoinhibitory pathways such as Abs to programmed death-1 (PD-1) (7), 2B4, LAG-3, and programmed death-1 on tumor-infiltrating MAA-specific CD8+ T cells elicited by the gD-adjuvanted vaccine, suggesting that CD8+ T cells induced in presence of gD are less susceptible to tumor-driven exhaustion.

The N-terminus of HSV-1 gD binds to a site on HVEM that is close to the BTLA/C1D160 binding site and thereby blocks immunoinhibitory but not costimulatory HVEM signaling (15).

As we have shown previously, vaccines that express Ags fused into the C terminus of gD elicit enhanced T cell responses, which is linked to blockade of the immunoinhibitory HVEM pathways (16). Adjuvanting vaccine Ags with gD is especially effective to augment CD8+ T cell responses in aging mice (17) and in mice with advanced cancers (18). Our previous cancer studies were based on human papilloma virus type 16–associated tumors, which express viral Ags that are foreign to the immune system. The current study was conducted to assess whether expressing “self” Ags from nonviral tumors within gD would enhance the immunogenicity and efficacy of a cancer vaccine. Experiments were conducted in a transplantable melanoma model, based on B16F10 cells that were stably transfected to express BrafV600E (B16BrafV600E). The vaccine Ag, termed Melapoly, was designed to express CD4+ and CD8+ T cell epitopes of melanoma-associated Ags (MAAs) including tyrosinase-related protein (Trp)-1, Trp-2, gp100 and mutated BrafV600E linked to the universal Th cell epitope PADRE and an endoplasmic reticulum targeting signal sequence. To test for the gD adjuvant effect, the Melapoly encoding sequence was fused into the C terminal domain of HSV-1 gD (gDMelapoly). The Melapoly and the gDMelapoly fusion proteins were expressed by a simian E1-deleted adenovirus vector of serotype 68 (AdC68).

As expected, the AdC68-gDMelapoly vector induced more potent MAA-specific CD8+ T cell responses, especially to subdominant epitopes, compared with the AdC68-Melapoly vector and provided superior protection if given before tumor challenge. In the same token, in a therapeutic vaccination model, the AdC68-gDMelapoly vector was superior in delaying tumor progression compared with the AdC68-Melapoly vector. To assess whether the improved efficacy of the gD-adjuvanted vaccine solely reflected differences in the magnitude of MAA-specific T cell responses, we vaccinated mice with different doses of the AdC68 vectors and selected subgroups with comparable frequencies of MAA-specific
CD8+ T cells. In a prechallenge vaccination model, vaccine efficacy was shown to depend on frequencies of MAA-specific CD8+ T cells. In contrast, in a postchallenge vaccination model, AdC68-gDMelapoly–vaccinated mice that had MAA-specific T cell frequencies comparable to those of AdC68-Melapoly–vaccinated mice survived significantly longer. This was not caused by differences in production of mediators by AdC68-gDMelapoly–induced T cells but rather by their increased resistance against differentiation toward exhaustion.

Materials and Methods

Mice

Female C57BL/6 mice (6–8 wk) were purchased from the National Cancer Institute and housed at the Wistar Institute Animal Facility. All procedures were performed under the guideline of protocols approved by the Institutional Animal Care and Use Committee of the Wistar Institute.

Cell lines

The B16BrafV600E cell line was derived from B16.F10 cells transduced with lentiviral vector pLU-EF1a-mCherry expressing mouse BrafV600E (provided by Dr. M Herlyn laboratory, Wistar Institute). The mutant cell line showed the same in vivo growth characteristics as unmodified B16.F10 cells. El-transfected HEK 293 cells were used to propagate Ad vectors. Cells were cultured with DMEM supplemented with 10% FBS.

Conconstruction of recombinant Ad vectors

We designed the Melapoly transgene to express a number of MAA-specific CD4+ T cell and CD8+ T cell epitopes. An endoplasmic reticulum targeting signal sequence was included at the N-terminal end of the sequence (19). Three human (h)Trp-2–specific CD4+ T cell epitopes (20) as well as the universal Th cell epitope PADRE (21) were incorporated into the Melapoly construct. CD8+ T cell epitopes were derived from human and mouse Trp-2, human gp100, mouse Trp-1, and BrafV600E. Epitopes from Trp-1 and BrafV600E were omitted to enhance their binding to MHC class I molecules (22). A Flag-tag was added at the C-terminal end. We designed the spacer sequences according to several analysis programs, including PAPROC I (http://www.paproc.de). Netcrop 3.1 (http://www.cbs.dtu.dk/services/NetChop/) and IEBD Analysis Resource (http://tools.immunopeptide.org/main/). The spacers were inserted between each CD4+ and CD8+ T cell epitope to reduce interference of epitope processing (23, 24). The Melapoly gene was codon optimized and inserted into the pUC57 vector (Genescipt, Piscataway, NJ). The Melapoly transgene was sequenced and cloned into the pShuttle vector by Eagl digestion. To construct the AdC68-gDMelapoly vector, the Melapoly transgene was fused into the N terminus of HSV-1 gD. The pShuttle gDMelapoly vector was generated by PCR using pShuttle Melapoly as the template, gDMelapolyFw 5′-GGGTTAGCTAAGTTTGTGGCCGCTTG-3′ and gDMelapolyRv 5′-GGGGTCCGGTGTG-3′. The PCR product was cleaved by AgeI and AvrII and cloned into the pShuttle gD-Flag vector. The inserts containing regulatory sequences of the pShuttle vector were subcloned into the El-deleted AdC68 viral molecular clone using I-CeuI and PstI-CceI sites. Recombinant AdC68 vectors were rescued, propagated on HEK 293 cells, purified by CsCl-gradient centrifugation and titrated as described previously (25).

Real-time PCR

We infected HEK 293 cells with 10^10 and 10^11 vp doses of AdC68-Melapoly and AdC68-gDMelapoly vectors. Cells were harvested 24 h later and total RNA was isolated from each sample using RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). cDNA samples were obtained by reverse transcription using 2 μg RNA/sample with high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Twenty nanograms of cDNA per sample was used for real-time PCR using Fast SYBR green Mastermix (Applied Biosystems). To obtain standards for real-time PCR, we performed regular PCR for Melapoly and GAPDH (housekeeping gene as internal control) using the cDNA samples. We ran the PCR products on 1% agarose gel, purified the PCR products of the expected sizes (MiniElute gel extraction kit; Qiagen) and measured the DNA concentration using spectrometry. The PCR products were template for the purified PCR products and GAPDH DNAs from 5 to 0.156 ng for real-time PCR standards. For real-time PCR, cDNA samples were replicated for both Melapoly and GAPDH using the 7500 Fast Real-Time PCR machine (Applied Bio-

systems). The primer sequences are as follows: regular PCR MelapolyFw 5′-ACAGGAAATCTCCGGCGTGC-3′, MelapolyRv 5′-TGGCATATCGCCAGGGTTGCTG-3′, real-time PCR MelapolyFw 5′-TGATCCGTCGTCGACCACGTCTG-3′, regular PCR GAPDHFw 5′-GGGTTCCGGTGTGAAAGGATTAG-3′, GAPDHRv 5′-AATGCCAAAATGTGCTGATGATGACCC-3′ and real-time PCR GAPDHFw 5′-TGCCCCCATGGTTGAGTGGTTG-3′. The final concentrations of Melapoly cDNA from both vector-infected samples were analyzed and compared after normalization to GAPDH.

Immunization and challenge of mice

Groups of C57BL/6 mice were vaccinated i.m. with the AdC68 vectors diluted in PBS into the triceps anterior muscle of each hind limb, with doses ranging from 3 × 10^9 to 10^11 vp per mouse. In tumor challenge experiments, B16BrafV600E cells suspended in serum-free medium were inoculated s.c. into the right flank of mice. For pre- and postchallenge tumor challenge experiments, 5 × 10^6 cells were injected and mice were vaccinated 14 d before or 3 d after tumor cell challenge. We monitored tumor growth by measuring the perpendicular diameters of tumors at least three times a week. Mice were euthanized once tumors exceeded a surface area of 100 mm^2. Mice were monitored for a period of 60 d posttumor challenge.

Isolation of lymphocytes

PBMCs and splenocytes were harvested as described before (18). To tumor-infiltrating lymphocytes (TILs), tumors were cut into small fragments and treated with 2 mg/ml collagenase P and 1 mg/ml DNase I (Life Technologies, Carlsbad, CA) dispersed in Hank’s balanced salt solution (GIBCO) containing 10% FBS (Thermo Fisher Scientific, Pittsburgh, PA) with agitation for 1 h. Tumor fragments were homogenized and filtered, and lymphocytes were purified by Percoll-gradient centrifugation.

Tetramer and intracellular cytokine staining

To detect tetramer-specific CD8+ T cells, we stained cells with a PE-labeled Trp-1–specific MHC class I (H-2D^d) tetramer carrying the TATFDNLGYM peptide and an Alexa647–labeled tyrosinase-related protein 1–specific MHC class I (H-2K^b) tetramer carrying the TAPA-DNLGYM peptide and an Alexa647–labeled tyrosinase-related protein 2 MHC class I (H-2K^d) tetramer carrying the SVYDFFVWL peptide (26). The concentration of each virus batch was determined by measuring virus particles (vp) by spectrophotometry at 260 nm. The volume of each virus batch was determined by measuring virus particles (vp) by spectrophotometry at 260 nm.

Statistical analysis

We compared the difference of immune responses between two groups using one-way ANOVA followed by Holm–Sidak’s multiple comparisons. Immune responses affected by two factors (vaccine and
tissue type or vaccine and cell type) were analyzed by two-way ANOVA with Holm–Sidak’s multiple comparisons. We performed Holm–Bonferroni correction for multiple comparisons within the same dataset. Survival rates between different treatment groups were compared using Gehan–Breslow–Wilcoxon test. Significance was set at \( p \leq 0.05 \). All statistical analyses were performed using Graphpad Prism6. Throughout the paper, \( p \) values adjusted for type 1 errors are shown.

**Results**

The gD adjuvant enhances CD8+ T cell responses to dominant and subdominant epitopes

Levels of transgene expression can affect the immunogenicity of viral vectors. We therefore tested the levels of transgene product expression in transfected cells and showed by real-time PCR that both vectors expressed comparable levels of transgene-derived mRNA (data not shown).

We compared the magnitude of MAA-specific CD8+ T cell responses induced by the vaccines in young female C57BL/6 mice. Each group (\( n = 5 \)) of mice received either the AdC68-Melapoly or the AdC68-gDMelapoly vector given i.m. at doses of \( 10^{10} \) or \( 10^{11} \) vp. Two and four weeks after vaccination, we measured CD8+ T cell responses to two MAA epitopes (i.e., Trp-1455 and Trp-2180) by staining T cells with the corresponding MHC class I tetramers (Supplemental Fig. 1). Both vaccines induced stronger CD8+ T cell responses to Trp-1455 than Trp-2180 (mean frequencies of positive CD44+CD8+ cells for Trp-1455 versus Trp-2180: \( 10^{10} \) vp: week 2: Melapoly: 5.1 versus 0.56%, gDMelapoly: 5.8 versus 0.58%; week 4: Melapoly: 2.5 versus 0.25%, gDMelapoly: 5.3 versus 0.42%; \( 10^{11} \) vp: week 2: Melapoly: 8.7 versus 0.86%, gDMelapoly: 14.7 versus 0.51%; week 4: Melapoly: 7.8 versus 0.18%, gDMelapoly: 8.5 versus 0.56%; naive CD44+ T cells: 0.043 versus 0.056 and 0.043 versus 0.078% for weeks 2 and 4, respectively, Fig. 1A, 1B). All groups mounted significant responses to the Trp-1455 and Trp-2180 tetramer compared with the internal CD44+CD8+ T cell controls (adjusted \( p \) values are listed in legend to Fig. 1) or to naive mice (data not shown). At the \( 10^{11} \) vp dose at week 2 and the \( 10^{10} \) vp dose at week 4 Trp-1455-specific CD8+ T cell responses were significantly higher in AdC68-gDMelapoly–vaccinated animals than in AdC68-Melapoly–vaccinated mice. Responses to Trp-2180 were significantly higher in mice vaccinated with \( 10^{11} \) vp AdC68-gDMelapoly at both weeks 2 and 4. At the \( 10^{10} \) vp dose at week 4 Trp-2180 responses were significantly higher in Melapoly-vaccinated mice.

To further assess differences, we immunized mice (\( n = 7–8 \)) with \( 10^{11} \) vp of the AdC68-Melapoly vector or \( 10^{10} \) vp of the AdC68-gDMelapoly vector. We collected blood at different time points after vaccination and measured the immune responses to individual MAA epitopes by ICS for production of IFN-\( \gamma \), TNF-\( \alpha \), and IL-2 (Fig. 2, Supplemental Fig. 2). For these experiments, blood had to be pooled to allow for testing the PBMC samples stimulated with the eight different peptides carrying CD8+ T cell epitopes expressed by the vaccines. Both vectors induced significantly higher tumor-Ag–specific CD8+ T cell responses to both dominant and subdominant epitopes compared with naive controls (CD44+ CD8+ T cells). Despite the lower dose, there was a clear trend of the AdC68-gDMelapoly vaccine to induce higher responses mainly to the subdominant epitopes (Trp-1455 epitope: mean frequencies averaging all time points: 1.9 and 2.9% for Melapoly and gDMelapoly, respectively, Fig. 2A; subdominant epitopes: mean frequencies of the sum of responses averaging all time points 1.3 and 3.0% for Melapoly and gDMelapoly, respectively, Fig. 2B). The preferential increase of responses to subdominant epitopes by gD was confirmed by comparing responses to the dominant Trp-1455 epitope to those against the subdominant Trp-2180 epitope by tetramer staining at 4 and 26 wk after vaccination with \( 10^{11} \) vp AdC68-Melapoly or \( 10^{10} \) vp AdC68-gDMelapoly (Fig. 2C, 2D). At both early and late time points, the responses to Trp-1455 epitope were comparable, whereas the responses to Trp-2180 epitope were significantly higher in mice immunized with AdC68-gDMelapoly (week 4, mean tetramer frequencies for Trp-1455: Melapoly versus gDMelapoly: 7.8 versus 5.3%; Trp-2180: 0.18 versus 0.42%, \( p = 0.0053 \); week 26, Trp-1455: Melapoly versus gDMelapoly: 4 versus 5.9%, Trp-2180: 0.35 versus 0.66%, \( p = 0.033 \)).

We selected three time points (weeks 6, 12, and 24) to compare the overall response pattern (Fig. 2E–J). Responses to the subdominant epitopes developed with a delay as compared with Trp-1455 responses. At all three time points the gD-adjuvanted vector induced a more prominent CD8+ T cell response to the subdominant epitopes compared with the AdC68-Melapoly vector (sum of frequencies to Melapoly and gD-Melapoly, respectively: week 6: 3.8 and 4.4%; week 12 and 6.7%; and week 24, 4.5 and 6.6%).

![FIGURE 1](Image)  Induction of MAA-specific CD8+ T cells by different vector doses. Groups (\( n = 5/\text{group} \)) of C57BL/6 mice were vaccinated i.m. with \( 10^{10} \) or \( 10^{11} \) vp of either AdC68-Melapoly (○) or AdC68-gDMelapoly (■) vector. PBMCs were collected 2 and 4 wk later, and (A) Trp-1455- and (B) Trp-2180-specific CD8+ T cell responses were compared in individual animals, the lines indicate means ± SEs. *, Significant differences between groups connected by lines (\( p = 0.01–0.05 \), ** \( p = 0.001–0.01 \), *** \( p = 0.0001–0.001 \), **** \( p < 0.0001 \)). The following differences had significant \( p \) values: all groups Trp-1455 compared with CD44+ CD8+ controls: \( p < 0.0001 \); Trp-1455: Melapoly versus gDMelapoly: \( 10^{11} \) vp, wk 2: \( p = 0.012 \), \( 10^{10} \) vp, wk 4: \( p < 0.0001 \); Trp-2180 compared with controls: Melapoly, \( 10^{11} \) vp: wk 2: \( p < 0.0001 \), wk 4: \( p = 0.016 \); \( 10^{11} \) vp: wk 2: \( p < 0.0001 \), wk 4: \( p = 0.034 \); gDMelapoly: both doses and both time points: \( p < 0.0001 \); Trp-2180: Melapoly versus gDMelapoly: \( 10^{11} \) vp: wk 2: \( p = 0.027 \), \( 10^{10} \) vp: wk 2: \( p = 0.036 \), wk 4: \( p < 0.0001 \).
In a pretumor challenge vaccination model, gD adjuvant efficacy is solely linked to magnitude of the MAA-specific CD8+ T cell responses.

To determine whether vaccination with the AdC68-gDMelapoly vector resulted in superior protection against tumor growth than vaccination with the AdC68-Melapoly vector, we immunized groups of mice ($n = 5-18$) with $10^{10}$ vp AdC68-gDMelapoly, $10^{11}$ vp AdC68-Melapoly or $10^{11}$ vp AdC68-gD, the latter as a control for nonspecific effects of the vector or gD. Fourteen days later, mice were challenged with B16BrafV600E cells given s.c. Total cytokine+CD8+ T cell responses to the eight CD8+ T cell epitopes were measured before tumor challenge. As shown in Fig. 3A, both vaccines induced significantly higher responses when compared with background stains of naive T cells (mean frequencies 4.5 and

FIGURE 2. MAA-specific CD8+ T cell responses to individual epitopes. Groups of C57BL/6 mice ($n = 7-8$) were vaccinated i.m. with $10^{11}$ vp AdC68-Melapoly or $10^{10}$ vp AdC68-gDMelapoly. (A and B) CD8+ T cell responses to individual epitopes were measured by ICS for IFN-γ, TNF-α, and IL-2 upon stimulation of cells with peptides representing the T cell epitopes expressed by the vaccines over a course of 24–26 wk. Graphs show the sum of cytokine responses. (A) shows responses to the dominant Trp-1-455 peptides. (B) shows the sum of responses over time for the other peptide; responses to individual peptides (but for Trp-1-455) were totaled. (C) and (D) shows Trp-1-teramer+ and Trp-2 tetramer+CD8+ T cell responses 4 and 26 wk after vaccination in tumors of vaccinated mice. Differences between the two vaccines were not significant for Trp-1–specific CD8+ T cells ($p = 0.12$ and 0.18, respectively) but significant for Trp-2–specific CD8+ T cells ($p = 0.0053$ and 0.035, respectively). (E–J) The patterns of the sum of cytokine responses to the eight CD8+ T cell epitopes at three time points [i.e., week 6 (E, F), 12 (G, H) and 24 (I, J)] after vaccination.
FIGURE 3. Vaccine efficacy in mice vaccinated before tumor cell challenge. Groups of C57BL/6 mice (n = 5–18) were vaccinated with 10^{11} vp AdC68-gD, 10^{13} vp AdC68-Melapoly, or 10^{10} vp AdC68-gDMelapoly vectors. Fourteen days later, PBMC samples were tested for CD8+ T cell responses to all of the MAA epitopes present in the vaccine. (A) The sum of tumor Ag-specific CD8+ T cell percentages for individual mice. *, Significant differences as detailed in legend to Fig. 1. Melapoly versus gDMelapoly: p < 0.0001, Melapoly versus naive: p = 0.0014, gDMelapoly versus naive: p < 0.0001. (B) The sum of tumor Ag-specific CD8+ T cell percentages in the two subcohorts that showed comparable CD8+ T cell responses. Subcohorts were selected by excluding animals with frequencies of MAA-specific CD8+ T cells below the mean of the Melapoly group or above the mean of the gDMelapoly group. Melapoly versus gDMelapoly, p = 0.1, Melapoly versus naive: p < 0.0001, gDMelapoly versus naive: p < 0.0001. (C) Survival rates of the entire group, measured for 60 d. Melapoly versus naive: p < 0.0001, gD-Melapoly versus naive: p < 0.0001, Melapoly versus gDMelapoly: p = 0.0018. (D) Survival rates of the of the Melapoly subcohort in comparison with the gDMelapoly and the control group. Melapoly versus controls: p < 0.0001, gD-Melapoly versus controls: p = 0.0003. Melapoly versus gDMelapoly: p = 0.08. In (C) and (D), control animals are shown as X, AdC68-Melapoly-vaccinated mice are shown as open squares, AdC68-gDMelapoly-vaccinated mice are shown as closed squares.

9.3% for Melapoly and gD-Melapoly, 0.02% for control cells). The AdC68-gD-Melapoly vector induced significantly higher CD8+ T cell responses compared with the AdC68-Melapoly vector (p < 0.0001), and this was linked to enhanced survival rates upon tumor challenge (p = 0.0018 by Gehan–Breslow–Wilcoxon test; Fig. 3C). Specifically, >60% of AdC68gD-Melapoly–vaccinated mice as compared with <20% of AdC68-Melapoly–vaccinated mice remained tumor free. When we compared subgroups with similar frequencies of MAA-specific CD8+ T cells by removing the low responders from the Melapoly group and the high responders from the gD-Melapoly group (mean frequencies of the Melapoly subcohort: 5.7% and the gD-Melapoly subcohort: 6.5% p = 0.1; Fig. 3B), survival rates of the two vaccine groups ceased to be significantly different (p = 0.08; Fig. 3D). This suggests that in the pretumor challenge model vaccine efficacy is significantly influenced by the magnitude of MAA-specific CD8+ T cell responses.

In a posttumor challenge vaccination model, gD adjuvant efficacy is not solely linked to the magnitude of MAA-specific CD8+ T cell responses

We further compared the efficacy of the two vectors in a postchallenge vaccination model. B16Braf_{V600E} cells grow very rapidly in vivo; after tumor cell inoculation, most control mice require euthanasia within 14–18 d. AdC68-induced T cell responses on the other hand do not peak till 12–14 d after vaccination. Therefore, we chose a fairly short interval of 3 d between tumor cell inoculation and vaccination for our therapeutic vaccine studies. Accordingly, groups of mice (n = 5–10) were challenged with B16Braf_{V600E} tumor cells on day 0 and vaccinated 3 d later with either 10^{11} vp of the AdC68-Melapoly vector (n = 9) or 3 × 10^{4} vp and 10^{10} vp of the AdC68-gDMelapoly vector (n = 8 and 10 for 3 × 10^{9} and 10^{10} vp, respectively). A control group (n = 28) was vaccinated with 10^{11} vp of the AdC68-gD vector. CD8+ T cell responses were measured 2 wk later from blood by ICS for production of IFN-γ, TNF-α, granzyme B, and perforin. In addition, we assessed CD8+ T cell responses by staining with the Trp-1455– and Trp-2180–specific tetramers. MAA-specific CD8+ T cell responses tested by ICS were significantly higher compared with background stains for both vaccine groups, and higher in gD-Melapoly than Melapoly–vaccinated mice (means: Melapoly: 3.6%, gD-Melapoly: 5.2%, controls: 0.27%, adjusted p values are shown in the figure legend; Fig. 4Aa). The AdC68-gDMelapoly and Melapoly vector induced significantly higher frequencies of Trp-1455 (means: Melapoly: 3.6%, gD-Melapoly: 5.2%, controls: 0.27%, adjusted p values are shown in the figure legend; Fig. 4Ba) and Trp-2180 tetramer CD8+ T cells (means: Melapoly: 0.19% and gDMelapoly: 0.14% control cells: 0.042%; Fig. 4Ca) compared with controls. Both vaccines significantly delayed tumor growth and prolonged overall survival compared with control mice (p < 0.0001; Fig. 4Da). Comparing the two vaccine groups showed that AdC68-gDMelapoly–vaccinated mice survived significantly longer than AdC68-Melapoly–vaccinated mice (p = 0.0039; Fig. 4Ad).

Because MAA-specific CD8+ T cell responses were higher in the AdC68-gDMelapoly group than in the Melapoly group, we selected eight mice from the AdC68-Melapoly vaccine group and...
parable frequencies of MAA-specific CD8+ T cell responses, the given s.c. 3 d before vaccination with $10^{11}$ vp AdC68-gD, $10^{11}$ vp AdC68-Melapoly or 3

mor growth and prolonged survival compared with control mice again, in the subcohorts, both vaccines significantly delayed tu-

Selected by excluding animals that had frequencies of MAA-specific CD8+ T cells below the mean of the Melapoly group divided by the fold difference

1.5, and 3.7%; gD-Melapoly: 1.2, 1.4, and 2.1%; controls: 0.049,

14 mice from the AdC68-gDMelapoly vaccine group with comparable frequencies of total cytokine+CD8+ T cell responses

(mean, Melapoly: 3.9%, gDMelapoly: 4.0%; Fig. 4Ab), Trp-1455
tetramer+CD8+ T cells (means, Melapoly: 3.0% and gDMelapoly: 3.1%; Fig. 4Bb) and Trp-2180 tetramer+CD8+ T cell responses

(means, Melapoly: 0.19% and gDMelapoly 0.14%; Fig. 4Cb).

Again, in the subcohorts, both vaccines significantly delayed tumor growth and prolonged survival compared with control mice ($p < 0.0001$). Although mice had been selected based on comparable frequencies of MAA-specific CD8+ T cell responses, the AdC68-gDMelapoly–vaccinated subcohort showed significantly prolonged survival rates compared with the AdC68-Melapoly–vaccinated subcohort ($p = 0.015$; Fig. 4Db).

To assess whether vaccine-induced T cell responses differed in tissues, we analyzed MAA-specific CD8+ T cell responses to all epitopes by ICS in the blood, spleens, and tumors at the time of necropsy when the size of tumor exceeded a surface area of ~100 mm². Interestingly, total cytokine+CD8+ T cell responses in the blood and tumors of AdC68-Melapoly–vaccinated mice were significantly higher than those of AdC68-gDMelapoly–vaccinated mice (mean frequencies: blood, spleens, tumors: Melapoly: 2.2, 1.5, and 3.7%; gDMelapoly: 1.2, 1.4, and 2.1%; controls: 0.049, 0.12, and 0.027%; Fig. 4Ac, $p$ values are shown in the figure legend). For Trp-1455–specific CD8+ T cell responses, both vaccines induced significant responses in the different compartments compared with naive cells. The AdC68-Melapoly group again showed significantly higher responses in tumors compared with mice immunized with AdC68-gDMelapoly (mean frequencies: blood, spleens, and tumors: Melapoly: 2.7, 2.0, and 7.8%; gDMelapoly: 1.6, 1.0, and 2.7%; controls: 0.085, 0.043, and 0.14%; Fig. 4Bc; gating strategies Supplemental Fig. 3). Trp-2180–specific responses were insignificant in blood but reached significance for both vaccine groups for cells from spleens and tumors compared with naive samples (mean frequencies: blood, spleens, and tumors: Melapoly: 0.18, 0.25, and 0.66%; gDMelapoly: 0.14, 0.33, and 0.89%; controls: 0.016, 0.02, and 0.13%; Fig. 4Bc). MAA-specific CD4+ T cell responses were analyzed in parallel; they showed no differences before challenge but after challenge were higher in spleens of Melapoly-vaccinated mice (mean frequencies in spleens 1.5 and 0.59% for Melapoly and gDMelapoly, respectively, $p = 0.012$; data not shown). Overall, these results indicated that in the postchallenge vaccine model better protection achieved with the gD adjuvanted vaccine could not solely be explained by induction of higher frequencies of MAA-specific CD8+ T cells.
MAA-specific CD8+ T cell phenotypes

Chronic exposure to Ag can lead to T cell exhaustion, which is characterized by progressive loss of T cell functions and eventual cell death (26, 27). To determine whether gD affected the differentiation pathways of T cells, we assessed the expression of several exhaustion markers (i.e., 2B4, LAG-3, and PD-1) on Trp-1455- and Trp-2180-specific CD8+ T cells in spleens and tumors at the time of necropsy, using the two subcohorts with comparable CD8+ T cell responses. Expression of exhaustion markers was also assessed on CD4+CD8+ (naive) T cells and CD4+CD8+ (memory) T cells. The latter population, negative for Trp-1455 and Trp-2180 tetramer–specific CD8+ T cells, presumably contained CD8+ T cells to other MAAs in the vaccine and to unrelated Ags encountered previously. Fig. 5A shows expression levels of the three exhaustion markers as mean fluorescence intensity (MFI) of the different dyes attached to the marker–specific Abs. Significant differences were found between CD4+CD8+ and CD4+CD8+ T cells in spleens and tumors only for 2B4 (Fig. 5A). Differences between CD4+CD8+ or CD4+CD8+ T cells and vaccine-inducted tetramer–specific CD8+ T cells were seen for all markers with the exceptions of 2B4 on Trp-1455- and Trp-2180–specific CD8+ T cells, 2B4 and PD-1 on Trp-2180–specific CD8+ TILs. We also observed significant differences between the two vaccine subcohorts as well as between CD8+ T cells to the two epitopes. In particular, 2B4 showed higher expression on Trp-2180- than Trp-1455–specific CD8+ T cells induced by either vaccine in spleens (mean MFI Melapoly: Trp-1455 versus Trp-2180: 158 versus 269, gDMelapoly: Trp-1455 versus Trp-2180: 166 versus 284, adjusted p values are shown in the legend to Fig. 5A). In both vaccine groups LAG-3 expression in spleens and in tumors was higher on Trp-1455– than Trp-2180–specific CD8+ T cells. In tumors, Trp-1455–specific CD8+ T cells showed significantly lower level of LAG-3 expression in AdC68-gDMelapoly–vaccinated animals compared with those from AdC68-Melapoly–vaccinated mice (mean MFI: spleen: Melapoly: Trp-1455 versus Trp-2180: 436 versus 224, gDMelapoly: Trp-1455 versus Trp-2180: 401 versus 313; tumors: Melapoly: Trp-1455 versus Trp-2180: 875 versus 504, gDMelapoly: Trp-1455 versus Trp-2180: 728 versus 609). In spleens, PD-1 was expressed at higher levels on Trp-2180- than Trp-1455–specific CD8+ T cells, and this reached significance for those induced by AdC68-gDMelapoly. Within tumors again Trp-1455–specific CD8+ T cells that had been elicited by the AdC68-gDMelapoly vaccine showed lower PD-1 expression compared with those elicited by the AdC68-Melapoly vaccine. In both vaccine groups, Trp-1455–specific CD8+ T cells expressed higher levels of PD-1 compared with those specific for Trp-2180 (mean MFI: spleen: Melapoly: Trp-1455 versus Trp-2180: 97 versus 126, gDMelapoly: Trp-1455 versus Trp-2180: 89 versus 151; tumors: Melapoly: Trp-1455 versus Trp-2180: 1190 versus 400, gDMelapoly: Trp-1455 versus Trp-2180: 721 versus 297).

In addition, we determined the percentages of Trp-1455- and Trp-2180–specific CD8+ TILs that were positive for either of the exhaustion markers and compared the results with those on naive (CD44+ or memory tetramer–CD8+ T cells (Fig. 5B). Supplemental Fig. 4). Only very low percentages of CD44+ CD8+ T cells expressed any of the three exhaustion markers (<2%). Percentages of marker–CD44+CD8+ T cells were intermediate (2B4, 19 and 15%; LAG-3, 17 and 12%; and PD-1, 23 and 41% for Melapoly and gDMelapoly-vaccinated mice). Expression of individual markers was significantly higher on CD44+CD8+ cells than on naive cells. Percentages of Trp-1455– or Trp-2180–specific CD8+ T cells positive for any of the three exhaustion markers were again significantly higher when compared with CD44+ CD8+ or tetramer CD44+CD8+ cells. Comparing the two vaccine subcohorts or Trp-1455– and Trp-2180–specific CD8+ T cells within each vaccine group showed no significant differences for percentages of 2B4+ cells (means: Melapoly: Trp-1455 versus Trp-2180: 52 versus 39%, gDMelapoly: Trp-1455 versus Trp-2180: 44 versus 33%). LAG-3 cells were significantly more common within Trp-1455–specific CD8+ T cells in both vaccine groups (means: Melapoly: Trp-1455 versus Trp-2180: 87 versus 48%, gDMelapoly: Trp-1455 versus Trp-2180: 81 versus 58%) as were PD-1+CD8+ cells in Melapoly-vaccinated mice. Interestingly percentages of PD-1+ Trp-1455–specific CD8+ cells were significantly lower in the AdC68-gDMelapoly group than in mice immunized with Melapoly (means: Melapoly: Trp-1455 versus Trp-2180: 84 versus 67%, gDMelapoly: Trp-1455 versus Trp-2180: 63 versus 57%).

Exhaustion leads to sequential expression of several markers (27). Accordingly, we further analyzed tumor-derived T cells for coexpression of PD-1, LAG-3, and 2B4 (Fig. 5B). Again we found that higher percentages of Trp-1455– and Trp-2180–specific CD8+ T cells coexpressed two or three markers compared with CD44+ CD8+ or tetramer CD44+CD8+ cells. Trp-1455–specific CD8+ T cells had in general higher frequencies of PD-1/LAG-3+ and PD-1/LAG-3+2B4+ cells compared with Trp-2180–specific CD8+ T cells. Trp-1455–specific CD8+ cells expressing two or three of the markers were present at significantly lower frequencies in gDMelapoly than Melapoly-immunized mice (means: Melapoly versus gDMelapoly: PD-1/LAG-3+: Trp-1455: 47 versus 35%, Trp-2180: 32 versus 24%; PD-1/LAG-3*: Trp-1455: 69 versus 53%, Trp-2180: 29 versus 24%; PD-1/2B4/LAG-3*: Trp-1455: 45 versus 34%, and Trp-2180: 25 versus 20%).

Markers that are indicative for exhaustion are also upregulated upon activation. To ensure that AdC68-gDMelapoly–induced TILs showed better preservation of function as would be expected of less exhausted CD8+ T cells, we compared the proportions of Trp-1 specific CD8+ T cells that produced only IFN-γ or IFN-γ together with TNF-α between the two vaccine groups. For these comparisons cells were isolated from tumors at days 30–35 after vaccination. AdC68-gDMelapoly–induced Trp-1–specific CD8+ T cells had significantly higher proportions of Trp-1–specific CD8+ TILs that produced both cytokines compared with AdC68-Melapoly–induced TILs (mean AdC68-gDMelapoly: 0.47, AdC68-Melapoly: 0.3, p = 0.04 by two-tailed t test). To further ensure that Trp-1–specific TILs with increased expression of PD-1 or PD-1 and LAG-3 were indeed exhausted rather than recently activated, we tested PD-1+ and PD-1+ TILs for production of IFN-γ and TNF-α upon stimulation with the Trp-1455 peptide. As shown in Fig. 6A, slightly higher frequencies of Trp-1–specific CD8+ T cells from blood produced IFN-γ together with TNF-α than IFN-γ only. In tumors this proportion shifted and ~70% of Trp-1–specific TILs produced only IFN-γ suggesting loss of function (mean frequencies of Trp-1–specific CD8+ T cells: blood: IFN-γ/TNF-α: 0.32 versus 0.57%; tumor: 1.85 versus 0.84%). C sostains for PD-1 showed significantly higher PD-1 expression on monofunctional Trp-1–specific CD8+ TILs as compared with those that produced both cytokines (mean PD-1 MFI values: blood: IFN-γ/TNF-α : versus IFN-γ/TNF-α*: 0.32 versus 0.57%; tumor: 1.85 versus 0.84%). An analysis for expression of T-bet, which controls transcription of effector molecules and inhibits expression of PD-1, showed that T-bet levels were low on naive circulating CD8+ T cells and markedly increased on blood-derived Trp-1–specific CD8+ T cells. Tumor-infiltrating Trp-1–specific CD8+ T cells showed a marked reduction in T-bet expression (Fig. 6C), which corresponded to expression levels of exhaustion/activation markers (mean T-bet MFI values: CD44+ CD8+ T cells: 6.2; Trp-1 specific CD8+...
FIGURE 5. MAA-specific CD8+ T cell phenotypes. Phenotypes of CD8+ T cells isolated from spleens and tumors of the two subcohorts shown in Fig. 4 were tested for expression of 2B4, LAG-3, and PD-1. (A) MFI of 2B4 (a), LAG-3 (b, e), and PD-1 (c, f) fluorochrome-labeled Abs on CD44+CD8+ and tetramer+CD44+CD8+ T cells from spleens (a–c) and tumors (d, f). Open boxes show results for CD8+ T cells from AdC68-Melapoly–vaccinated mice, gray boxes indicated CD8+ T cells from AdC68-gDMelapoly vaccinated mice. *, Significant differences as detailed in legend to Fig. 1 between groups connected by lines (dashed lines for differences between control cells or between control cells and Ag-specific CD8+ T cells, solid lines for differences between specific CD8+ T cells). The following p values were obtained for Trp-1455 and Trp-2180 comparisons: (Aa) Trp-1455 versus Trp-2180: Melapoly: \( p = 0.0001 \), gDMelapoly: \( p = 0.0001 \); (Ad) none; (Ab) Trp-1455 versus Trp-2180: Melapoly: \( p = 0.0001 \), gDMelapoly: \( p = 0.0056 \); (Ac) Trp-1455 versus Trp-2180: Melapoly: \( p = 0.0001 \), gDMelapoly: \( p = 0.028 \), Melapoly versus gDMelapoly: Trp-1455: \( p = 0.0008 \). (B) Percentages of tumor-derived naive CD44+CD8+, tetramer+CD44+CD8+, and tetramer+CD44+CD8+ T cells that were positive for a given marker or combinations of exhaustion markers. Cells from AdC68-Melapoly–vaccinated mice are shown as open boxes, and cells from AdC68-gDMelapoly vaccinated mice are shown as gray boxes. Whiskers show 5–95th percentile, lines show 50th percentile, + within boxes indicates means. *, Significant differences between groups as described in legends to Fig. 1. (Ba) (Figure legend continues)
T cells blood versus TILs: 464.2 versus 204.6); Trp-1-specific CD8+ TILs that expressed high levels of PD-1 or PD-1 and LAG-3 had reduced levels of T-bet as compared with those with low PD-1 or PD-1 and LAG-3 expression (Fig. 6D–F) (mean T-bet MFI values: PD-1+ versus PD-1-: 191.4 versus 286.2; PD-1+LAG-3+ versus PD-1-LAG-3-: 181.3 versus 256). Overall these results show that increased expression of PD-1 was associated with loss of polyfunctionality and downregulation of T-bet, confirming that MAA-specific CD8+ T cells induced by the vaccines were differentiation toward exhaustion within the TME.

Taken together, these results demonstrate complex patterns of up-regulation of exhaustion markers during tumor progression that were affected by the vaccines, the TcR specificity as well as the anatomic sites from which T cell had been isolated. Within tumors, MAA-specific CD8+ T cells induced by the AdC68-Melapoly vector were less exhausted compared with those induced by the AdC68-Melapoly vector, fewer cells expressed exhaustion markers, and they were able to delay tumor progression for longer periods of time. In addition, tumor-infiltrating CD8+ T cells specific to the dominant Trp-1455 epitopes showed more evidence of exhaustion compared with those specific to the subdominant Trp-2180 epitope.

Discussion

T cell activation is finely tuned upon binding of the TcR to its cognate Ag-MHC class I molecule complex through additional interactions with costimulatory and coinhibitory receptors (28, 29). Coinhibitory receptors include CTLA-4, PD-1, BTLA, and others. BTLA, unlike PD-1 and CTLA-4, is expressed on both naive and activated T cells (30) and thereby presumably regulates T cell activation at several stages. PD-1 and CTLA-4 inhibit downstream signals transmitted upon TcR ligation as such induction of the
protein kinase B pathway, albeit through distinct pathways (31, 32). Protein kinase B pathway in turn regulates cell survival as well as glucose metabolism and is therefore crucial for successful activation and expansion of T cells (33). The exact signaling pathway downstream of BTLA remains unknown. Similar to PD-1 and CTLA-4, the cytoplasmic domain of BTLA contains immune receptor tyrosine–based inhibitory motifs (34) that may dampen TcR signaling.

Although modulators of HVEM signaling have not yet been tested in humans, the TNFRSF14 locus, which encodes HVEM, has been linked to increased risks for ulcerative colitis (35), systemic lupus erythematosus (36), and rheumatoid arthritis (37), stressing the importance of this regulatory pathway. Similarly, genetic deletions of BTLA or HVEM cause exacerbated inflammatory reactions (38) and accelerated rejection of partially mismatched transplants in mice (39).

It has been shown that HVEM is expressed on tumor cells such as B cell lymphomas (40) or metastatic melanomas (41). BTLA on the other hand is highly expressed on melanoma-specific CD8+ T cells and it has been argued that the impaired proliferative capacity of MAAspecific T cell in melanoma patients is linked to immunoinhibitory signals from HVEM+ tumor cells (41). In another study, NY-ESO-1–specific CD8+ T cells from end-stage melanoma patients were shown to express elevated levels of PD-1 and BTLA (42). T cells were defective in their ability to proliferate or secret factors in response to antigenic stimulation, which could in part be restored by blocking PD-1 and/or BTLA signaling. This suggests that inhibitors of BTLA should be explored as additives to active immunotherapy of melanoma.

As we showed previously, blockade of the BTLA–HVEM pathway through HSV-1 gD during Ag-driven T cell stimulation numerically augments CD8+ T cell responses (17, 18). This was confirmed in the current study using vaccines expressing multiple epitopes derived from MAAs. In a prophylactic melanoma model, the superior protection achieved upon gD-mediated blockade of the HVEM pathways at the time of T cell induction was correlated with the magnitude of MAAspecific CD8+ T cell responses. Using different doses of the two vaccines (i.e., AdC68-Melapoly and AdC68-gDMelapoly) mice that developed approximately equal frequencies of MAAspecific T cells showed comparable levels of protection against tumor protection. In contrast, in mice that were inoculated first with melanoma cells and then vaccinated with either of the two vectors, the magnitude of the vaccine-induced MAAspecific CD8+ T cell response was not the sole factor that determined vaccine efficacy; in mice with approximately comparable frequencies of MAAspecific CD8+ T cells following vaccination with the AdC68-Melapoly or AdC68-gDMelapoly vectors, those that received the latter survived significantly longer. Cytokine profiles of Ag-specific CD8+ T cells were largely comparable between the two vaccine groups before and after challenge with responses being dominated by CD8+ T cells producing IFN-γ alone or in combination with TNF-α (data not shown) as is typical for T cells induced by Ad vectors.

Paradoxically, at the time of necropsy AdC68-Melapoly–vaccinated animals had significantly higher frequencies of MAAspecific CD8+ T cells in some tissues compared with those vaccinated with AdC68-gDMelapoly. This again confirms that protection did not solely correlate with frequencies of vaccine-induced T cells. Further analysis of MAAspecific CD8+ T cells for expression of activation/exhaustion markers showed that within tumors lower frequencies of AdC68-gDMelapoly–induced CD8+ T cells expressed LAG-3, PD-1, and PD-1 together with LAG-3 or 2B4 or all three markers as compared with AdC68-Melapoly–induced CD8+ T cells. These markers are not only indicative for exhaustion but they are also increased upon recent CD8+ T cells activation. Ags derived from the tumor may have provided further activation signals to the MAAspecific CD8+ T cells, which then contributed to their elevated levels on tumor-infiltrating MAAspecific CD8+ T cells. Nevertheless, as these activation signals should have been provided in both vaccine cohorts, we assume that the differences in expression levels on tumor-infiltrating MAAspecific CD8+ T cells in Melapoly and gDMelapoly-vaccinated reflected differences in their stage of exhaustion. This was further confirmed by loss of polyfunctionality and T-bet expression on cells with increased expression of immunoinhibitory markers. In turn, our finding suggests that the relative resistance to tumor-driven exhaustion of MAAspecific CD8+ T cells generated in presence of gD resulted in prolonged control of tumor progression.

T cell exhaustion, a consequence of continued Ag-driven stimulation of T cells, was initially described in chronic viral diseases such as those caused by lymphocytic choriomeningitis virus infection of mice (26) or HIV (43) or hepatitis C virus infection (44) of humans and subsequently in cancer patients (45, 46). Differentiation toward exhaustion is initially characterized by increased expression of PD-1 on the T cell surface, which over time is joined by other coinhibitors (47). Expression of PD-1 is regulated by TcR ligation (48) and at the transcriptional level by NFAT1c (49). T cells with high-affinity TcRs appear to be more sensitive to Ag-driven exhaustion compared with those with lower affinity TrRs (50). This is supported by our finding that within tumors CD8+ T cells specific to the subdominant Trp-2180 epitope appeared less exhausted; vaccine-induced Trp-2180–specific CD8+ T cells expressed lower levels of PD-1 or LAG-3 as compared with CD8+ T cells specific to the more dominant Trp-1155 epitope. The same pattern was seen for MAAspecific CD8+ T cells that were double or triple positive for PD-1, 2B4, and/or LAG-3. Notwithstanding, it should be pointed out that the finding that higher TcR affinity promotes exhaustion remains debatable, although it is compatible with the highly reproducible finding that exhaustion is primarily driven by continued TcR ligation (51). Other pathways that prevent Ag-driven exhaustion of CD8+ T cells used by the gD-adjuvanted vaccine may have contributed the results, but cannot be explored without additional knowledge of the BTLA/CD160 signaling pathways.

Active cancer immunotherapy with traditional vaccines that boost tumor-specific T cell responses has performed poorly in patients with advanced cancer. Additional strategies that block the immunoinhibitory pathways may improve the efficacy of cancer vaccines. In this study, we show that adjuvantage a tumor vaccine with HSV-1 gD could induce tumor Ag-specific CD8+ T cells with increased resistance to exhaustion. This approach, unlike other reagents that block immunological checkpoints such as Abs to PD-1 or CTLA-4, selectively affects tumor Ag-specific T cells and presumably will not globally perturb the exquisitely fine-tuned balance of the immune system.

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The authors have no financial conflicts of interest.

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ADJUVANT EFFECT ON TUMOR-DRIVEN T CELL EXHAUSTION


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SUPPLEMENTAL FIGURE 1
Gating scheme for tetramer^+CD8^+ T cells. Samples were initially gated on lymphoid cells (A) and then singlets (B) using forwards and side scatters. Singlets were further gated on live cells (C). Live cells were gated on cells positive for CD8 (D). CD8^+ cells were blotted into CD44^- and CD44^+ cells (E) with the former serving as intrinsic controls. Using the CD44 gate CD44^- (F,H) and CD44^+ (G,I) cells were gated over the Trp-1 (F,G) and Trp-2 (H,I) tetramers. Counts for live CD8^+ cells as well as tetramer^+CD44^- and tetramer^+CD44^+ cells were recorded and used to calculate frequencies of tetramer^+ cells over all CD8^+ cells.
SUPPLEMENTAL FIGURE 2

Gating scheme for cytokine producing CD8⁺ T cells. Cells were stimulated with a control peptide or the MAA peptides present in the Ad vector encoded antigen. Cells were then stained for surface expression of CD44 and CD8 and intracellular cytokines such as IGN-γ, TNF-α and IL-2. Cells were analyzed and blots were generated. Cells were gates onto lymphoid, single, live CD8⁺ cells as described in Fig. 1. They were then gated onto CD44⁺ and CD44⁺CD8⁺ cells (A). Both populations were gated on cells positive for TNF-α and/or IFN-γ (B-I) and TNF-α over IL-2 (not shown). To better illustrate the gates, blots of cells stimulated with the peptides carrying the subdominant epitopes (B,C) were concatenated. (B,C,F,G) show gates for cells stimulated with the control peptides, in B and F blots were concatenated, (C) and (G) show a single blot, (B) and (C) show blots for CD44⁺ cells, (F) and (G) show blots for CD44⁺ cells. D and H show concatenated blots for CD44⁺ (D) and CD44⁺ (H) cells stimulated with peptides carrying subdominant epitopes. (E) and (I) show blots for the same populations stimulated with the peptide carrying the immunodominant Trp-1455 epitope. Boolean gates were set by Flowjo to determine counts and frequencies of cells producing the different combinations of cytokines. Counts were recorded and those obtained for each possible combination of cytokines for cells stimulated with the control peptide were subtracted from those obtained with peptides expressing MAA-specific epitopes (these results were obtained with original blots rather than concatenated blots). Counts obtained for CD8⁺ T cells were then used to determine frequencies of cytokine producing cells over CD8⁺ cells. Frequencies for the 7 possible combinations of cytokines were summed to provide overall frequencies of responding cells. In Fig. 2 the frequencies of CD44⁺ and CD44⁺ cells producing cytokines over all CD8⁺ cells in response to the Trp-1455 epitope are shown while Fig. 2B shows the sum of frequencies to the other 7 peptides carrying subdominant epitopes.
SUPPLEMENTAL FIGURE 3
Gating scheme for MAA-specific TILs TILs were gated on lymphoid, live, single
CD8+ T cells. CD8+ T cells were gated on CD44+ and CD44+ cells (A, F). For tetramer
stains CD44+CD8+ (B,D) and CD44+CD8+ (C, E) cells were gated on cells positive for
the Trp-1 (B, C) and Trp-2 (D, E) tetramer. For ICS CD44+CD8+ (G, I) and CD44+CD8+
cells were gated on TNF-α over IFN-γ (G, H) or perforin over granzyme (I, J). Frequencies
were calculated after subtraction of background data obtained with cells cultured
with a control peptide.
SUPPLEMENTAL FIGURE 4

Gating scheme for expression of markers on CD8\(^+\) T cells. Cells were gated on lymphoid, single, live CD8\(^+\) cells as shown in Fig. 1. Cells were then gated onto CD44\(^-\) and CD44\(^+\) cells (A). CD44\(^+\) cells were gated on cells positive for either of the two tetramers and on cells negative for either of the two tetramers (B). CD44\(^-\) (C,D,G,H,K), tetramer\(^+\)CD44\(^+\) (E,F,I,J,L) and tetramer\(^-\)CD44\(^+\) cells were then gated onto LAG-3 (C,E), 2B4 (D,F) and PD-1 (G,I). The gates were adjusted so that CD44\(^-\)CD8\(^+\) cells had less than 2% of marker positive cells. Cells were then gated onto 2B4 over PD-1 to identify cells positive for both markers (H,I). Cells double positive for PD-1 and 2B4 were then gated onto LAG-3 (K,L) to identify cells positive for all three markers.