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Killing of Normal Melanocytes, Combined with Heat Shock Protein 70 and CD40L Expression, Cures Large Established Melanomas

Luis Sanchez-Perez,2*† Timothy Kottke,2* Gregory A. Daniels,* Rosa Maria Diaz,*† Jill Thompson,* Jose Pulido,‡ Alan Melcher,§ and Richard G. Vile3*†§

Previously, we showed that nine intradermal injections of a plasmid in which the HSVtk suicide gene is expressed from a melanocyte-specific promoter (Tyr-HSVtk), combined with a plasmid expressing heat shock protein 70 (CMV-hsp70), along with systemic ganciclovir, kills normal melanocytes and raises a CD8+ T cell response that is potent enough to eradicate small, 3-day established B16 tumors. We show in this study that, in that regimen, hsp70 acts as a potent immune adjuvant through TLR-4 advertisement. This article must therefore be hereby marked in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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vitiligo in tumor cured mice (13). In addition, cured mice did not develop memory against the melanocyte Ags and were unable to reject a subsequent challenge of B16 tumor cells 6 mo after the initial treatment regimen. However, autoimmune vitiligo could be induced in treated animals, but only if they were not challenged with tumor, and this autoimmune disease was significantly more aggressive if the animals also were depleted of CD25+ T cells before plasmid administration (13). Presumably these mechanisms of suppression of anti-self T cell responses exist in vivo to safeguard against the development of autoimmune disease in circumstances where pathological killing of normal cells occurs (22).

In these previous studies, we were able to cure large percentages (70–100%) of mice carrying 3-day established tumors using a total of nine injections of the Tyr-HSVtk/CMV-hsp70 mixture (13). When we attempted to treat more established tumors seeded 9 days previously, we observed therapeutic delays in tumor progression with plasmid injections, but the percentage of cures decreased to between 0 and 10% (23). In those mice where tumor growth was delayed but not cured, there was a potent in vivo immunoselection by a CD8+ T cell response of tumor cell variants that had lost expression of a specific subset of known melanocyte Ags (23).

In this study, we have investigated both the mechanisms by which inflammatory killing of melanocytes leads to the priming of CD8+ T cell responses against B16 melanomas and methods by which it can be enhanced to treat larger, more established tumors. Taken together, our data show that, by manipulating in vivo mechanisms that normally restrain autoimmune responses, increased antitumor responses can be induced following the killing of normal cells.

Materials and Methods

Cell lines, plasmids, and viruses.

The murine melanoma B16.F1, tumor cell line has been described previously (24). Plasmids used in these studies have been described previously (13). Briefly, the Tyr-HSVtk plasmid uses a hybrid promoter of three tandem copies of a 200-bp element of the murine tyrosinase enhancer (25) upstream of a 270-bp fragment of the tyrosinase promoter (26) to drive expression of the HSVtk gene (27). In CMV-hsp70, the murine hsp70 gene (16) is driven by the CMV promoter in pCR3.1 (Invitrogen Life Technologies). In pCD40L, the murine CD40L gene is driven by the CMV promoter. The adenovirus expressing murine TNF-α was a gift from Dr. Zhou Xing, (McMaster University, Hamilton, Ontario, Canada).

RT-PCR

Skin samples at the site of plasmid injection were snap-frozen in liquid nitrogen. RNA was prepared with the Qiagen RNA extraction kit. One micrograms of total cellular RNA was reverse transcribed in a 20-μl volume using oligo(dT) as a primer. cDNA equivalent of 1 ng RNA was amplified by PCR for a variety of murine cytokines or melanoma/melanocyte Ags as described previously (24, 28) (details of the primers are available upon request).

Splenocyte preparation

Splenocytes enriched in lymphocytes were prepared from spleens by standard techniques (29) and Lympholyte-M density separation (Cedarlane Laboratories). CD8+ T cells were purified from spleens using the MACS CD8a (Ly-2) Microbead magnetic cell sorting system (Miltenyi Biotec). Ag presentation assays and tetramers

Tumor treatment protocols

For protocols aimed at treating established s.c. tumors, 2 × 105 B16 cells were seeded subcutaneously in the right flank of C57BL/6 mice (day 0). At the appropriate day following tumor seeding, 20 or 30 μg of plasmid DNA was injected intradermally on the contralateral flank. GCV at 50 mg/kg was administered i.p. In our hands, a 3-day established tumor was usually palpable under the skin; a 9-day established tumor was usually ~0.3–0.4 cm in its longest diameter.

Statistics

Data from the animal studies were analyzed by the logrank test (33). Statistical significance was determined at the level of p < 0.05.

Results

Hsp70 induces anti-tumor immunity through TLR-4-dependent signaling

We have reported previously (13, 23) that three rounds of Tyr-HSVtk/CMV-hsp70/GCV treatment (a total of 9 intradermal plasmid injections and 15 i.p. injections of GCV) cures 70–100% of mice bearing 3-day established s.c. B16 tumors on the contralateral flank (Fig. 1A). Both the Tyr-HSVtk and the CMV-hsp70 plasmids are required for this therapy to be effective (Fig. 1A and Ref. 13). Consistent with anti-tumor therapy, i.d. injection of the hsp70 plasmid is required for the priming of TRP-2-specific responses in the spleens of vaccinated mice (Fig. 1B). In Fig. 1B, confirming our previous data that TRP-2 and tyrosinase, but not gp100, are targets of the murine melanocyte Ags (23).

Hsp70 has been reported to act as a chaperone of immunogenic peptides (34–39), a cytokine (39–41), an immunogen (42–44), a maturation agent for dendritic cells (20), and as an inducer of proinflammatory cytokines from monocytes (45) following ligation to TLR 2 and TLR 4 (40, 41). Therefore, to understand which of these possible activities hsp70 is exerting in our system, we tested our protocol of inflammatory melanocyte killing in mice lacking key elements of these effector responses. Whereas C57BL/6 mice bearing 3-day established B16 tumors were cured...
Local expression of hsp70 induces priming of anti-melanoma/melanocyte responses through induction of TNF-α

Because of the striking dependence upon TLR-4 for hsp70-mediated therapy, and because we and others have reported that hsp70 released from dying tumor cells induces proinflammatory cytokines (TNF-α, IL-1β, and IL-6) from macrophages (18, 40, 41, 46), associated with anti-tumor immune responses (17, 18, 46), we screened the site of hsp70 injection for the expression of such cytokines. Of the seven different cytokines, we tested TNF-α correlated consistently with the expression of hsp70 (Fig. 2A). In contrast, hsp70 expression at the site of melanocyte killing was unable to induce local TNF-α expression in C57BL/10ScNJ mice lacking TLR-4 signaling (Fig. 2B).

Therefore, we hypothesized that hsp70 expression induces local immune activation through TNF-α induction as a critical element to the in vivo, CD8+ T cell-mediated therapy of B16 tumors. In this respect, we observed that, whereas the Tyr-HSVtk/CMV-hsp70/GCV treatment effectively primed TRP-2 specific responses in C57BL/6 mice (Figs. 1B and 2C), these effects were lost in B6;129S6-Tnftm1Gkl/J TNF-α−/− mice (Fig. 2C). However, the local provision of TNF-α by delivery of an adenovirus expressing TNF-α at the site of plasmid injection was able to rescue the ability of these mice to generate TRP-2 specific responses, but only if hsp70 was provided in the plasmid injections (Fig. 2C).

Hsp70 expression induces trafficking of an APC-like population to the draining LN

We hypothesized that local immune activation by hsp70, through TLR-4-mediated signaling and TNF-α induction, induces migration of APC from the site of plasmid injection to the LN. To test this hypothesis, we tracked Cell Tracker Green-labeled cells from the site of injection to the draining LN. No CTG+ cells could be detected in draining LN following i.d. injection of CTG alone (data not shown), or with any plasmid combination in which hsp70 was not present (Fig. 3A and B). However, when hsp70 was expressed locally, MHC class II (MHC-II)hi, CTG+ cells trafficked to the LN, irrespective of whether GCV was administered as well (red arrows Fig. 3C and D), consistent with this being a population of activated APC. This CTG+/MHC-IIhi population was further characterized and shown to consist of between 55 and 60% MAC3+ cells (Fig. 3F) and ~40% Mac3−, CD11c+ cells (data not shown).

To test the functional relevance of this LN migration, we codedelivered a plasmid (Tyr-ova) in which the cDNA of the model chick ova Ag, expressed from the tyrosinase promoter, is only expressed in melanocytes. CD8+ T cells specific for the H-2Kb-restricted SIINFEKL epitope of ova could be detected in LN by tetramer analysis, but only if pTyr-ova was coinjected with Tyr-HSVtk/GCV (to kill melanocytes and release ova Ag) and CMV-hsp70 (consistent with migration to the LN of a putative APC population) (Fig. 4A). (We were unable to detect priming of naive T cell responses to the TRP-2 Ag in these assays).

Because the Mac3 marker is not truly specific for macrophages, we used transgenic OT-I T cells (specific for H-2Kb-restricted SIINFEKL) to monitor which of the Mac3+, or CD11c+, cell populations migrating to the LN are presenting the melanocyte-derived (ova) Ag. Fig. 4B shows that the SIINFEKL epitope of the ova Ag, expressed from the melanocyte-specific tyrosinase promoter, was presented almost exclusively by the CD11c+ population of cells, which hsp70 induces to migrate to the draining LN.
Hsp70-induced LN trafficking is critical to therapeutic efficacy

When we replaced the Tyr-HSVtk plasmid with a CMV-HSVtk plasmid (HSVtk cDNA expressed by the CMV promoter) in the therapeutic protocol of Fig. 1A, we consistently observed a complete loss of therapy of established B16 tumors (Fig. 5A), even though levels of expression of HSVtk were directly comparable between the two plasmids in melanocyte-derived cell types (data not shown). PCR from genomic DNA of LN cells following i.d. plasmid injections showed that the injected HSVtk gene could be detected in draining LN cells (Fig. 5A), but only in mice in which hsp70 had been present (Fig. 5A). These findings were consistent with our cell tracking studies in Fig. 3 above showing the importance of hsp70 in promoting LN migration of APC dependent on TLR-4-mediated, TNF-α mechanisms. However, consistent with the observation of loss of therapy when the Tyr-HSVtk plasmid was replaced with the CMV-HSVtk plasmid, no PCR signal for the HSVtk transgene could be detected in LN of mice injected with CMV-HSVtk/CMV-hsp70 and given GCV treatment (Fig. 5A). Similarly, CTG-labeled, class II Hi cells could be detected in the LN following i.d. plasmid injections with the CMV-HSVtk plasmid only if hsp70 were coexpressed (Fig. 5D), and if PBS was administered (Fig. 5D). However, this LN migrating population was lost, even if hsp70 was present, if GCV was used instead of PBS (Fig. 5E). Finally, the HSVtk transgene also could not be detected by PCR in the LN following i.d. Tyr-HSVtk/CMV/hsp70/GCV injections into C57BL/10ScNJ mice, which carry a deletion of the Tlr4 gene (Fig. 5F), confirming the importance of hsp70/TLR-4 signaling in vivo to promote the migration of cells carrying melanocyte derived Ag to the LN. Overall, these data are consistent with the hypothesis that CMV-HSVtk/GCV kills hsp70-activated APC carrying melanocyte Ags from the site of melanocyte destruction to the LN and explain why Tyr-HSVtk/hsp70/GCV, but not CMV-HSVtk/hsp70/GCV, induces tumor regressions.
MHC-II for CD8 assayed by flow cytometry with SIINFEKL-loaded H-2Kb MHC-I tetramers. Because it has been suggested that hsp70 works through binding of hsp70 expression induces priming of CD8+ T cells specific for the ova Ag. Tetramer-positive cells (1.1%) were detected only in animals injected with Tyr-HSVtk/CYT-ova/CMV-hsp70/GCV and no positive staining cells were detected using tetramers specific for TRP-2. Although we were unable to detect TRP-2 specific cells in the LN we could detect them in the spleen. We assume that this inability to detect TRP-2 specific T cells reflects a balance between the sensitivity of the detection system and the immunogenicity of the two Ags (ova is foreign while gp100 is self in this system). B, Mice were given a single cycle of intradermal plasmid injections of Tyr-HSVtk/CMV-hsp70/Tyr-ova or Tyr-HSVtk/CMV-LacZ/Tyr-ova along with GCV, and LN from three similarly injected animals were recovered at day 6. By pooling LN from three similarly injected animals, we could recover at day 6. By pooling LN from three similarly injected animals, we could detect 105 and 2×106 CTG+ /MHC-II+ cells were separated by flow cytometry sorting into CD11c+ or CD11c− populations. A total of 5×105 of these cell populations was assayed directly with transgenic OT-1 T cells specific for the SIINFEKL epitope of ova (at ratios of 1:1, 1:50, and 1:100) and assayed after 72 h by IFN-γ ELISPOT assays to show the potency of T cell activation. Results shown are from a OT-1 to LN cell ratio of 100:1. The positive control, OT-1 incubated with SIINFEKL peptide gave >1000 spots per well under these conditions (data not shown).

Inflamatory killing of melanocytes is enhanced by additional T cell costimulation

Because it has been suggested that hsp70 works through binding of CD40 on APC (20, 47), we investigated whether addition of CD40 ligation could replace hsp70, or whether it would enhance the quality, and/or quality, of the T cell response against melanocyte Ags. Therefore, we added a plasmid expressing CD40L from the CMV promoter (pCD40L) to the regimen of plasmid injections. When pCD40L was added to the curative protocol of Fig. 1A, there was no significant inhibition of treatment efficacy, compared with using hsp70 alone (Fig. 6A). Moreover, the presence of pCD40L in the plasmid treatments gave no significant added survival benefit to C57BL/10ScNJ TLR-4−/− mice bearing 3-day established B16 tumors, compared with treatment with Tyr-HSVtk/CMV-hsp70/GCV alone (Fig. 6A). Taken together, these data indicate that pCD40L could neither inhibit, nor replace, the activity of hsp70 in either wild-type or TLR4−/− mice.

Next, we tested whether addition of pCD40L could enhance the activity of hsp70-mediated inflammatory melanocyte killing. We have reported previously that animals bearing 9-day established s.c. B16 tumors treated with only two rounds of Tyr-HSVtk/CMV-hsp70 and 10 i.p. injections of GCV, typically survive longer than controlled treated animals but nearly all eventually succumb to disease (13, 23) (Fig. 6B). In contrast, over several different experiments, 80% of animals treated with Tyr-HSVtk/CMV-hsp70/pCD40L (10 μg of each plasmid) rejected their tumors and survived >60 days following tumor seeding (Fig. 6B). We also confirmed that pCD40L was unable to substitute for hsp70. Thus, whereas mice treated with 9 injections of Tyr-HSVtk/CMV-hsp70 plasmid are cured of 3-day tumors (Fig. 1) 0/20 mice treated with Tyr-HSVtk/pCD40L were cured, and tumors grew as rapidly as in control-treated animals.

Coexpression of pCD40L increases the number and potency of TRP-2 specific T cells

We have shown previously that hsp70-mediated inflammatory killing of melanocytes primes T cell responses specific to the TRP-2, but not gp100, Ags (13, 23). Consistent with the increased therapeutic potential of expression of CD40L at the injection site, ELISPOT data indicated that there was a modest, but consistently significant (p < 0.01), increase in the frequency of TRP-2-specific splenocytes generated in vivo 8 days following the first of three injections of Tyr-HSVtk/CMV-hsp70/pCD40L + GCV, compared with treatment with Tyr-HSVtk/CMV-hsp70 + GCV (Fig. 6C). However, combined with IFN-γ ELISA analysis, close to a 3-fold increase was observed in the specific activity of the TRP-2 specific splenocytes generated following i.d. injection of Tyr-HSVtk/CMV-hsp70/pCD40L + GCV, compared with Tyr-HSVtk/CMV-hsp70 + GCV (Fig. 6D). Inclusion of CD40L in the plasmid regimen also enhanced epitope spreading (48) in that splenocytes specific for gp100 could now be detected in Tyr-HSVtk/CMV-hsp70/pCD40L/GCV-treated mice (Fig. 6C), whereas we have never been able to detect gp100-reactive T cells in Tyr-HSVtk/CMV-hsp70/GCV-treated mice (Fig. 6C and Refs. 13 and 23).

Injection of pCD40L did not increase the number of Cell Tracker Green cells, or CD11c+ cells, detected in the LN using either the PCR detection method (Fig. 6E) or by flow cytometry in the cell migration assay described in Fig. 2 (data not shown). However, inclusion of the pTyr-ova plasmid into the plasmid injection regimen significantly increased the number of SIINFEKL-specific T cells detected in the LN, compared with when pCD40L was absent (p < 0.001) (Fig. 6F), confirming again that ligation of CD40 on putative APC increases the number of Ag-specific T cells primed in the draining LN.
In animals cured of 3-day established tumors by 9 injections of TyrHSVtk/CMV-hsp70/GCV, development of autoimmune disease was difficult to detect (13) (Fig. 7A); only mice depleted of CD25+ T cells, which received TyrHSVtk/CMV-hsp70/GCV treatment, but never saw tumors, developed localized areas of depigmentation (data reported in ref. (13)). In addition, long-term survivors (>100 days) could not reject rechallenge with B16 (data in Ref. 13). Thus, we concluded that the CD8+ T cell response from TyrHSVtk/CMV-hsp70/GCV therapy is short lived, due at least in part to the induction of putative suppressor cells in the CD4+CD25+ compartment (13). In contrast, animals cured by TyrHSVtk/CMV-hsp70/pCD40L, intradermal injections developed alopecia-like symptoms with often severe but patchy hair loss across their abdomens (Fig. 7A). In addition, these mice were often unable to regrow their hair in the shaved areas where the initial injections had been performed. Moreover, mice surviving the 9-day established tumors following TyrHSVtk/CMV-hsp70/pCD40L/GCV treatment developed stringent memory in 100% of the survivors (Fig. 7B), and none of the cured mice have developed recurrent tumor growth up to 9 mo following tumor challenge. Interestingly, systemic administration of an anti-CD40 Ab (FGK45 at 50 µg i.p.) was ineffective (Group B; Fig. 7B). These data indicate that pCD40L enhances the development of autoimmune disease that we observe following TyrHSVtk/CMV-hsp70/GCV therapy alone, and which we have demonstrated previously is controlled in part by both the presence of tumor and the activity of regulatory T cells (13, 23).

Discussion

The data presented in the current report are consistent with a model in which local hsp70 expression at the site of plasmid injection induces the migration of a population of MHC-IIhi/CD11c+ APC from the site of injection to the draining LN. Once at the LN, these APC, previously loaded with melanocyte Ags derived from the HSVtk/GCV-induced killing by the TyrHSV plasmid (13), present these Ags directly to naive T cells leading to activation of anti-melanocyte immune responses. In support of this model, we show in this study that hsp70 expression is required to prime T cell responses against the TRP-2 Ag, which is shared between both normal melanocytes and melanomas, and against which we have previously observed that the CD8+ T cell response is directed (13, 23). Moreover, these MHC-IIhi/CD11c+ cells, recovered from draining LN, could present a model OVA Ag, expressed exclusively in the melanocytes, to activate naive OT-1 T cells in vitro.

We show in this study that hsp70 expression induces TNF-α expression locally at the site of plasmid injection. In the absence of hsp70, the MHC-IIhi/CD11c+ APC could not be detected in the draining LN, correlating with a lack of therapeutic efficacy if hsp70 is absent (13, 23). Similarly, in mice lacking TNF-α, the TyrHSVtk/CMV-hsp70 plasmid combination was unable to raise TRP-2-specific responses in the spleens of treated animals. However, replacement of TNF-α locally by adenoviral-mediated delivery restored the priming of this anti-melanocyte response. The importance that we attribute to the LN migration of this population of CD40L enhances anti-melanocyte responses and immunological memory in vivo

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We show in this study that hsp70 expression induces TNF-α expression locally at the site of plasmid injection. In the absence of hsp70, the MHC-IIhi/CD11c+ APC could not be detected in the draining LN, correlating with a lack of therapeutic efficacy if hsp70 is absent (13, 23). Similarly, in mice lacking TNF-α, the TyrHSVtk/CMV-hsp70 plasmid combination was unable to raise TRP-2-specific responses in the spleens of treated animals. However, replacement of TNF-α locally by adenoviral-mediated delivery restored the priming of this anti-melanocyte response. The importance that we attribute to the LN migration of this population of
FIGURE 6. Expression of pCD40L with inflammatory killing of melanocytes enhances the ability to cure large established disease. A, C57BL/6, or TLR-4/C57BL/10ScNJ, mice (10 per group) were seeded with B16 tumors s.c. on day 1. On days 4–6, 11–13, and 18–20 mice were injected on the right flank i.d with (Tyr-HSVtk/CMV-hsp70/CMV-LacZ) (10 µg each), (Tyr-HSVtk (10 µg) + CMV-LacZ (20 µg)), or (Tyr-HSVtk + CMV-hsp70 + pCD40L (10 µg each) plasmids. GCV was administered i.p. on days 4–8, 11–15, and 18–22 as described previously (13, 23). The percentage of long-term survivors (mice surviving for 60 days) is shown along with the day at which the last mouse in each group was euthanized. Treatment with either Tyr-HSVtk/CMV-hsp70 or Tyr-HSVtk/CMV-hsp70/pCD40L were both statistically significantly different from treatment with Tyr-HSVtk/CMV-LacZ (p < 0.0001), but there was no significant difference between treatment with Tyr-HSVtk/CMV-hsp70/CMV-LacZ and Tyr-HSVtk/CMV-hsp70/pCD40L. B, Different treatment regimens have different efficacies to treat small (3-day established) or larger (9 day established) disease in C57BL/6 mice (p < 0.005 between Tyr-HSVtk/CMV-hsp70 and Tyr-HSVtk/CMV-hsp70/pCD40L in the 9-day model of established disease). C, Splenocytes were recovered from C57BL/6 mice 9 days following the first of three daily i.d injections of Tyr-HSVtk/CMV-hsp70/pCD40L, Tyr-HSVtk/CMV-hsp70, or Tyr-LacZ/CMV-hsp70 and five daily injections of GCV. Splenocytes from each treatment group were divided into three separate cultures and stimulated with either Ova SIINFEKL (31) (ova) (control) or with the synthetic, H-2Kb-restricted peptides hgp10025–33, KVPRNQDWL (gp100), or TRP-2180–188SVYDFFVWL (30) (TRP-2) and were used to seed IFN-γ-coated ELISPOT wells at 250,000 splenocytes per well in triplicate. Seventy-two hours later, spot numbers were determined as shown. Error bars represent SDs. D, Splenocytes recovered from C57BL/6 mice 9 days following the first of three daily i.d. injections of Tyr-HSVtk/CMV-hsp70/pCD40L, Tyr-HSVtk/CMV-hsp70, or Tyr-LacZ/CMV-hsp70 and five daily injections of GCV. Splenocytes from each treatment group were divided into three separate cultures and stimulated with either Ova SIINFEKL (31) (ova) (control) or with the synthetic, H-2Kb-restricted peptide TRP-2180–188SVYDFFVWL (30) (TRP-2) at 100,000 splenocytes per well in triplicate in IFN-γ ELISPOT wells. Seventy-two hours later, supernatants were recovered and assayed for IFN-γ by ELISA. The activity of TRP-2 reactive cells in the splenocytes of mice from different treatment groups was calculated from the total amount of IFN-γ detected by ELISA divided by the mean number of IFN-γ producing cells as determined by the ELISPOT analysis. This experiment was repeated three times. (p < 0.02 for Tyr-HSVtk/CMV-hsp70/GCV, compared with Tyr-HSVtk/CMV-hsp70/pCD40L/GCV).
cells from the site of plasmid injection was corroborated by the fact that replacement of the Tyr-HSVtk plasmid by the CMV-HSVtk plasmid caused both complete loss of therapeutic efficacy and abrogation of our ability to detect any migrating cells by both PCR and flow cytometry following CTG labeling. Overall, these observations are explicable by proposing that intradermal plasmid injection of CD8+ T cells also may help differentiate presentation of self Ags, which are shared with tumor cells. As well as acting with cytokine like properties as described in this study (39–41), hsp also can enhance immunogenicity by serving as chaperones of immunogenic peptides (34–39). We have not directly addressed this mechanism in the current study; but additional chaperoning of self peptide Ags from the dying melanocytes into the MHC class II/CD11c+ APC, which travel to the LN, would significantly increase the efficiency of priming of anti-melanocyte T cell responses.

However, when we used only six plasmid injections to treat larger, 9-day established B16 tumors, typically only between 0 and 10% of mice were cured of these larger tumors. Moreover, we observed the emergence of aggressively growing tumors that had lost expression of the TRP-2 and tyrosinase Ags, while apparently retaining full expression of other tumor cell-associated Ags such as gp100(23). These data suggested that the quality and/or quantity of the T cell response induced by inflammatory melanocyte killing was inadequate to clear a larger, more established tumor burden (23).

Priming of naive CTL by APC requires a helper T cell-dependent signal provided by ligation of CD40 on the APC surface by its ligand CD40L (CD154), (“licensing” of APC) (49–52), for development of humoral immunity (53, 54) and CD4-independent CD8+ T cell responses (50–52, 55). CD4 helper cell-mediated ligation of CD40 on activated CD8+ T cells also may help differentiation of memory CD8+ effector T cells (56–58). Therefore, agonists to CD40, including Abs or CD40L, can increase long-term T cell survival, thereby enhancing anti-tumor T cell responses (55, 59, 60). There is also now impressive evidence that a combination of TLR activation with CD40 ligation can synergize to stimulate CD8+ T cell responses (61). It also has been reported that hsp70 may exert some of its biological activities through binding of CD40 (20, 47). Therefore, to test whether CD40 ligation could replace hsp70, or whether we could also increase the potency of the T cell response raised in vivo by hsp70-mediated inflammatory killing of normal melanocytes, we incorporated a plasmid expressing CD40L into the i.d. plasmid injections. Our data indicate that pCD40L could neither replace, nor inhibit, the activity of hsp70 in either wild-type or TLR4−/− mice. However, addition of pCD40L to the Tyr-HSVtk/CMV-hsp70/GCV regimen converted a cure rate of 0–10% into >80% over several experiments in which 9-day established tumors were treated by plasmid injection.
Surprisingly, the results of Fig. 7B show that only addition of the plasmid form of CD40L, rather than the anti-CD40 Ab, conferred memory to mice cured of 3-day disease by the Tyr-HSVtk/CMV-hsp70 treatment, which pCD40L alone was unable to achieve (Group D). We hypothesize that both temporal and spatial location of CD40 ligation on the APC being loaded with melanocyte-derived Ags, which subsequently migrate to the LN, may be critical in mediating the effects of pCD40L. Thus, it may be that the anti-CD40 Ab cannot access the local site of melanocyte killing, where the CD40L is expressed from the injected plasmids. Ligation of CD40 on the APC at that site may be important before these APC reach the LN where, presumably, the anti-CD40 Ab is readily accessible.

Moreover, inclusion of pCD40L increased the severity of autoimmune manifestations in treated mice, which were only observed following treatment with Tyr-HSVtk/CMV-hsp70 alone following prior depletion of CD4+ CD25+ T reg cells (13). We observed alopecia-like autoimmune hair loss, along with occasionally localized intense vitiligo concentrated around the eyes, the top of the head and shoulders, but with no obvious ocular involvement. Such symptoms are reminiscent of Vogt-Koyanagi-Harada disease, considered to be an autoimmune disease directed against melanocytes and specifically the TRP-1 and 2 Ags (62, 63). In addition, mice cured of large established tumors by Tyr-HSVtk/CMV-hsp70/pCD40L/GCV treatment developed almost complete and long-term immunological memory against tumor rechallenge. Once again, such memory was never observed in mice cured of smaller tumors by the Tyr-HSVtk/CMV-hsp70/GCV regimen (13).

These data increased T cell-mediated therapy, autoimmunity, and immunological memory, are all consistent with the predicted actions of CD40L on activating APC (49–52) and on differentiation of memory CD8+ effector T cells (56–58). Thus, we observed that, although pCD40L did not increase the numbers of MHC-II+/CD11c+ APC that migrated to the LN under the influence of hsp70 expression, these APC primed more naive T cells against the melanocyte-expressed ova Ag in vitro than did APC recovered from LN following Tyr-HSVtk/CMV-hsp70/GCV treatment. In addition, inclusion of pCD40L not only induced both greater numbers of TRP-2-specific splenocytes than Tyr-HSVtk/CMV-hsp70/GCV treatment, but those splenocytes also secreted greater amounts of IFN-γ per cell. These results suggest that CD40L binding to CD40 expressed on the APC, which migrate to the LN, licenses these APC to activate T cells both more potently and in higher numbers. Nonetheless, the increases in numbers of effector T cells were rather modest suggesting that CD40L expression may have additional effects as well as enhancing the priming of Ag-specific T cells in the LN. Consistent with our demonstration that the activity of the activated T cells was increased in CD40L-treated animals, we have recently observed that CD40L may be exerting potent immunostimulatory effects through modulation of regulatory T cell activity in vivo through mechanisms that are currently under investigation in our laboratory (L. Sanchez-Perez, manuscript in preparation).

In summary, we show in this study that CD40L-enhanced, hsp70-mediated inflammatory melanocyte killing can be used to cure large established tumors and to confer immunological memory against tumor cells, although a concomitant increase in autoimmune sequelae also is produced.

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Disclosures
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