Peritumoral administration of immunomodulatory antibodies as a triple combination suppresses skin tumor growth without systemic toxicity

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ABSTRACT

Background Skin cancers, particularly keratinocyte cancers, are the most commonly diagnosed tumors. Although surgery is often effective in early-stage disease, skin tumors are not always easily accessible, can reoccur and have the ability to metastasize. More recently, immunotherapies, including intravenously administered checkpoint inhibitors, have been shown to control some skin cancers, but with off-target toxicities when used in combination. Our study investigated whether peritumoral administration of an antibody combination targeting PD-1, 4-1BB (CD137) and VISTA might control skin tumors and lead to circulating antitumor immunity without off-target toxicity.

Methods The efficacy of combination immunotherapy administered peritumorally or intravenously was tested using transplantable tumor models injected into mouse ears (primary tumors) or subcutaneously in flank skin (secondary tumors). Changes to the tumor microenvironment were tracked using flow cytometry while tumor-specific, CD8 T cells were identified through enzyme-linked immunospot (ELISPOT) assays. Off-target toxicity of the combination immunotherapy was assessed via serum alanine aminotransferase ELISA and histological analysis of liver sections.

Results The data showed that local administration of antibody therapy eliminated syngeneic murine tumors transplanted in the ear skin at a lower dose than required intravenously, and without measured hepatic toxicity. Tumor elimination was dependent on CD8 T cells and was associated with an increased percentage of CD8 T cells expressing granzyme B, KLRG1 and Eomes, and a decreased population of CD4 T cells including CD4 “FoxP3+” cells in the treated tumor microenvironment. Importantly, untreated, distal tumors regressed following antibody treatment of a primary tumor, and immune memory prevented growth of subcutaneous flank tumors administered 50 days after regression of a primary tumor.

Conclusions Together, these data suggest that peritumoral immunotherapy for skin tumors offers advantages over conventional intravenous delivery, allowing antibody dose sparing, improved safety and inducing long-term systemic memory. Future clinical trials of immunotherapy for primary skin cancer should focus on peritumoral delivery of combinations of immune checkpoint antibodies.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Intravenously administered antibodies against checkpoint molecules such as PD-1 and CTLA-4 are being used in the clinic to treat skin cancers. However, a subset of tumors can be resistant to this therapy and off target toxicities can be problematic particularly when combinations of antibodies are used. There is a clear clinical need for optimizing new combinations of checkpoint antibodies with improved efficacy that can be delivered in a safe manner for the treatment of skin cancer.

WHAT THIS STUDY ADDS

⇒ We have identified a triple combination of antibodies targeting inhibitory and stimulatory checkpoint molecules that can successfully eliminate three different cutaneous tumors after peritumoral administration. Local administration enabled the use of lower antibody doses, prevented off target toxicities in the liver and was associated with a systemic, memory immune response which protected against tumor challenge at distant skin sites.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Peritumoral administration of novel checkpoint antibody combinations could prove to be a potent therapeutic option in the future as standard clinical practice in the treatment of primary skin cancers.

BACKGROUND

Cutaneous cancers such as squamous cell carcinoma (SCC) and melanoma represent a spectrum of disease from localized in situ carcinoma of the skin through to widely dispersed, metastatic tumors. The global incidence of these cancers is increasing with non-melanoma skin cancer (NMSC) being the most common malignancy in individuals with Fitzpatrick skin types I–III. While treatment of early-stage cancer with surgery is often effective, the 5-year recurrence rates for...
cutaneous SCCs are 4%-8% and highly susceptible patients often have multiple lesions, some of which may not be easily accessed. Other treatment options such as topical chemotherapeutics (imiquimod) and radiotherapy have varying effectiveness and undesirable side effects and may not confer lasting protection. A role for the immune system in controlling skin tumors is implied by the higher incidence of NMSC in immunocompromised individuals, particularly organ transplant recipients where the incidence of cutaneous SCC is 63–250 times higher than the general population. These cancers also have high mutational burdens (induced by UV light) suggesting the possibility of unique cancer antigens which can be targeted by the adaptive immune system. Recently, tools for manipulating the immune response have expanded with the identification of surface checkpoint molecules which signal to inhibit (eg, PD-1) or costimulate (eg, 4-1BB) immune cells. Food and drug administration approvals for the use of checkpoint inhibitor antibodies, which block PD-1 and CTLA-4 signaling, in cutaneous cancers have ushered in a new era of therapy, pivoting the focus of treatment from targeting tumors or cancer cells, to harnessing the immune system, as means to eradicate tumors. While these antibodies have shown promising efficacy in skin cancer, treatment resistance to monotherapies can occur and treatment is generally applied to advanced or metastatic cancer due to initial failure of “standard-of-care” treatments or concerns related to systemic toxicities when using antibodies against molecules such as CTLA-4 or 4-1BB. Autoimmune activation is particularly problematic when combinations of antibodies change the signaling in multiple non-redundant signaling pathways. Intravenous delivery of antibodies against both 4-1BB and PD-1 exacerbated the liver toxicity seen with anti-4-1BB antibody alone in a mouse model, suggesting that safer delivery methods for combinations of checkpoint antibodies are needed.

To enable the use of combination antibody therapy targeting checkpoint molecules in skin cancer, our study has investigated the peritumoral injection of a novel combination of antibodies that block the inhibitory signals from PD-1 and VISTA while simultaneously promoting activation signals through 4-1BB. These targets were chosen on the basis of (1) their expression within human, cutaneous SCC (2) a role for VISTA in resistance to PD-1 therapy and (3) their non-redundant signaling pathways in immune cell activation. Overall, our data demonstrate that peritumoral triple therapy is highly effective in regresssing different types of cutaneous tumors with the establishment of systemic immune memory and protection against distant, simultaneous tumors. Importantly, therapy can be achieved at lower antibody doses than required intravenously and with no evidence of systemic toxicity. Together, this suggests a new paradigm for the clinical treatment of early stage, primary skin cancer with multiple antibodies against immune checkpoint molecules.

**METHODS**

**Mice**

C57BL/6 or RAG1KO (B6.129S7-Rag1tm1.Mom/J) adult (6–8 weeks old) mice were sourced from the Animal Resource Centre (Perth, Australia) or the University of Queensland Biological Research Facility (UQBRF) at the Translational Research Institute (Brisbane, Australia). All mice were bred and housed under specific pathogen free (SPF) conditions at the UQBRF. Mice were regularly monitored for signs of distress and any adverse events were reported immediately to the ethics committee.

**Tumor cell lines and in vivo establishment of ear and subcutaneous tumors**

TC-1 lung epithelial, B16F10 melanoma and 8101-PRO UV-induced fibrosarcoma cell lines have been previously described. These cells were maintained for 3–4 passages in complete RPMI media containing FCS prior to injection. For establishment of tumors, TC-1 cells were trypsinized and washed to prepare a single cell suspension (>85% viability). TC-1 cells resuspended in PBS were mixed with an equal volume of Matrigel (Phenol red free, Corning, cat#356237) before injection into the ventral ear pinnae of C57BL/6 mice at 5×10⁶ cells in a 20µL total volume. For B16F10 and 8101-PRO tumors, 1×10⁶ cells were injected into the ear in Matrigel. Tumor volumes in the ear were monitored daily and measured using digital calipers. For tumors grown in back skin, 5×10⁶ tumor cells were placed in a 200µL volume of PBS and injected subcutaneously into the shaved lower back. Subcutaneous placement of the tumor was confirmed with histology. Tumor volumes were calculated using the following equation as previously described: volume (mm³)= (length×width²)/2. For histological analysis, mice were euthanized and excised, tumor injected ears (or flank tumors) and livers were placed in 4% paraformaldehyde before processing into H&E sections by the histology core facility of the Translational Research Institute (Brisbane, Australia).

**Antibodies, in vivo therapy and flow cytometry**

For tumor therapy experiments, unlabelled antibodies against PD-1 (Clone RMP1-14; Rat IgG2A)), 4-1BB (Clone 3H3; Rat IgG2A), VISTA (Clone 13F3; Armenian Hamster IgG), Rat IgG2a isotype control (Clone 2A3), Armenian hamster isotype IgG, CD8β (Clone 53-5.8; Rat IgG1) and Rat IgG1 isotype control (Clone HRPN) were all purchased from BioXcell (New Hampshire, USA). For multiparameter flow cytometry, fluorescently conjugated antibodies targeting CD45 (Clone 30-F11; BUV563), CD49b (Clone HM-62; BV786), TC-Rb (Clone H157-597; BUV737), CD4 (Clone GK1.5; BUV496), CD8a (Clone 53-6.7, BV805) and live/dead stain (BUV450/50) were all purchased from BD (New Jersey, USA). Antibodies
against FoxP3 (Clone FJK-16s; PE/Cy5), KLRG-1 (Clone 2F1; FTTC), and Eomes (Clone Dan11mag; PE/Cy7) were purchased from Thermofisher (Massachusetts, USA) and anti-granzyme B antibody (Clone QA16A02; PE/Dazzle 594) was purchased from Biologend (San Diego, USA).

For flow cytometry staining of cells from ear tumors, ears were excised from euthanized mice and ventral/ dorsal surfaces were separated mechanically, minced and then placed in 100 μg/mL Liberase (Roche, Basel, Switzerland) and 50 μg/mL DNase I (Sigma, St. Louis, USA) in DMEM solution for 40 mins at 37°C. Finally, the cell suspension was passed through a 70μm cell strainer and incubated for 20min with the live/dead stain (BD) and 1μL of TruStain FcX (Biolegend) to block Fc binding. Cells were then incubated with antibodies targeting surface molecules for 20 mins in 100 μL of PBS before being washed and analyzed in the flow cytometer. For intracellular FoxP3 staining, cells were fixed and permeabilized using the FoxP3 Transcription factor staining kit as per manufacturer’s instructions (Thermofisher) and incubated overnight with anti-FoxP3 antibody. Multicolour cell fluorescence and compensation beads (Ultra-comp beads; Thermofisher) were acquired on a BD LSR Fortessa X-20 (BD) and the data analyzed using Flowjo V.10 (BD).

For in vivo antibody therapy experiments, antibodies were injected on three separate occasions spaced 3 days apart beginning on day 3 when a small tumor mass was palpable (days 3, 6, 9 post-tumor inoculation). For ear injections, antibodies (10 μg per antibody; mixed together for combination treatment) were injected beside the tumor mass in a volume of 20 μL PBS while intravenous injection (10 μg or 100 μg per antibody) was administered in the tail vein in a total volume of 200 μL. In ascobolp experiments, tumor was injected into both ears of an individual mouse but antibody therapy was applied to the right ear only. In experiments requiring CD8 T cell depletion, 250 μg of antibody targeting CD8β (Lyt 3.2; clone 53-5.8, BioXcell) was administered intraperitoneally 3 days prior to TC-1 tumor inoculation and again 4 days after tumor inoculation. CD8 T cell depletion in the blood (>95% depletion at the time of tumor inoculation) was confirmed by flow cytometry.

**ELISPOT assay**

Harvested spleens were processed into single cell suspensions. Millicell Multiscreen-HA 96-well plates precoated with 4 μg/mL anti-IFN-γ mAb (eBioscience #14-7313-85), were washed and blocked with sterile RPMI 1640 full media (RPMI 1640/penicillin/streptomycin/glutamine/sodium pyruvate+50μM 2-mercaptoethanol+10% fetal bovine serum), for 2 hours. Blocking solution was then removed and 2.5×10^7 splenocytes were added to triplicate wells for peptide and no peptide control conditions, in a volume of 50 μL. A 50 μL of RPMI full media was added to no peptide controls. A 50 μL of the immunodominant, HPV16E7 peptide (Auspex; RAHYNIVTF), was added to peptide stimulated wells at a concentration of 20 μg/mL. PMA/ionomycin (50 ng/mL and 2 μg/mL, respectively) served as an assay positive control and was added in triplicate at 50 μL/well. Cells were incubated for 24 hours at 37°C, 5% CO₂. The contents of the plate were then aspirated and washed 3X with PBS/Tween (PBS-T). Biotinylated IFN-γ detection antibody (eBioscience #13-7312-85) was added to the plates at a concentration of 1 μg/mL in 2% PBS-FBS-PBS-T. The plate was incubated for 4 hours at room temperature and later washed 3X with 2% PBS-PBS-T. Avidin-HRP (Sigma cat # A-3151, 1 mg/mL stock) was applied to the plate at a concentration of 2.5 μg/mL and incubated in the dark for 60 min at room temperature. The plate was washed 3X with PBS-T and 3X with PBS before adding DAB substrate (Sigma cat # DO426). Plates were developed for approximately 90–120s. Color development was stopped by removing the plate backing and washing thoroughly in water. The plate was then allowed to dry prior to being read on a ELISPOT counter.

**Serum alanine transaminase assay**

Changes in serum alanine transaminase (ALT) levels were assessed by ALT protein ELISA (Abcam, ab282882), according to manufacturer’s instructions. Mean baseline mouse serum ALT levels were defined as 67.60 ng/mL.

**Study design and statistical analysis**

Sample sizes for tumor experiments (n=4-13) were chosen to minimize mouse usage while providing sufficient statistical power given the large effect sizes observed with treatment. No mice were excluded from the data analysis, the studies were not blinded and mice were randomized to study groups prior to tumor injection. Confounders such as order of treatments and measurements were not controlled. All statistical analyses were performed using Graphpad prism and summarized data are presented as mean±SD. Details of specific statistical tests are included in the figure legends. In experiments to compare the effect of monotherapy and combination therapies on mouse survival, a Kaplan-Meier survivability curve was generated. A Mantel-Cox test was applied to determine which treatments imparted significant survivability to test subjects compared with isotype control. P values less than 0.05 were considered significant and were numerically annotated in figures. Where asterisks were used, p<0.05 (*), p<0.01 (**), p<0.001 (***) and p<0.0001 (****).

**RESULTS**

**Peritumoral injection of a combination of 4-1BB agonist and PD-1/VISTA checkpoint inhibitors induces superior antitumor responses when compared with monotherapy**

To evaluate the efficacy of antibodies specific for 4-1BB, PD-1 and VISTA for clearance of a syngeneic tumor (TC-1) expressing a defined tumor antigen (HPV-16 E7 protein), each antibody was injected peritumorally around a small TC-1 tumor grown under the skin of the ear. Tumors in mice recipient of isotype control
the mice recipient of VISTA antibody whose ear tumors had regressed, two developed secondary tumors in tumor-draining lymphatics within the neck (figure 1A). We next investigated the efficacy of peritumoral administration of a combination of antibodies specific for 4-1BB, PD-1 and VISTA. Of TC-1 tumor-bearing mice treated with this combination, all (8/8) became tumor free and remained so for a period of at least 60 days, whereas treatment with a combination of isotype control antibodies had no impact on tumor growth (figure 1B). Differences between anti-4-1BB monotherapy and triple antibody combination did not reach statistical significance. However, survival data suggested that combination treatment was superior to monotherapy (figure 1C). The combination of three antibodies was used in subsequent experiments.

**CD8 T cells are required for the antitumor efficacy of triple antibody treatment**

CD8 T cells play a central role in the clearance of subcutaneous TC-1 tumors.\(^2\) To determine whether T and B cells played a part in clearing TC-1 tumor in our model, combination antibody therapy was administered to TC-1 ear tumor bearing immunocompetent C57BL/6 mice, and to T and B cell-deficient, RAG1\(^{-/-}\) (RAG1KO) mice. As expected, in control immune competent mice, tumors grew progressively until ethical limits after isotype antibody treatment, whereas tumor was cleared after triple antibody treatment (figure 2A). In contrast, tumors in RAG1KO mice treated with triple antibody therapy demonstrated delayed growth, relative to isotype control treated animals, but continued to grow (figure 2A). To establish whether CD8 T cells contributed to tumor clearance, antibodies against the CD8\(\beta\) chain were used to deplete CD8 T cells from immunocompetent mice. This resulted in a >95\% reduction in blood CD8 T cells (data not shown). CD8 T cell depleted animals given triple antibody therapy failed to clear TC-1 tumors (figure 2B), showing that tumor clearance in the ear induced by combination antibody therapy is CD8-dependent. In addition, we have shown that tumor antigen-specific, CD8 T cells are generated by day 12 after treatment of TC-1 tumors as evidenced by IFN-\(\gamma\) producing T cells in an ELISPOT assay after HPV7 peptide stimulation (online supplemental figure 1).

**Combination antibody therapy delivered peritumorally is efficacious at lower doses than intravenous delivery and results in an improved toxicity profile**

Immunomodulatory checkpoint inhibitor antibodies (eg, anti-PD-1 antibody) are generally delivered intravenously. We, therefore, compared the efficacy of triple antibody (10\(\mu\g per antibody) given peritumorally or intravenously. Triple antibody therapy given intravenously to mice with TC-1 ear tumors resulted in tumor regression in 2/10 mice (figure 3A), and a further 2/10 mice showed delay in tumor growth, suggesting that intravenous delivery of antibody was, at the same total dose as peritumoral administration (10\(\mu\g/antibody), less effective at promoting
TC1 tumor clearance. To confirm whether this difference was a consequence of triple antibody dose, the triple combination was administered at 10 µg per antibody peritumorally, or 100 µg per antibody intravenously, to mice bearing TC1 tumors (figure 3B). The higher intravenous dose proved as effective at eliminating tumor as the lower dose delivered peritumorally (figure 3B). However, the larger intravenous dose was associated with significant adverse effects including raised serum ALT, inflammatory liver infiltrates (figure 3C), kidney damage and inflammation of the small intestine (data not shown; as detailed in necropsy report) that was not seen with the lower peritumoral dose. Together, these data suggest that while a higher dose of intravenous antibodies could limit TC1 tumor growth to a similar extent as a 10-fold lower dose of peritumoral antibodies, this was associated with severe off-target toxicity.

Local combination therapy drives tumor regression at a distant site and augments systemic immune memory

Patients with keratinocyte cancers often present with multiple simultaneous cancers at different skin sites or with recurrence of cancer. To investigate whether a skin cancer at a second site could be impacted by treatment of the cancer at the primary site, and whether immune memory could be developed after treatment of a primary cancer, TC1 tumors were inoculated into both ears of mice with combination antibody treatment limited to one tumor site (right ear) (figure 4A) This resulted in tumor clearance in both ears in 5/6 mice over two experiments (one further mouse developed a neck tumor). In one mouse that showed initial regression of tumor in both ears, the tumor subsequently returned in both ears, and the recurrent tumors regressed with further combination antibody treatment, suggesting that the tumor had not become treatment resistant (figure 4A, right panel). Eventually, the left ear tumor began to grow again in this mouse despite two rounds of antibody treatment. Using fluorescently labeled antibody injected peritumorally and IVIS imaging, antibody was seen to distribute to the draining lymph nodes and spleen, although at lower levels, after 24 hours suggesting some systemic leakage of the injected antibody from the tumor site (online supplemental figure 2). This distribution is consistent with published literature using IVIS imaging of intratumoral antibody injection in the dorsal flank.

To investigate whether successful triple therapy treatment of tumors generated immune memory, we rechallenged mice that had successfully cleared tumors in a single ear after treatment, and tumor naïve mice, with TC1 tumor placed subcutaneously in the back skin. Inoculations of TC1 tumor cells in tumor naïve mice grew progressively as expected (figure 4B, left panel) whereas inoculations in previously treated mice failed to grow, suggesting a circulating, memory immune response (figure 4B, left panel) whereas inoculations in previously treated mice failed to grow, suggesting a circulating, memory immune response (figure 4B, left panel) whereas inoculations in previously treated mice failed to grow, suggesting a circulating, memory immune response (figure 4B, left panel). Eventually, the left ear tumor began to grow again in this mouse despite two rounds of antibody treatment. Using fluorescently labeled antibody injected peritumorally and IVIS imaging, antibody was seen to distribute to the draining lymph nodes and spleen, although at lower levels, after 24 hours suggesting some systemic leakage of the injected antibody from the tumor site (online supplemental figure 2). This distribution is consistent with published literature using IVIS imaging of intratumoral antibody injection in the dorsal flank.

The immediate immunotherapeutic tumor response generated by combination treatment is associated with increased granzyme B+ KLRG1+ Eomes+ CD8 T cells and reduced CD4 T cells (including CD4+ FoxP3+ cells) at the tumor site

Having demonstrated a pivotal role for the immune system in the success of our treatment, we characterized the immune response within the tumor site by analyzing
CD4 and CD8 T cell immune populations within the tumor at day 12 post-tumor inoculation (figure 5A), a time point at which tumor was being eliminated by combination antibody treatment. The fraction of CD8 T cells among the total T cell population within the tumor was increased with combination antibody treatment, relative to isotype control treatment (figure 5B), and the proportion of CD4 T cells including FoxP3$^{+}$ T cells (figure 5B,C) was decreased. These results are consistent with a role for CD8 T cells in clearing tumor, while also suggesting that...
induced reduction in CD4+ FoxP3+ T cells can contribute to treatment efficacy. Among the CD8 population, combination treatment was associated with an increase in granzyme B expressing T cells, consistent with increased cytotoxic potential (figure 5D). Granzyme B-expressing CD8 T cells also included more Eomes+KLRG1+ cells in the combination treated tumors relative to isotype treated tumors (figure 5E). Overall, the phenotypic profile of T cell populations within the treated tumors was consistent with increased effector CD8 T cell function.

**Peritumoral delivery of combination antibody treatment is effective in eliminating B16F10 and 8101-PRO tumor growth**

Given the success of peritumoral, combination antibody treatment against TC-1 tumors within the ear, we next tested for broader efficacy against two further, unrelated cutaneous tumor models. B16F10 (a melanoma cell line) and 8101-PRO (a UV-induced skin tumor lacking immunodominant antigen expression) were inoculated into the ear of mice to establish a small tumor that was then treated peritumorally with a combination of antibodies against PD-1, 4-1BB and VISTA. In isotype-treated controls, B16F10 tumor grew progressively in all mice (figure 6A; upper left panel). In combination antibody treated mice however, 11/13 mice were tumor-free at day 25 post-tumor inoculation (figure 6A; upper right panel) showing that the therapeutic effects of the antibody combination delivered peritumorally extended to a melanoma cell line.
Similarly, combination antibody prevented 8101-PRO tumor growth in 5/8 animals at day 25 (figure 6B), and one of three animals with continued tumor growth showed slowed growth. Consequently, we have demonstrated that local therapy with antibodies targeting PD-1, 4-1BB and VISTA are effective in eliminating a range of cutaneous tumors, suggesting that peritumoral delivery of our checkpoint antibodies may offer therapy against heterogenous skin tumors.

**DISCUSSION**

Our current study addressed two important clinical needs in skin cancer: (1) development of a safe delivery method for combination antibody treatment in primary skin tumors and (2) identification of a novel combination of checkpoint antibodies inducing potent antitumor immunity including systemic memory cells. Given the accessibility of skin cancers, we have demonstrated that local, peritumoral administration of small doses of a triple...
A combination of antibodies targeting PD-1, 4-1BB and VISTA leads to long term, cutaneous tumor regression with systemic immune memory and no observed toxicity in the liver. Antibody combinations involving the targeting of 4-1BB and PD-1 have stalled in clinical trials due to hepatotoxicity. This study provides a novel disease indication in which therapeutically relevant doses of previously toxic antibody combinations can treat disease, potentially restarting a process to getting regulatory approval for the safe use of these drugs in skin cancers.

A key feature of our combination immunotherapy was the peritumoral administration of antibodies. Therapies targeting multiple immune checkpoints are often complicated by the induction of autoimmune T cells or other immune-related adverse events particularly for systemically delivered therapy. Intravenous administration of immune checkpoint therapies is the gold standard in the clinic for many tumors but our study challenges this paradigm suggesting that local and systemic immune responses can be generated in a safe manner by peritumoral antibody administration for skin cancers. It is not clear in our studies if the antibody combination acts on tumor-infiltrated immune cells, immune cells in the draining lymph node (or beyond) or both although it is clearly possible to separate antitumor immune responses from systemic immune pathology in our system. The importance of checkpoint antibodies, such as those targeting PD-1, acting at tumor draining lymph nodes has been recently described. Our data also show that peritumoral injection allows for lower doses of therapy to achieve similar tumor efficacy to higher doses of intravenous antibody. It is likely that intravenous delivery at low antibody dose leads to antibody binding to cells within the bloodstream or other organs such that insufficient therapy reaches the cutaneous tumor site. While there are many literature studies of intratumoral/peritumoral delivery of immunotherapeutic reagents to tumors, the delivery of multiple checkpoint antibodies via this route is an emerging area of interest.

Intratumoral delivery of antibodies against CTLA-4 resulted in delayed tumor growth and protection against distant tumors in an MC-38 subcutaneous tumor model with limited systemic side effects. While tumor growth was significantly delayed in this model, it suggested that addition of other immune stimulators might enhance the effect of CTLA-4 monotherapy. In a subcutaneous model using MB49 bladder carcinoma, local administration of anti-PD-1 and CTLA-4 antibodies reduced tumor growth more effectively than either antibody alone. Our immunotherapeutic strategy resulted in clearance of B16F10 tumors known to be immunologically “cold” due to reduced MHC-I expression. Furthermore, our therapy produced delayed growth of 8101-PRO tumor, a cutaneous tumor cell line derived after chronic UV exposure of C57Bl/6 mice and lacking an immunodominant antigen. To our
knowledge, this is the first report of an immunotherapeutic immune response to 8101-PRO tumor.

Consistent with previous tumor studies using the TC-1 model, CD8+ cells were necessary for tumor clearance.33 34 Tumor therapy was associated with high Granzyme B levels in CD8 T cells, Eomes+KLRG1+CD8 T cells and a high CD8/FoxP3 ratio was observed. All of these properties have been associated with either anti-4-1BB antibody therapy in melanoma or tumor survival in the literature.35 36 In addition, tumor antigen-specific CD8 T cells were detectable at day 12 in the TC-1 tumor system consistent with the role for CD8+ cells in tumor therapy. Importantly, local delivery also led to long lasting, systemic antitumor immune responses evidenced in our study by tumor antigen-specific CD8 T cells in the spleen at day 150 after peritumoral treatment and protection against tumor rechallenge at distant sites. In our experiments with concurrent ear tumors, only one of which is treated, it is difficult to determine if therapeutic effects in the untreated tumor was due to antibody leakage or a mobile immune response that had spread from the treated ear. Certainly, we have shown that peritumoral antibody can become systemic (online supplemental figure 2), although at lower concentrations, but whether it accumulates at a sufficient dose to effect therapy in the untreated ear is unknown. One future approach to address the effects of antibody leakage would be to use two different tumors (with no common tumor antigens but susceptible to our combination therapy) in each ear, only one of which is treated with antibody. This is an important issue in cutaneous SCC where susceptible individuals frequently have multiple, simultaneous tumors or have reoccurrence of the tumor over time. With respect to tumor reoccurrence, our studies suggest that local delivery can provide protection against distant, homologous tumors at a later time although again we do not address the significant issue of tumor heterogeneity and whether induced immune responses against one tumor are capable of protecting against a genetically distinct, untreated tumor at a distant point in time. In the future, it will be important to determine if combination antibody treatment elicits CD8 T cell immunity against shared tumor antigens to protect against heterologous tumors.

While antibodies against PD-1 are currently approved for clinical use in metastatic and locally advanced cutaneous SCC,37 combination with antibodies against 4-1BB and VISTA is less well studied. Both PD-1 and VISTA act predominantly as inhibitory molecules for T cell activation in a non-redundant fashion.39 40 Blocking VISTA interactions alone in tumors using inhibitory antibodies has led to delays in tumor growth, while combination with anti-PD-1/CTLA-4 antibodies improves immunotherapeutic outcomes.30 40 VISTA can contribute to resistance to PD-1 checkpoint immunotherapy and plays a role in skin inflammation.41 42 In our study, both PD-1 and VISTA targeted antibodies as monotherapy cleared tumors in a limited number of individual mice but the underlying cause of this variability in tumor response was not determined and may relate to small differences in tumor size at the time of first therapy. Anti-PD-1 antibody therapy also led to delays in tumor growth consistent with previous studies using subcutaneous TC-1 tumor and intraperitoneal delivery of antibody.43 44 As a monotherapy, the dominant antitumor effect seen in the TC-1 ear tumor model was with agonist antibody targeting 4-1BB. While antibodies against 4-1BB are known to costimulate effector T cells, the efficacy of this monotherapy may also have been due to regulatory T cell depletion within the tumor, as evidenced in our work by decreased FoxP3 cells after treatment, or anti-4-1BB antibody effects on other immune cells such as DC, macrophages or NK cells.45-47 Studies targeting 4-1BB alone in keratinocyte cancers are limited but successful immunotherapy was been achieved in melanoma.48 One study using subcutaneous TC-1 tumors demonstrated a delayed tumor growth in some mice with 4-1BB monotherapy (intraperitoneal delivery) with significant impact on tumor growth when combined with a vaccine.49 A combination of antibodies against 4-1BB and PD-1 delivered intraperitoneally was also shown to be effective in treating subcutaneous B16F10 and MC38 tumors although hepatotoxicity was observed.50 Consequently, the success of our 4-1BB monotherapy may be related to the tumor site (cutaneous in the ear), tumor size at treatment or high local concentrations of antibody after peritumoral injection. Together, the combination of anti-PD-1, anti-4-1BB and VISTA antibodies was superior to monotherapy in the TC-1 tumor model although we acknowledge the dominant role of antibodies targeting 4-1BB. Reducing the concentration of anti-4-1BB antibody within the triple antibody combination or dissecting the effects of double antibody combinations might more clearly demonstrate the benefits of triple therapy in the TC-1 model. However, given that the triple antibody combination does not provide complete therapy in other tumors such as 8101-PRO, our future approach is to supplement the triple therapy rather than dissect the minimal requirements for therapy of TC-1 tumors alone. Using combinations of checkpoint antibodies in tumor immunotherapy may be important in overcoming tumor resistance to monotherapies into the future. In this regard, it would be interesting to combine peritumoral antibodies against 4-1BB and VISTA with the standard clinical treatment of intravenous antibody against PD-1 or PD-L1 for cutaneous cancers.

In conclusion, our study has highlighted the effectiveness and safety of peritumoral delivery of checkpoint antibodies in the therapy of skin tumors, particularly when a unique combination of antibodies targeting PD-1, 4-1BB and VISTA is used. This provides novel preclinical evidence to support a paradigm shift from intravenous to peritumoral combination antibody delivery for safe immunotherapy of primary tumors in human cSCC patients with the potential for protection against cancer recurrence at the same or distant skin sites.

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