Anti-PD-1 antibody therapy potently enhances the eradication of established tumors by gene-modified T cells

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Running Title: Anti-PD-1 therapy enhances tumor rejection by CAR T cells

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Translational Relevance

In this study, we demonstrate that blockade of the PD-1 immunosuppressive pathway using an anti-PD-1 antibody can significantly enhance the anti-tumor efficacy of genetically modified T cells expressing a chimeric antigen receptor (CAR). This combination therapy was shown to significantly inhibit tumor growth in two different mouse models leading to eradication of disease in a proportion of mice. Both of these approaches have been used singly in the clinic demonstrating good safety profiles where objective and complete responses have been reported against various cancer types. However many patients do not respond to either treatment alone. The current study shows that combining these two modalities can dramatically increase anti-tumor effects against established disease. Furthermore we show that the increased effects from combination therapy did not cause pathology in mice and that therapeutic responses strongly correlated with a decrease in MDSCs.
Abstract

Purpose: To determine the antitumor efficacy and toxicity of a novel combination approach involving adoptive T cell immunotherapy utilizing chimeric antigen receptor (CAR) T cells with an immunomodulatory reagent for blocking immunosuppression.

Experimental Design: We examined whether administration of a PD-1 blocking antibody could increase the therapeutic activity of CAR T cells against two different Her-2^+^ tumors. The use of a self-antigen mouse model enabled investigation into the efficacy, mechanism and toxicity of this combination approach.

Results: In this study we first demonstrated a significant increase in the level of PD-1 expressed on transduced anti-Her-2 CD8^+^ T cells following antigen-specific stimulation with PD-L1^+^ tumor cells and that markers of activation and proliferation were increased in anti-Her-2 T cells in the presence of anti-PD-1 antibody. In adoptive transfer studies in Her-2 transgenic recipient mice, we demonstrated a significant improvement in growth inhibition of two different Her-2^+^ tumors treated with anti-Her-2 T cells in combination with anti-PD-1 antibody. The therapeutic effects observed correlated with increased function of anti-Her-2 T cells following PD-1 blockade. Strikingly a significant decrease in the percentage of Gr1^+^ CD11b^+^ myeloid derived suppressor cells (MDSC) was observed in the tumor microenvironment of mice treated with the combination therapy. Importantly, increased anti-tumor effects were not associated with any autoimmune pathology in normal tissue expressing Her-2 antigen.

Conclusion: This study demonstrates that specifically blocking PD-1 immunosuppression can potently enhance CAR T cell therapy that has significant implications for potentially improving therapeutic outcomes of this approach in cancer patients.
Introduction

Adoptive immunotherapy utilizing gene-modified T cells expressing antigen-specific chimeric antigen receptors (CARs) is a promising approach for the treatment of cancer [1-3]. Recent clinical trials using CARs that target cell surface tumor associated antigens such as CD20 for the treatment of non-Hodgkin lymphoma [4], GD2 in neuroblastoma [5] and CD19 in chronic lymphocytic leukemia [6, 7] have all displayed encouraging results to date resulting in objective anti-tumor responses in a proportion of patients. These trials clearly illustrate the broad range of tumors that can be targeted by CAR T cells in a HLA-independent manner [8].

Despite encouraging results in preclinical models and in patients, the existence of a number of different immunosuppressive pathways can restrict the full potential of adoptive T cell therapy. This includes increased expression of inhibitory immune receptors such as T cell membrane protein-3 (TIM-3), cytotoxic T lymphocyte associated antigen 4 (CTLA-4) and/or programmed death-1 (PD-1) on T cells following T cell activation, which can limit the duration and strength of the adaptive immune response [9]. However, the recent development of checkpoint inhibitors such as ipilimumab targeting CTLA-4 [10], provide diverse opportunities to enhance anti-tumor immunity with the potential to produce durable clinical responses [9].

The PD-1 pathway has emerged as another promising target for cancer therapy. PD-1 binds to two known ligands; PD-L1, the predominant mediator of immunosuppression which is upregulated on many different tumor types, and PD-L2 which has restricted expression on macrophages and dendritic cells [11]. Engagement of the PD-1/PD-L1 pathway results in the phosphorylation of tyrosine-based motifs in the cytoplasmic tail
of the PD-1 inhibitory receptor, which promotes the recruitment of SHP2 phosphatase leading to dephosphorylation of PI3K. As a result, inhibition of PI3K effects downstream activation of Akt kinase reducing T cell activation, proliferation and survival [12, 13]. Recent trials utilizing a fully human IgG4 PD-1 monoclonal antibody (mAb) (BMS-936558) have reported durable clinical responses in patients with advanced melanoma, non-small-cell lung, renal cell, as well as hematologic malignancies [11, 13-15]. Furthermore, a significant correlation between level of PD-L1 expression on tumor cells and objective responses was reported [14]. Another clinical trial utilized a human IgG4 anti-PD-L1 mAb which demonstrated promising efficacy against a range of cancers with objective responses reported in a proportion of patients [16]. Given the early success of these checkpoint inhibitors in enhancing T cell immunity, it opens the possibility that these reagents could be utilized to enhance adoptive T cell strategies.

Hence, in this study we hypothesized that CAR T cell therapy in combination with PD-1 blockade may overcome PD-L1⁺ tumor immunosuppression, thereby leading to improved therapeutic efficacy. Herein, we utilized self-antigen Her2 transgenic mice and employed two different Her-2⁺ tumor targets to examine both anti-tumor efficacy and safety of CAR T cells and anti-PD-1 antibody in a combined immunotherapeutic approach.
Materials and Methods

Cell culture, antibodies and mice

The C57BL/6 mouse 24JK sarcoma cell line was kindly provided by Dr. Patrick Hwu (NIH, Bethesda, MD) and maintained at 37°C in 5% CO₂ in RPMI-1640 media supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life technologies, Grand Island, NY). The B6 mouse e0771 (LMC variant) breast cancer line was kindly provided by Professor Robin Anderson (Peter MacCallum Cancer Centre, VIC, Australia) and maintained at 37°C in 10% CO₂ in DMEM media, supplemented as above with 5% FCS. These lines were retrovirally transduced with the human Her-2 antigen (24JK-Her-2/e0771-Her-2) as described previously [17]. The anti-PD-1 (clone RMP1-14, IgG, Protein G purified, endotoxin-free) and isotype (clone UB8-1B9) antibodies were derived from BioXCell and in-house, respectively. C57BL/6 human Her-2 transgenic mice and congenic Thy1.1⁺ Her-2 mice were bred at the Peter MacCallum Cancer Centre and used for experimentation at 6 to 16 weeks of age. All animal experimentation was approved in advance by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee.

Generation of gene-modified T cells

The GP+E86 packaging line expressing the chimeric antigen receptor containing the extracellular scFv-anti-Her-2 human monoclonal antibody (mAb) region fused to the transmembrane and intracellular costimulatory domain CD28 and intracellular TCR-ζ domain was generated as previously described [18, 19]. For transduction, splenocytes from C57BL/6 Her-2 or Thy1.1⁺ Her-2 mice transgenic mice were gene modified as
previously described [20]. Following transduction, anti-Her-2 T cells were cultured in RPMI-1640, supplemented with 100 U/mL of IL-2 and 2 ng/mL of IL-7, and utilized for *in vitro* and *in vivo* experiments.

**Flow cytometry**

Transduction efficiency of T cells expressing the chimeric anti-Her-2 receptor was determined by direct immunofluorescence with a myc-tag Alexa Fluor 488–conjugated immunoglobulin (Cell Signaling Technology, Beverly, MA). Background immunofluorescence was assessed using an IgG2a Alexa Fluor 488–conjugated mouse isotype immunoglobulin (Invitrogen). Phenotypic characterization of transduced cells was determined using direct staining with myc-Tag (clone 9B11; Cell Signaling), TCRβ (clone H57-597; Jomar Diagnostics), CD4 (clone RM4-5; BD Pharmingen), CD8 (clone 53-6.7; BD Pharmingen) and PD-1 (clone J43; Jomar Diagnostics) cell surface markers. Intracellular staining for IFNγ (clone XMG1.2; Jomar Diagnostics) granzyme B (clone GB11; BD Bioscience) and Ki67 (clone B56; BD Bioscience) was carried out using a Cytofix/Cytoperm, Fixation/Permeabilization Solution Kit in accordance with manufacturers' instructions (BD Bioscience). Tumors were examined for PD-L1 cell surface expression by double staining with biotinylated PD-L1 (clone MIH5; Jomar Diagnostics) and Streptavidin-APC (Jomar Diagnostics). For T cell function and localization studies, tumors and blood were removed from mice at day 2 and 8 post-treatment (n=6-8 per group) and processed as previously described [21]. Cells were stained with a cocktail of antibodies for identifying presence of various immune cell populations (TCRβ, Thy1.1, CD4, CD25, FR4, CD11b, Gr-1, F4/80) and intracellular IFNγ. Gr1+ cells were examined for PD-L1 and PD-1 cell surface expression by double staining with biotinylated PD-L1 (clone
MIH5; Jomar Diagnostics) and Streptavidin-Pe-Cy7 (Jomar Diagnostics) or PE-conjugated PD-1 (clone J43; Jomar Diagnostics). Samples were incubated for 25 min at 4°C and analyzed by flow cytometry (LSR-II, BD Biosciences, North Ryde, NSW, Australia).

**Adoptive cell therapy experiments**

C57BL/6 human Her-2 transgenic mice (n = 5-8) were injected subcutaneously with $1 \times 10^6$ 24JK-Her-2 cells or orthotopically with $5 \times 10^5$ e0771-Her-2 breast carcinoma cells into the fourth mammary fat pad. Mice were then preconditioned (5 Gy) on Day 7 post-tumor injection, prior to transfer of scFv-anti-Her-2–transduced T cells or control LXSN–transduced T cells alone ($1 \times 10^7$ per dose on days 7 and 8) or T cells in combination with either anti-PD-1 or isotype antibody (250 µg/injection on days 7, 11 and 15). Control groups were left untreated. Tumor growth in mice was monitored by twice weekly measurements using electronic calipers (length × height) and the area of the tumor determined for each mouse. The mean tumor area ± SEM was recorded for each treatment group. In some experiments, recipient mice received transduced donor T cells from congenic Thy1.1+ Her-2 mice to investigate the function and localization of adoptively transferred T cells in vivo. Mice were also given twice daily intraperitoneal injections of recombinant human IL-2 (Biological Resource Branch, National Cancer Institute, Frederick, MD) involving 9 doses of 50,000 IU/200 µL given subsequent to T-cell transfer.

**Histology**

Mammary and brain tissue from non-treated and treated Her-2 transgenic mice were removed, fixed with 10% neutral buffered formalin, embedded in paraffin, and
sectioned. Slides were stained with Haematoxylin and Eosin (H & E) to evaluate any potential signs of autoimmunity caused by the therapy. Images were visualized with an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan), acquired using a RT SE Diagnostics Instruments SPOT camera (Diagnostic Instruments, Sterling Heights, MI) in conjunction with SPOT Advanced Version 4.6 (Diagnostic Instruments), and compiled with Adobe Photoshop and Illustrator CS5 (Adobe systems, San Jose, CA).

**Statistical analysis**

The difference in tumor growth and survival distributions between the treatment groups was analyzed by two-way ANOVA with p<0.05 considered significant. The difference in mean percentage ± SEM of each immune cell type in the blood and tumors between treatment groups was analyzed by two-way ANOVA and Student t-test.
Results

Expression of the anti-Her-2 chimeric receptor in Her-2 transgenic mouse T cells

Splenic T cells from Her-2 transgenic mice were activated and retrovirally transduced with the anti-Her-2 CAR. We observed reproducible levels of anti-Her-2 receptor expressed on transduced T cells compared to control LXSN vector-transduced T cells (Fig. 1A) (39.75 ± 8.23% SEM, n = 9). T cells transduced with the anti-Her-2 receptor or control vector alone were predominantly CD8+ (83.78 ± 2.50% SEM, n = 6), with only a small percentage of CD4+ T cells present in the culture (8.99 ± 14.72% SEM, n = 6) (Fig. 1B). Further phenotypic analysis revealed that the majority of CD4+ c-myc tag+ (Fig. 1C) and CD8+ c-myc tag+ (Fig. 1D) gated T cells displayed a CD62LhiCD44hi activated/memory T cell phenotype.

Increased PD-1 expression on anti-Her-2 T cells following antigen stimulation

Activation of T cells through TCR recognition of MHC/peptide leads to enhanced PD-1 expression which can reduce T cell function and ultimately lead to T cell exhaustion [13]. To determine whether stimulation of T cells through the chimeric antigen receptor similarly leads to increased expression of PD-1, we first evaluated the expression of the PD-1 ligand (PD-L1) on Her-2+ (24JK-Her-2, e0771-Her-2) and parental (24JK, e0771) tumor targets. All of these tumor lines displayed high level expression of the PD-L1 surface marker (Supplementary Fig. 1A-D). To determine whether CAR-stimulation upregulated PD-1 expression, anti-Her-2 T cells were co-cultured overnight with 24JK and 24JK-Her-2. Interestingly, we observed a significantly increased level of PD-1 receptor expression on the total transduced (Tag+) T cell population when co-cultured with Her-2+ target cells (11.86 ± 3.14% SEM) compared to stimulation with 24JK parental tumor cells (6.45 ± 0.45% SEM).
A similar finding was also demonstrated when T cells were co-cultured with e0771-Her2 tumor cells (Supplementary Fig. 2). Further analysis revealed that increased PD-1 expression was only observed on CD8$^+$ T cells when co-cultured with 24JK-Her-2 target cells ($9.23 \pm 3.77\%$ SEM), compared to stimulation with parental tumor cells ($3.40 \pm 0.58\%$ SEM) or in media alone ($5.16 \pm 0.84\%$ SEM) (Fig. 2A). In contrast, there was no significant increase in PD-1 receptor expression on transduced CD4$^+$ T cells after antigen stimulation ($2.31 \pm 0.38\%$ SEM) (Fig. 2A).

**PD-1 blockade enhances the proliferative and functional capacity of anti-Her-2 T cells in vitro**

The functional capacity of anti-Her-2 T cells co-cultured with 24JK cells expressing human Her-2 antigen (24JK-Her-2) in combination with anti-PD-1 or isotype antibody was determined by staining for intracellular markers of activation by flow cytometry. Following overnight stimulation, we found that blockade of PD-1 significantly enhanced the intracellular expression of the proliferation marker Ki-67, as well as IFN$\gamma$ and granzyme B, compared to isotype control antibody or transduced T cells co-cultured with parental 24JK tumor cells (Fig. 2B-D). These results suggest that blocking PD-1 immunosuppression can increase important functional parameters in CAR T cells following stimulation through the chimeric antigen receptor.

**Adoptive transfer of anti-Her-2 T cells in combination with PD-1 blockade enhances regression of established tumor**

We have previously shown that adoptive transfer of T cells gene-modified with the scFv-anti-Her-2 receptor could specifically mediate regression of 24JK-Her-2 experimental lung metastasis in Her-2 transgenic mice, although this therapy was less
effective against established subcutaneous disease [20]. Given that PD-1 expression was upregulated on CAR T cells following antigen stimulation \textit{in vitro} this raised the possibility that anti-PD-1 blockade may combine to enhance anti-tumor effects mediated by adoptively transferred anti-Her-2 T cells \textit{in vivo}. In this experiment, we compared the tumor growth and survival of Her-2 transgenic mice bearing day 7 established 24JK-Her-2 subcutaneous tumors (~20-30 mm$^2$) treated with anti-Her-2 or control LXSN–transduced T cells with or without anti-PD-1 antibody. Treatment of mice with anti-Her-2 T cells and anti-PD-1 antibody led to the strongest growth inhibition of 24JK-Her-2 tumors (Fig. 3A) and long term survival (~63%) of mice (Fig. 3B) that was statistically significant to mice treated with anti-Her-2 T cells alone or in combination with a control isotype antibody (Fig. 3A,B). Mice treated with anti-PD-1 alone or control T cells with anti-PD-1 had no effect on tumor growth or survival of mice (Fig. 3A,B). To demonstrate the broad utility of this combined therapy, we demonstrated that anti-Her-2 T cell and anti-PD-1 antibody treatment significantly reduced the growth of day 7 established e0771-Her-2 breast carcinoma tumors injected orthotopically into Her-2 transgenic mice compared to anti-Her-2 T cells alone or in combination with isotype control antibody (Fig. 3C). Collectively, these data demonstrate for the first time that the combination of adoptive immunotherapy using gene-modified CAR T cells with PD-1 blockade could significantly enhance anti-tumor effects against established cancer in an immune competent self-antigen setting.
Anti-tumor effects correlate with increased function of anti-Her-2 T cells but not localization at the tumor site following combination therapy

To determine whether the enhanced anti-tumor effects observed following combined CAR T cell and anti-PD-1 antibody therapy correlated with increased function and localization of anti-Her-2 T cells at the tumor site, mice were challenged with 24JK-Her-2 tumor and irradiated prior to receiving anti-Her-2 Thy1.1+ T cells with either anti-PD-1 or control isotype antibody. In this experiment and consistent with our in vitro data, we observed significantly increased expression of intracellular IFNγ in adoptively transferred Thy1.1+ T cells at day 2 following anti-PD-1 blockade compared to isotype control treated mice (Fig. 4A). However, although Thy1.1+ donor T cells were detectable in both the tumor (Fig. 4B,C) and blood (Fig. 4D) of mice that received anti-Her-2 T-cells, there was no difference in the percentage of Thy1.1+ T cells between groups of mice that received anti-PD-1 compared to isotype control antibody (Fig. 4B-D). Given that there were no impact of PD-1 blockade on the percentage of adoptively transferred T cells at the tumor site, our data raised the possibility that anti-PD-1 therapy may also be enhancing CAR T cell anti-tumor responses through an indirect mechanism.

Anti-tumor effects correlate with a decrease in myeloid derived suppressor cells at the tumor site following combination therapy

Given that PD-1 is known to be expressed on immunosuppressive cells including T regulatory cells (Tregs) [22] and myeloid derived suppressor cells [23, 24], we next examined the effect of combined anti-Her-2 CAR T cell and anti-PD-1 therapy on these different cell populations. In this experiment, tumor and blood were taken at day 1 and 8 post-treatment from control mice and mice that received anti-Her-2 T cell
with or without anti-PD-1 antibody and analyzed by flow cytometry. Although no significant difference in the percentage of CD4+ T cells, Tregs or Gr-1+ cells was observed at the tumor site at day 1 post-therapy between treatment groups (Fig. 5A), we strikingly found a significant reduction in percentage of CD11b+ Gr-1+ cells (MDSC’s) in tumors of mice treated with CAR T cells and anti-PD-1 antibody, compared to non-treated controls at day 8 post-therapy (Fig. 5B). In contrast, there was no significant difference between mice treated with anti-Her-2 T cells and control isotype antibody and non-treated mice at this time point. Our data suggests that the addition of anti-PD-1 mAb with CAR T cells has contributed to the significant decrease in percentage of MDSC’s given the decrease was greater than that observed with CAR T cells combined with isotype control antibody. Nevertheless, our experiments do not formally distinguish the relative contribution of either CAR T cells or anti-PD-1 mAb. Interestingly, we also observed a significant decrease in percentage of CD11b+ Gr-1+ cells in the blood of mice that received combination therapy compared to non-treated mice at day 1 post-therapy (Fig. 5C) and a moderate decrease in the blood at day 8 (Fig. 5D). This data fits well with the fact that these Gr-1+ cells isolated from 24JK-Her-2 tumors were found to express both PD-1 (albeit at low levels) and PD-L1 (Supplementary Fig. 3). In contrast to the effects on MDSCs, there was no additional modulation of Treg cells at the tumor site following combination therapy at day 8 (Fig. 5B). Notably though, the percentage of total CD4 T cells and Treg cells in the tumor at day 8 post-therapy were similarly decreased in both anti-Her-2 treated mice with or without anti-PD-1 antibody compared to untreated control mice (Fig. 5B). This correlated with a concomitant increase in number of Thy1.1+ donor T cells in the tumor of these mice (Fig. 5A). Collectively, our data demonstrate a strong correlation between decreased numbers of MDSC’s and
increased therapeutic responses mediated by combined CAR T cell and anti-PD-1 antibody therapy.

**Adoptive transfer of CAR T cells with PD-1 blockade does not cause autoimmunity in mice**

The human Her-2 antigen is reportedly expressed as a self-antigen in parts of the brain (cerebellum) and mammary tissue of Her-2 transgenic mice [20, 25]. We have previously shown that adoptive transfer of anti-Her-2 T cells alone did not induce pathology to normal tissue expressing Her-2 antigen in these mice [20]. However, given the significantly increased anti-tumor effects observed in Her-2 transgenic mice following combination therapy, it was important to examine potential toxicity to Her-2⁺ normal tissue in this model. In this experiment we performed H&E staining of brain and mammary sections from Her-2 transgenic mice that were irradiated and challenged with Her-2⁺ tumor and received anti-Her-2 T cells in combination with or without anti-PD-1, as previously described. Brain and mammary tissues were then thoroughly assessed for any potential signs of T cell–mediated pathology such as inflammation caused by immune infiltration. Interestingly, all mice treated with T cells expressing the anti-Her-2 receptor either alone or in combination with anti-PD-1 antibody demonstrated no tissue damage, as shown by H&E staining of mammary or brain sections from representative mice at day 8 post-treatment (Fig. 6). The morphologic appearance of sections from these tissues was comparable with sections from non-treated mice (Fig. 6). Thus, the transfer of CAR T cells with PD-1 blockade did not induce any significant autoimmunity after early treatment indicating this combination therapy to be both effective and safe.
Discussion

Adoptive immunotherapy utilizing gene-modified T cells has emerged as a promising approach for the treatment of a broad range of cancers [8]. However, the presence of an immunosuppressive microenvironment induced by tumors and host regulatory cells can limit the full potential of adoptive T cell immunotherapy. One such regulatory pathway includes PD-1/PD-L1 which serves to act as a negative feedback loop to switch off adaptive immunity after the initial immune response [13]. Recently, the use of checkpoint inhibitors targeting the PD-1/PD-L1 pathway has shown remarkable anti-tumor responses in patients with advanced melanoma and against other cancers with durable clinical responses reported in a proportion of cases [9, 14-16]. Given that PD-1 receptor expression is increased on activated T cells following engagement of PD-L1 ligand on target cells [26], we hypothesized that blocking this pathway may further enhance the anti-tumor activity of gene-modified T cells. Indeed, we first demonstrated that expression of the PD-1 receptor was significantly increased on CAR T cells following co-culture with PD-L1⁺ Her-2⁺ expressing tumor targets and that PD-1 blockade enhanced functional parameters of CAR T cells in vitro and in vivo. In adoptive transfer experiments, we demonstrated that combined CAR T cell therapy with anti-PD-1 antibody significantly increased the growth inhibition of two different Her-2⁺ tumors and led to enhanced survival of transgenic Her-2 recipient mice. Interestingly we found that increased anti-tumor responses mediated by this combined therapy correlated well with a significant decrease in numbers of CD11b⁺ Gr-1⁺ myeloid derived suppressor cells (MDSC’s) but not Treg cells at the tumor site compared with non-treated control mice. Importantly CAR T cell treatment combined with anti-PD-1 antibody administration was well tolerated causing no signs of autoimmunity in recipient mice. Overall, this is the first study to demonstrate that blocking a major
immunosuppressive pathway such as PD-1 can significantly enhance adoptive immunotherapy utilizing gene-modified T cells. Although the question of whether PD-L1 ligand expressed on the tumor cells was critical for the therapeutic effects observed was not determined in this study, a significant correlation between the level of PD-L1 expression on tumor cells and objective responses in patients has been previously reported [14].

We have previously shown that CAR T cell therapy alone could elicit significant anti-tumor effects on the growth of established 24JK-Her-2+ lung metastases leading to long term survival without causing any signs of pathology in vivo [20]. However in this current study, CAR T cell therapy alone was not as effective against established subcutaneous disease. Nevertheless, we were able to demonstrate in our in vivo experiments striking growth inhibition of both 24JK-Her-2+ sarcoma cells and E0771-Her-2+ breast carcinoma cells when inoculated subcutaneously following CAR T cell therapy in combination with PD-1 blockade compared to mice treated with CAR T cells and isotype control antibody. We found both in vitro and in vivo that blocking PD-1 significantly enhanced the function of CAR T cells leading to a more lethal hit against the tumor. Unlike in other studies that utilized MHC class I restricted T cells [26, 27], we found no increase in the percentage of adoptively transferred Thy1.1+ T cells in the blood or tumor site following combined therapy compared to control treated mice. Future studies employing other qualitative approaches such as luciferase transduced T cells would enable the kinetics of T cell infiltration to be better defined [27].
An interesting aspect of the current study was that the strong anti-tumor effects observed with CAR T cells combined with anti-PD-1 antibody correlated with a significant decrease in the percentage of Gr-1$^+$ CD11b$^+$ MDSC’s at the tumor site. Given that the percentage of Gr-1$^+$ CD11b$^+$ cells expressing PD-1 was found to be low and that anti-PD-1 has been reported to be a blocking antibody rather than a depleting antibody [28, 29] we believe that the increased anti-tumor effects observed utilizing anti-PD-1 in our tumor models were most likely due to an indirect effect on the MDSC population. Our results are consistent with another study demonstrating a reduction of myeloid cells in tumors following anti-PD-1 therapy [30]. Previous studies utilizing anti-PD-1 alone or in combination with anti-CTLA-4 have demonstrated a reduction in the suppressive activity of MDSC’s through inhibition of arginase I [24]. Other studies have shown that MDSC’s can suppress anti-tumor responses through production of nitric oxide synthase [31], reactive oxygen species [32] and cytokines such as TGFβ and IL-10 [33, 34]. It remains to be determined in future studies whether the increased anti-tumor effects of CAR T cell therapy in combination with PD-1 blockade was through modulation of the immunosuppressive effects of MDSC’s. In contrast to the effects on MDSC numbers, we observed no additional effect on percentage of Treg cells in the blood or tumor site following combined CAR T cell and anti-PD-1 treatment. The decreased percentage of Treg cells and concomitant increase in Thy1.1$^+$ T cells in Her-2 recipient mice was most likely due to the effects of irradiation which has been reported to decrease host immunosuppressive cell populations enabling expansion of transferred T cells [35]. Although PD-1 blockade alone has previously been reported to decrease Treg numbers [30], our findings are consistent with another report demonstrating that anti-PD-1 antibody treatment did not modulate Treg cell numbers following adoptive
immunotherapy [27]. It is important to note that these studies do not preclude an effect of PD-1 blockade on the function of Treg cells within the tumor microenvironment.

Another important aspect of our work was that the use of self-antigen Her-2 transgenic mice allowed us to assess whether CAR T cell therapy combined with anti-PD-1 caused any pathology to normal tissue expressing the Her-2 antigen. Although we have previously reported no toxicity of anti-Her-2 CAR T cells alone in this model [20], it remained possible that significant increases in therapeutic activity using anti-PD-1 could have concomitantly led to autoimmunity. In this current study, detailed immunohistochemical analysis revealed no damage to either Her-2+ mammary or brain tissue following combined therapy. This may have been due to the lower levels of Her-2 antigen expressed on normal tissue compared to 24JK-Her-2 tumor cells as we have reported previously [20] or due to insensitivity of the Her-2 transgenic mouse model for detecting such autoimmune events. Nevertheless, our data is encouraging for moving towards testing this combined approach in the clinic. A number of recent clinical trials involving adoptive transfer of CAR T cells alone has shown promising results in patients with chronic lymphoid leukaemia and neuroblastoma with reports of long term remission in some cases [5, 7]. Nevertheless, other trials using CAR T cells have reported on target toxicity following treatment [36-38]. Similarly, recent clinical trials using antibodies targeting the PD-1 receptor and its ligand PD-L1 have reported durable responses against various solid cancers [14-16]. In these trials, treatment was generally well tolerated although some drug associated adverse events and pneumonitis was reported in a small percentage of patients [14]. Given these reported toxicities in some patients it will be important in future studies to further
optimize dose and timing regimens of CAR T cells with anti-PD-1 antibody in self-antigen mouse models prior to Phase I testing.

In conclusion, our studies have demonstrated for the first time that administration of an anti-PD-1 antibody can significantly enhance the therapeutic efficacy of CAR T cell therapy. The combination of these two modalities is likely to have a significant impact on increasing the effectiveness of immunotherapy against a number of cancers that are currently resistant to first line treatment.
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References

Figure Legends

Figure 1. Expression of the anti-Her-2 chimeric receptor antigen in transduced Her-2 transgenic mouse T cells.
Splenic T cells derived from Her-2 transgenic mice were retrovirally transduced with the scFv-anti-Her-2 CAR. (A) CAR expression was detected in T cells by flow cytometry following staining with an Alexa Fluor 488–conjugated anti-tag mAb (grey) or Alexa Fluor 488– conjugated mouse isotype control antibody (black) (top panel), and compared to T cells transduced with LXSN vector alone (bottom panel). (B) T cells were phenotypically analyzed for expression of CD4 and CD8 on tag⁺ T cells (top panel) and LXSN transduced control T cells (bottom panel) and their activation and memory status by expression of CD44 and CD62L on CD4⁺tag⁺ cells (C) and CD8⁺tag⁺ cells (D). Data is representative of 6-9 experiments.

Figure 2. Upregulation of PD-1 expression on CAR T cells following antigen-specific stimulation and enhanced T cell function following PD-1 blockade.
(A) Anti-Her-2 transduced T cells were stimulated overnight with 24JK or 24JK-Her-2 tumors alone or left in media alone. The percentage PD-1 expression on viable TAG⁺ (CAR⁺) total T cells (white bars), CD8⁺ (black bars) and CD4⁺ (grey bars) T cell populations were detected by flow cytometry following direct staining with FITC-conjugated myc-Tag, APC-e780-conjugated TCRβ, Pacific blue-conjugated anti-CD4, PE-Cy7-conjugated anti-CD8 and PE-conjugated anti-PD-1 monoclonal antibodies. *p<0.05, data is representative of 3 experiments. To examine the effects of PD-1 blockade on T cell function, anti-Her-2 T cells stimulated overnight with 24JK-Her-2 in combination with anti-PD-1 or isotype control antibody, or 24JK alone were stained intracellularly for (B) Ki-67, (C) IFNγ and (D) granzyme B as determined by
flow cytometry. Results are expressed as mean ± SEM of GFP-CD8+ T cells from triplicate samples. *p<0.05, **p<0.01.

**Figure 3. Adoptive transfer of anti-Her-2 T cells in combination with PD-1 blockade enhances growth inhibition of established tumor.**

Her-2 transgenic mice (n=8 mice/group), were injected subcutaneously with 1 × 10^6 24JK-Her-2 sarcoma or orthotopically with 5 × 10^5 e0771-Her-2 breast carcinoma cells and grown to 20-30 mm^2 for 7 days. Mice were then irradiated (5 Gy) prior to the adoptive transfer of anti-Her-2 or control (LXSN) T cells alone (1 × 10^7/dose on days 0 and 1 of treatment) or in combination with anti-PD-1 or control isotype antibody at 250 μg/injection (on days 0, 3 and 7) or left untreated. Mice were treated with IL-2 (9 doses of 50,000 IU between days 0 and 4). (A) Anti-Her-2 T cells with anti-PD-1 antibody significantly enhanced growth inhibition of 24JK-Her-2 subcutaneous tumors compared to combined anti-Her-2 T cell and isotype control antibody or anti-Her-2 T cell treatment alone (***p<0.001). (B) Percentage survival of mice treated with anti-Her-2 T cells and anti-PD-1 antibody was significantly increased compared to anti-Her-2 T cells alone or in combination with isotype antibody (***p<0.001) (C) Anti-Her-2 T cell and anti-PD-1 antibody treatment significantly enhanced growth inhibition of e0771-Her-2 orthotopic tumors compared to anti-Her-2 T cell treatment alone or in combination with isotype control antibody (***p<0.001). Results are represented as the mean tumor size (mm^2) ± SEM. Data from two combined experiments is shown.
Figure 4. Anti-tumor effects correlate with increased function of anti-Her-2 T cells but not localization at the tumor site following combination therapy

The frequency of anti-Her-2 Thy1.1⁺ T cells in the tumor expressing intracellular IFNγ (A), and total (black bars, TCRβ⁺) and anti-Her-2 Thy1.1⁺ (white bars, TCRβ⁺ Thy1.1⁺) T cell numbers in the tumor (B, C) and blood (D) of irradiated 24JK-Her-2 tumor-bearing mice that were treated with or without anti-PD-1 or isotype control antibody and IL-2 was investigated by flow cytometry at day 2 and 8 post-therapy. A significant increase in the percentage of IFNγ-expressing Thy1.1⁺ donor T cells in the tumor of mice following anti-PD-1 blockade was observed compared to isotype control treated mice (*p≤0.05). A significant increase in the percentage of Thy1.1⁺ donor T cells in the tumor and blood of mice that received anti-Her-2 T-cells was also observed compared to non-treated mice. There was no significant difference in the percentage of donor T cells following administration of anti-PD-1 or isotype antibody (*p≤0.05, **p<0.01, ***p<0.001). Data shown are the mean percentage ± SEM of total viable GFP⁺ cells (non-tumor cells) from 6-8 mice analyzed.

Figure 5. Anti-tumor effects correlate with a decrease in myeloid derived suppressor cells following combined CAR T cell and anti-PD-1 therapy.

Tumors (A, B) and blood (C, D) were taken at day 1 and 8 from mice treated with anti-Her-2 T cells in combination with anti-PD-1 or isotype control antibody and non-treated mice, and examined by flow cytometry (n=6 mice/group). (A-D) Mean percentage of CD4⁺ T cells (CD4⁺ TCRβ⁺), regulatory T cells (CD4⁺ TCRβ⁺ CD25⁺ FR4⁺) and Gr-1⁺ myeloid cells (Gr-1⁺ CD11b⁺) at day 1 (A, C) and 8 (B, D). Gr1⁺ cells were significantly reduced in tumor (day 8) and blood (day 1) following combined CAR T cell and anti-PD-1 therapy compared to non-treated mice. Treg
cells were significantly reduced in CAR T cell treated mice with or without anti-PD1 compared to non-treated control mice (*p<0.05, **p<0.01 and ns; not significant between treatment groups). Data shown are the mean percentage ± SEM of viable GFP− cells (non-tumor cells) from 6 mice analyzed.

**Figure 6. Adoptive transfer of anti-Her-2 T cells combined with PD-1 blockade does not cause autoimmunity in Her-2 transgenic mice.**

Her-2 transgenic mice bearing 24JK-Her-2 subcutaneous tumors were irradiated (5 Gy) and treated with adoptively transferred anti-Her-2 T cells (1 × 10^7/dose on days 0 and 1 of treatment) or in combination with anti-PD-1 or isotype control antibody at 250 μg/injection or left untreated. Mammary (left panel) and brain (right panel) tissues were harvested and stained with H&E at day 8 post-treatment. Analysis of tissue sections revealed no structural damage or increased immune cell infiltration for mice treated with anti-Her-2 T cells and anti-PD-1 or isotype antibody in mammary or brain tissues compared to non-treated mice. Mammary and brain sections are representative of 6 mice analyzed per treatment group.
FIGURE 1

A

Anti-Her2

CD62L

CD44

CD8

Relative cell number

Anti-Her-2 receptor expression

B

Anti-Her2

CD4

CD8

LXSN

LXSN

C

CD44

CD62L

D

CD44

CD62L

Anti-Her2

LXSN

Anti-Her2

LXSN

Research. on October 16, 2017. © 2013 American Association for Cancer Research.
FIGURE 3

A. 24JK-Her2

B. 24JK-Her2

C. e0771-Her2

- Non-treated
- LXSN T cells + anti-PD-1
- Anti-PD-1
- Anti-Her-2 T cells
- Anti-Her-2 T cells + isotype
- Anti-Her-2 T cells + anti-PD-1

Days Post-Tumor Injection

Days Post-Treatment

Tumor Area (mm²)

Percent survival

Tumor Area (mm²)
FIGURE 4

A  TUMOR D2 Post-therapy

B  TUMOR D2 Post-therapy

C  TUMOR D8 Post-therapy

D  BLOOD D8 Post-therapy
FIGURE 5

A  TUMOR D1 Post-therapy

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B  TUMOR D8 Post-therapy

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C  BLOOD D1 Post-therapy

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D  BLOOD D8 Post-therapy

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* p < 0.05
** p < 0.01
ns: not significant
Anti-PD-1 antibody therapy potently enhances the eradication of established tumors by gene-modified T cells

Liza B. John, Christel Devaud, Connie M. Duong, et al.

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