Regulation of anti-tumor T cell immunity by the B7 family member B7-H4

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stories). I even cherish the moments of conflict (in hindsight at least), where our disagreements would ultimately result in my growth and emergence into an independent and critical thinker. For all the effort you've made in understanding me, I can only hope that I was a good enough student who fulfilled your expectations and maybe even provided some unique insights. Most importantly, while we may not always see eye to eye on all issues, I will always admire your steadfastness and commitment, qualities that I have benefited from and hope to emulate – it is no surprise that the loyalty of the students in your lab is a reflection of your own personal attitude towards us. Thank you for breaking the mold when it comes to being a supervisor; you've managed to incorporate the best aspects of traditional and modern conventions, no doubt through your own tribulations, and whereas many PI's would feel more comfortable being at arm's length with their lab members, you have willingly stepped outside your comfort zone to get to know all of us and it has had tremendous positive ramifications. No words are needed to describe all we've been through, and a quick glance between us speaks volumes. An unspoken bond has no doubt formed between us, and no matter where life takes me, I will always be a loyal and devoted member of the Suh lab.

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Abstract

Immune evasion is a hallmark of cancer, and immune checkpoint blockade has shown success in harnessing anti-tumor T cells to treat cancer. B7-H4, a B7 family inhibitor of T cell activity, is expressed on immune cells and is highly elevated in human tumors. B7-H4 overexpression in human cancers correlates with decreased infiltrating lymphocytes and poor prognosis, and in murine models, blockade of tumor-expressed B7-H4 rescues T cell activity and lowers tumor burden. So far, most studies have focused on the role of tumor-expressed B7-H4, implicating B7-H4 blockade as an immunotherapeutic option for B7-H4-positive tumors. Here, I hypothesized that inhibition of host B7-H4 could also augment anti-tumor immunity independent of its expression in the tumor. To test this, I investigated tumor-immune interactions using B7-H4-negative transplantable 4T1 murine mammary carcinoma cells in syngeneic hosts. Consistent with the current view, B7-H4 knock-out (KO) mice displayed augmented anti-tumor activity against 4T1 cells; however, tumors grew similarly between B7-H4 KO and wild-type (WT) hosts. I provide evidence that this may be due to the dual inhibition of both T cells and immunosuppressive myeloid cells by B7-H4 in the 4T1 model. In contrast, when a highly immunogenic 4T1 derivative (4T1-12B) was used, B7-H4 KO mice exhibited significant tumor reduction correlating with greater tumor-associated T cell responses. Moreover, B7-H4-deficiency synergized with the chemotherapeutic agent, gemcitabine, further slowing tumor growth, and in some cases, eradicating tumors and generating anti-tumor memory T cells. Collectively, these findings show that inhibition of host B7-H4 can enhance anti-tumor T cell immunity particularly against immunogenic tumors, and suggest that B7-H4 blockade may be combined with other anti-cancer therapies to treat human cancers regardless of B7-H4 tumor positivity.

Résumé

L'évasion immunitaire est une caractéristique du cancer et le blocage des checkpoints immunologiques a connu du succès en exploitant des cellules T anti-tumorales pour traiter le cancer. B7-H4, un membre de la famille B7 qui est un inhibiteur de l'activité des cellules T, s'exprime sur les cellules immunitaires et est très élevée dans les tumeurs humaines. La surexpression de B7-H4 dans les cancers humains est en corrélation avec une diminution des lymphocytes infiltrant les tumeurs et des pronostics défavorables, et dans des modèles murins, le blocage des B7-H4 exprimées dans la tumeur préserve l'activité des cellules T et réduit la charge tumorale. Jusqu'à présent, la plupart des études ont mis l'accent sur le rôle de B7-H4 exprimé dans la tumeur, voulant que le blocage de B7-H4 soit une option immunothérapeutique pour les tumeurs positives pour B7-H4. Ici, j'ai émis l'hypothèse que l'inhibition de l'hôte B7-H4 pourrait également augmenter l'immunité anti-tumorale indépendamment de son expression dans la tumeur. Pour en faire le test, j'ai étudié les interactions entre la tumeur et le système immunitaire en utilisant des cellules murines de carcinome mammaire transplantables, 4T1 (B7-H4-négatif), dans des hôtes syngéniques. Conformément aux vues actuelles, les souris knock-out (KO) B7-H4 ont affiché une augmentation de l'activité anti-tumorale contre les cellules 4T1; cependant, les tumeurs ont augmenté de la même façon chez les hôtes B7-H4 KO et les contrôles. Je fais la preuve que cela peut être dû à l'inhibition double des cellules T et des cellules myéloïdes immunosuppressives par B7-H4 dans le modèle 4T1. Au contraire, lorsqu'on a utilisé un dérivé de 4T1 hautement immunogénique (4T1-12B), les souris KO B7-H4 ont présenté une réduction tumorale significative en corrélation avec une plus importante réponse des tumeur-associée cellules T. De plus, l'absence de B7-H4 avec l'agent chimiothérapeutique, la gemcitabine, ralentie davantage la croissance tumorale et dans certains cas, éradique les tumeurs et génère des cellules mémoire T anti-tumorales. L'ensemble de ces conclusions démontre que l'inhibition de l'hôte B7-H4 peut améliorer l'immunité anti-tumorale des cellules T, en particulier contre les tumeurs immunogéniques, et propose que le blocage de B7-H4 peut être combiné avec d'autres traitements anticancéreux pour traiter les cancers humains indépendamment de l'expression de B7-H4 dans les tumeurs.

Preface

In accordance with McGill University's guidelines for thesis preparation, the candidate has chosen to present the results of her research in manuscript format. A general introduction is given in chapter I, and a final conclusion and summary is presented in chapter IV. A comprehensive review of relevant literature has been modified from a published article, and all results described in chapters II & III have been published, or will be submitted.

1. **Leung, J.**, and W. K. Suh. 2014. **The CD28-B7 Family in Anti-Tumor Immunity: Emerging Concepts in Cancer Immunotherapy.** *Immune Network* 14: 265-276.
2. **Leung, J.**, and W. K. Suh. 2013. **Host B7-H4 regulates antitumor T cell responses through inhibition of myeloid-derived suppressor cells in a 4T1 tumor transplantation model.***J Immunol* 190: 6651-6661.
3. **Leung, J.**, Stagg, J., Suh, W.K. 2015. **Host B7-H4 restricts anti-tumor T cell immunityto allow the growth of an immunogenic murine mammary carcinoma.** To be submitted.

Contribution of Authors

A version of chapter I has been published. Leung, J., and W. K. Suh. 2014. The CD28-B7 Family in Anti-Tumor Immunity: Emerging Concepts in Cancer Immunotherapy. Immune Network 14: 265-276.**Joanne Leung** compiled and conducted all the research, and wrote the manuscript. **Dr. Woong-Kyung Suh** created the figures, proofread, edited, and gave insightful comments and feedback.

Chapter II has been published. Leung, J., and W. K. Suh. 2013. Host B7-H4 regulates antitumor T cell responses through inhibition of myeloid-derived suppressor cells in a 4T1 tumor transplantation model. J Immunol 190: 6651-6661.**Joanne Leung** conducted all the research, including the design, optimization, acquisition and analysis of results. I also wrote the introduction, methods, and results of the manuscript. **Dr. Woong-Kyung Suh** generated B7-H4 KO mice, conceived the project, and gave valuableguidance regarding the planning and designing of experiments. He wrote the discussion section, proofread and edited the entire manuscript. **Marie-Claude Lavallée** and **JadeDussureault** of the IRCM animal facilities performed the intraperitoneal injections of antibodies on mice.

Chapter III is a version of a manuscript to be submitted. Leung, J., Stagg, J., Suh, W.K. 2015. Host B7-H4 restricts anti-tumor T cell immunity to allow the growth of an immunogenic murine mammary carcinoma.**Joanne Leung** conducted all the research, including the design, optimization, acquisition and analysis of results. I also wrote the manuscript. **Dr. Woong-Kyung Suh** previously generated B7-H4 KO mice, conceived the project, and gave valuable guidance regarding the planning and designing of experiments. He proofread and edited the entire manuscript. **Dr. John Stagg** provided the AH1-H2K^{d} tetramers and also gave insightful comments, suggestions, and feedback. **Marie-Claude Lavallée** from the IRCM animal facilities performed all intraperitoneal injections on mice.

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Chapter I

Introduction

1.1 Introduction

Since the discovery of T lymphocytes in the 1960s (1,2), the role of T cells in mediating tumor rejection has been extensively studied in the hopes of curing human cancers. Cancer immunotherapy, which aims to enhance adaptive immunity to reject tumors or prevent tumor recurrence, relies on the concept that patients can generate T lymphocytes capable of responding specifically to tumor-associated antigens. Given that adaptive immunity also contributes to the long-term benefits of canonical chemotherapy and radiotherapy (3), a clearer understanding of how tumor-specific T cell responses are regulated can provide meaningful insight into the development of more efficient cancer therapeutics.

1.2 Identification of murine and human cancer antigens leads to development of cancer immunotherapeutics

Initial studies in the 1940s to 1960s performed on mice first revealed that the host immune system can recognize cancer cells (1); following tumor resection, mice were conferred protection from a subsequent inoculation of the same tumor cells (4). Additionally, immunization with irradiated cancer cells afforded protection against tumor growth (1,5). To demonstrate the contribution of T cells to tumor rejection, adoptive transfer of T cells from mice capable of rejecting tumors could protect naïve irradiated mice from tumor development (1,6). While these experiments indicate the presence of strong T cell rejection antigens in tumors induced by chemical carcinogens or oncolytic viruses, similar experiments performed on mice with spontaneous tumors failed to show signs of any tumor control by the host's immune response (7). In the 1970s, further insight was gleamed when it was discovered that certain cancer cell clones (*tum-*), derived from a mutated carcinoma cell line, were rejected in syngeneic mice, despite the ability of the original parental cells to grow undeterred. Notably, mice that had rejected these *tum-* clones were protected from a secondary challenge of the non-immunogenic parental cancer cells (1,8-10). These observations point to the notion that T cell responses triggered by highly immunogenic antigens on the *tum-* variants also triggered a response against antigens found on the original parental cells, which were originally poorly immunogenic. These and other similar observations show that poorly

immunogenic tumors, such as those that are spontaneously derived, do express tumor antigens, but are incapable of stimulating strong T cell immunity on their own. This raised the possibility that spontaneously arising human cancers, then, may also express poorly immunogenic antigens which can be targeted.

Indeed, results obtained from human patients in the 1980s revealed the existence of cytotoxic T lymphocytes (CTLs) with enhanced lytic activity towards tumor cells relative to normal cells (1,11-14). CTL clones generated from melanoma patients helped to identify and characterize various tumor antigens, and in early 1990, the first human tumor-specific antigen, *MAGEA1,* was discovered to be distinctly expressed on a multitude of different tumors (15). Since then, various methods have been utilized to identify a wide panel of human cancer antigens with the aim of developing targeted therapeutics to enhance host immunity and inhibit tumor growth. Three major approaches have since been initiated, including the development of a therapeutic cancer vaccine, the adoptive transfer of anti-tumor T cells and the administration of reagents to stimulate T cell immunity. Of the three, only two methodologies have proven effective in ameliorating patient outcome, as therapeutic vaccines have so far revealed little success in improving clinical responses within the past decade (16,17). Adoptive cell transfer (ACT) of cultured tumor-infiltrating lymphocytes (TILs) from resected human metastatic melanomas was first demonstrated to elicit tumor regression in 1988, and was followed by subsequent clinical studies showing its efficacy in a significant portion of cancer patients (1,13,18). In 2002, lymphodepletion prior to adoptive transfer was discovered to enhance the anti-tumor capacity of transferred T cells, commemorating a landmark in ACT therapy with natural lymphocytes (19,20). Genetic engineering of the T cell receptor, or the insertion of chimeric antigen receptors (CARs) within a patient's T lymphocyte, further allowed researchers to bypass the obstacle of identifying immune cells with potent anti-tumor activity *in vitro* – the first successful adoptive transfer of genetically modified *MART-1*-specific T cells into metastatic melanoma patients was performed in 2006 (18,21), and in 2010, T cells bearing a chimeric receptor against CD19 showed efficacy in mediating lymphoma regression (22), the first successful clinical report to use CAR-transduced lymphocytes. Given the importance of adaptive immunity, direct stimulation of T cells, or the blockade of immune checkpoints, has also

been demonstrated to inhibit tumor growth. The efficacy of IL-2 administration in producing complete and durable cancer response was observed as early as 1985 (18), while the abrogation of the inhibitory CTLA-4 signaling pathway was recently observed in 2003 to enhance T cell function and show clinical efficacy in patients with metastatic melanoma (18,23). Consequently, the past decade has seen substantial advances in the development of antibodies capable of targeting various inhibitory receptors expressed on the surface of T cells; notably, combinatorial therapy involving the blockade of both CTLA-4 and PD-1 led to significant tumor reduction in the majority of patients administered (24). Not surprisingly, these encouraging results have prompted the identification of novel T cell inhibitory molecules belonging to the B7 family, some of which are distinctly and abundantly expressed on human cancers.

1.3The regulation of adaptive immunity & overcoming cancer immune evasion

The adaptive immune system plays a critical role in the protection of the host against pathogens and cancer cells while maintaining tolerance to self and innocuous environmental antigens. Based on the recognition of unique foreign antigens, the cellular arm of the adaptive immune response orchestrates a variety of effector functions such as cytotoxic CD8+ T cell responses which involve the lysis of virus-infected or tumor cells, or a variety of CD4+ T helper responses which include the initiation of humoral immunity and the production of pro- and anti-inflammatory cytokines. The regulation of T cell activity is largely achieved during activation, where three signals are required. First, T cell receptors must specifically engage peptide antigens presented by major histocompatibility complexes (MHCs) on antigen presenting cells (APCs); secondly, co-stimulatory CD28 receptors on T cells must bind B7-1 & B7-2 ligands expressed on APCs to prevent anergy (25,26). Once activated, CTLA-4 receptors are induced and outcompete CD28 for B7-1 and B7-2 ligands, thus representing a key checkpoint in the regulation of T cell immunity (25). Furthermore, depending on the nature of the immune response, T cells can also upregulate a broad range of co-stimulatory or co-inhibitory receptors in order to restrict T cell responses at different phases and to maintain peripheral tolerance. Lastly, T cells require inflammatory cytokines to facilitate the differentiation and expansion of T cell subsets,

whereby IL-12 and type I interferons have been shown to play a crucial role for the development and function of CD8 T cells, and whereas IL-1 was observed to be essential for antigen-specific responses in CD4 T cells (216).

In human tumors, the ability of cancer cells to evade immune destruction has been recently added to the list of cancer hallmarks, and represents a potential area of exploitation in the development of novel cancer therapeutics (27). The aberrant expression of numerous T cell co-inhibitory molecules in the tumor microenvironment has been attributed to the suppression of anti-tumor immunity and immune evasion (28). Naturally, attempts to block the interactions mediated by inhibitory B7 family ligands are currently being pursued in order to rescue and enhance T cell infiltration and effector functions, both of which have been demonstrated in a large majority of cancers to predict favorable outcome (29-32).

1.4Immune checkpoint blockade: CTLA-4 mechanism & expression

While the engagement of B7-1 and B7-2 molecules to CD28 receptors can lead to T cell activation, the binding of B7-1 and B7-2 to CTLA-4 results in the inhibition of T cell responses. Following T cell activation through the B7:CD28 signaling, CTLA-4 receptors are induced and bind to B7 molecules with higher affinity and avidity relative to CD28. By outcompeting CD28 for B7 ligands, CTLA-4 attenuates the T cell response, primarily through the inhibition of IL-2 production, IL-2 receptor expression and by blocking cell cycle progression. Similarly, CTLA-4 engagement can lead to transendocytosis of B7 molecules, once again reducing the availability of B7 ligands for CD28 and limiting the function of APCs (33). The significance of CTLA-4-mediated immune regulation is seen in mice deficient for CTLA-4, as these animals cannot survive past 4 weeks due to CD28-dependent lymphoproliferative disease (34).

Although the appearance of CTLA-4 on T cells during acute antigen exposure is transient, chronic antigen exposure, as in the case of cancer, leads to a sustained expression of CTLA-4 (35,36). This is in part believed to drive peripheral tolerance of cancer antigens, resulting in cancer immune evasion. Indeed, while T cells with specificity towards tumor-associated antigens do appear during tumor growth, they are incapable of mounting an immune attack and rejecting the tumor. This is thought to be

attributed to the highly immunosuppressive tumor environment, where high expression of tolerance-inducing factors are present, including IDO, arginase, IL-10, TGF-beta, VEGF, and even T cell co-inhibitors such as CTLA-4, PD-1 and B7-H4 (see below).

1.5Pre-clinical evidence of CTLA-4 blockade in murine tumor models

Given the role of CTLA-4 in subduing T cell responses, substantial effort has been made to investigate CTLA-4 blockade in the hopes of rescuing T cell responses during cancer growth. Treatment with anti-CTLA-4 antibodies has shown to be effective in ameliorating disease in multiple murine tumor models including fibrosarcoma, glioma, prostate cancer, T cell lymphoma, ovarian carcinoma and melanoma (37-42). Collectively, these studies demonstrated improved tumor regression and survival, with concomitant increases in T cell activity. There is also evidence to suggest that the effect of CTLA-4 inhibition is dependent on IFN-γ, as treatment with IFN-γ neutralizing antibodies abrogated the impact of CTLA-4 blockade. Of note, anti-CTLA-4 antibody therapy alone has been proven to be most effective with immunogenic tumor models, since low or non-immunogenic tumor models show no improvement upon CTLA-4 inhibition (43-47).

Combinatorial therapy has been successful at circumventing this obstacle, as administration of anti-CTLA-4 antibodies alongside cancer vaccines such as those targeting p53 (48,49), those expressing GM-CSF (46,50,51), dendritic cell vaccines (52,53), and peptide vaccines (54) create a synergistic effect, allowing weakly immunogenic tumors to be targeted by activated T cells. In particular, several notable studies examining the synergistic effects of CTLA-4 blockade and the engagement of another T cell co-stimulatory molecule, ICOS, have shown promising results (see below). In mice, engagement of ICOS by ICOSL-expressing tumor vaccines enhanced tumor rejection (55), and absence of ICOS reduced the anti-tumor T cell response mediated by CTLA-4 blockade (56).

1.6Clinical findings of CTLA-4 blockade in human patients

Two human anti-CTLA-4 antibodies have been in clinical investigations for the past decade, and have shown success as viable cancer immunotherapeutics

particularly in melanoma patients. While both antibodies have the ability to neutralize CTLA-4 and enhance T cell responses, the efficacy and response rate appears to differ dramatically. Ipilimumab (IgG1 isotype) was demonstrated to enhance the expression of activation markers on circulating lymphocytes (57) and augment antigen-specific immune responses (58) in patients with melanoma who were co-administered peptide vaccines. In addition, one study also found that ipilimumab was capable of enhancing the immune response to the NY-ESO-1 melanoma tumor-associated antigen, and observed a correlation between patients with detectable NY-ESO-1 CD8+ T cell responses and clinical benefit (59). In 2010, a seminal phase III clinical trial of ipilimumab monotherapy provided encouraging results, as previously treated metastatic melanoma patients receiving ipilimumab had increased overall survival (60). Another randomized phase III study in 2011 also observed similar results when untreated metastatic melanoma patients were administered ipilimumab in conjunction with dacarbazine. These patients had longer overall survival and enhanced survival rates compared to dacarbazine treatment alone (61). Based on these results, the U.S. FDA and European Medicines Agency approved ipilimumab for metastatic melanoma therapy in 2011 and currently, studies examining ipilimumab as a monotherapy and in conjunction with other therapies are underway, and aim to expand the use of ipilimumab beyond melanoma (62). Notably, combinatorial therapy involving ipilimumab and the blockade of another T cell co-inhibitor, PD-1, has shown exceptionally promising results, as a phase I trial showed that 53% of advanced melanoma patients had an objective response with tumor reduction of at least 80% (63). These observations have led to the initiation of phase III clinical studies, which is expected to yield encouraging data.

Studies involving tremelimumab (IgG2 isotype) have resulted in moderate success, yet have provided interesting and valuable information regarding the mechanism of CTLA-4 inhibition in humans. In one phase I clinical study, 4 out of 29 melanoma patients had responses that were maintained from 25+ to 34+ months upon tremelimumab treatment, and 5 patients experienced extended periods with no disease progression (64). While hopeful, these results were contrasted by a 2013 phase III clinical study, in which tremelimumab-treated melanoma patients had similar objective response rates to chemotherapy-treated control patients (65). The contrast in efficacy

between ipilimumab and tremelimumab has been puzzling, yet possible explanations may shed light on the mechanism of action between these two methods of CTLA-4 blockade. It is likely that these differences stem from the properties of the immunoglobulin isotype, as murine studies have demonstrated that anti-CTLA-4 antibodies expressing the IgG2a isotype (equivalent to human IgG1, ipilimumab isotype) have enhanced anti-tumor activity relative to treatment with anti-CTLA-4 antibodies expressing other isotypes (66). This was attributed to not only an increase in CD8+ T effector cells, but a rapid depletion of Tregs in the tumor and periphery, as the inhibition of CTLA-4 has also been shown to reduce Treg function. Presently, tremelimumab is being explored as an option for mesothelioma treatment, and alongside other immunotherapies including PD-L1 blockade and CD40-agonists for non-small-cell lung cancer and metastatic melanoma (62).

1.7Clinical biomarkers for CTLA-4 blockade

In light of studies in mouse models demonstrating the importance of tumor immunogenicity on the impact of CTLA-4 blockade, emerging clinical evidence also suggests that patients with ongoing immune responses prior to and during immunotherapy respond better upon CTLA-4 treatment. Patients who exhibited NY-ESO-1-specific T cell activity were better responders to ipilimumab-mediated CTLA-4 inhibition compared to patients that did not (59). Moreover, an increase in absolute lymphocyte counts, sustained ICOS expression, and upregulation of T cell activation markers have all been observed in responding patients administered ipilimumab (67,68). Collectively, these findings show that the most predictive indicator of response to CTLA-4 blockade consists of pre-existing or elicited T cell responses by anti-CTLA-4 treatment, and these observations are reflective of the results obtained in murine tumor models.

1.8CTLA-4 abrogation results in adverse autoimmune-like symptoms

Given the significant function of CTLA-4 in negatively regulating the immune response, it is not surprising to find that toxicity upon CTLA-4 blockade is common in patients, most commonly giving rise to inflammation-associated side effects termed “immune-related adverse events” (IRAE). While the symptoms can range from mild to

severe, and in some cases, life-threatening, the majority of patients have benefited from symptomatic therapies, corticosteroids, patient monitoring and hospitalization (69). Of significance, recent findings have linked the local delivery of anti-CTLA-4 antibodies to reduced toxicity in multiple murine tumor models; intratumoral injection of antibodies garnered no significant difference in overall survival compared to conventional methods of delivery, but diminished or abrogated adverse side effects due to reduced effective dosage (70,71). These observations provide basis for further investigation of local anti-CTLA-4 antibody delivery for the reduction of toxic side effects related to the mechanism of CTLA-4 inhibition.

2.1 Late-phase immune checkpoint blockade: PD-1 & inhibitory B7 ligands as targets for immunotherapy

Programmed death-ligand 1 & 2 (PD-L1 & PD-L2) are additional members of the B7 family, and represent the two known ligands for the PD-1 receptor. PD-L1 is found on hematopoietic cells and parenchymal cells, and PD-L2 is restricted to macrophages and dendritic cells (72). The PD-1 receptor is induced on activated T cells, but can also be found on B cells and NK cells (73,74). While CTLA-4 limits the amplitude of early T cell responses, PD-1 suppresses T cell function in peripheral tissues, particularly during chronic antigen exposure, thus inhibiting autoimmunity (75). As such, the PD-1 signaling axis represents another crucial immune checkpoint during disease progression. Since PD-1 is selectively upregulated in conditions of persistent antigen exposure and has been shown to drive cellular exhaustion, the expression of PD-1 on T cells has become a marker of T cell anergy (75-77). While tumor-specific T cells are found in numerous cancers, the fact that tumor growth is still observed indicates that their function may be compromised. Indeed, in multiple human tumors, a significant proportion of tumor-infiltrating lymphocytes have been shown to express PD-1, and in the majority of cases, this expression has been associated with impaired CD8+ T cell function (78-81). Interestingly, rescue of T cell activity via PD-1/PD-L1 blockade has been demonstrated in models of chronic viral infections (76,82), providing evidence for the use of PD-1 blockade to restore T cell function amongst PD-1+ TILs as a means to reduce tumor burden.

Although PD-1 is often associated with TILs in numerous cancers, PD-1 ligands are frequently observed on the surface of multiple human tumors and murine cancer cell lines, with PD-L1 as the ligand that is most commonly expressed (83-86). PD-L2 expression has mainly been documented in B cell lymphomas, such as primary mediastinal B-cell lymphoma and Hodgkin's lymphoma (87,88). Notably, PD-L1 has also been detected in tumor-infiltrating myeloid subsets including dendritic cells, macrophages and myeloid-derived suppressor cells (89-91). In certain cancers such as in hepatocellular carcinoma, melanoma and breast cancer, PD-L1 positivity was correlated with worse prognosis (92-94), consistent with its physiological role in negatively regulating T cell responses. Indeed, *in vitro* assays have demonstrated that the expression of PD-L1 on human tumors can dampen T cell effector functions, and that PD-L1 blockade was effective at enhancing the CTL activity and cytokine production of tumor-specific T cells (95). Despite these findings, other studies have found that PD-L1 status on tumors has either a positive or no correlation with patient prognosis (96-99). These observations may be attributed to differences in cancer type, stage of cancer, and/or patient treatment history, indicating the requirement for further studies examining PD-1 ligand positivity and patient outcome (96).

Evidence to support the development of reagents to target the PD-1:PD-L1/PD-L2 pathway stem from the physiological differences in mice deficient for CTLA-4, PD-1, PD-L1 and PD-L2. Whereas CTLA-4-deficient mice have dramatic lymphoproliferative and autoimmune disorders, PD-1-deficiency imparts a milder autoimmune phenotype, and these traits tend to manifest more often in mice strains prone to autoimmunity (100). PD-L1 KO mice appear normal and are viable, and show no signs of spontaneous T or B cell activation. However, these mice develop more severe EAE upon immunization (101). Similarly, PD-L2-deficient mice show no apparent signs of inflammation and appear to be largely normal relative to WT mice, but have abrogated oral tolerance (102). These findings suggest that blockade of the PD-1:PDL-1/PD-L2 signaling axis in human patients may manifest fewer immune-related toxicities, which appeared to be a concern when blocking CTLA-4 in clinical trials. Indeed, while anti-PD-1 reagents are still currently in clinical trials, immune-mediated adverse events appear milder than those observed with anti-CTLA-4 blocking antibodies, the most frequent

symptoms being mild fatigue, diarrhea, colitis, and rash, although cases of pneumonitis ranging from mild to life-threatening have been reported (103).

2.2 Pre-clinical evidence for PD-1, PD-L1 & PD-L2 blockade in murine tumor models

In numerous murine models of cancer, inhibition or absence of PD-1 has been demonstrated to rescue anti-tumor T cell responses, resulting in diminished tumor burden (104-106). Likewise, blockade of tumor-associated PD-L1 with monoclonal antibody could also rescue T cell function and enhance tumor regression in a variety of murine cancer models (107-110). Alternatively, since PD-L1 expression has also been noted on tumor-associated myeloid cells, the inhibition of PD-L1 on dendritic cells, macrophages, and MDSCs was similarly able to enhance T cell function and inhibit tumor growth (111-114).

Studies on PD-L2 blockade, however, have provided contradicting results regarding the inhibitory or stimulatory role of PD-L2. For instance, inhibition of PD-L2 in pre-established murine pancreatic cancers led to diminished IL-10 levels and tumor regression (110), revealing a T cell inhibitory and pro-tumor role for PD-L2 in this model. On the other hand, plasmacytomas engineered to express PD-L2 were rapidly rejected compared to PD-L2 negative tumors due to enhanced T cell activity; notably, this observation was also seen in PD-1-deficient mice, which may indicate the involvement of an undiscovered co-stimulatory T cell receptor specific for PD-L2 (115). While it is difficult to reconcile these contradictory findings, disparities regarding the function of PD-L2 may be attributed to differences in tumor models and mouse strains, all of which may influence the tumor microenvironment. Depending on the tumor cytokine milieu, the expression of PD-L2, PD-1, and other PD-L2-binding partners may vary, and these variations may provide some clues as to how PD-L2 can alter its function. Alternatively, administration of a GM-CSF-secreting whole-cell vaccine consisting of a fusion PD-L2 protein that does not bind PD-1 but likely engages an unknown co-stimulatory receptor resulted in enhanced tumor-specific CTLs, anti-tumor cytokine production, and the elimination of established tumors *in vivo*(116), indicating a potential method to enhance the efficacy of cancer vaccines.

2.3 Clinical findings of PD-1, PD-L1 & PD-L2 blockade

While the blockade of CTLA-4 has been characterised in human patients, clinical studies examining PD-1 signaling inhibition have only been initiated recently, and thus limited data is available. Nonetheless, initial clinical trials showed improved disease progression upon administration of anti-PD-1 antibodies. In the first clinical study involving a fully human IgG4 anti-PD-1 antibody (MDX-1106), PD-1 blockade elicited partial responses in patients with melanoma and renal cell carcinoma. Further, in 9 of 39 patients, tumor-associated expression of PD-L1 corresponded with likelihood of response (117). An additional study with another anti-PD-1 antibody (BMS-936558) has also demonstrated objective responses in patients with non-small cell lung cancer, melanoma and renal cell cancer, which did not occur in patients whose tumors were negative for PD-L1 (118). In 2013, the results of a clinical trial involving nivolumab (formerly MDX-1106) and ipilimumab co-treatment was published, demonstrating enhanced response rates compared to monotherapy alone in patients with advanced melanoma. Evidence of clinical activity was found in 65% of patients, and 53% of patients had an objective response with at least 80% tumor reduction (119). Interestingly, this was only observed in patients receiving concurrent therapy with nivolumab and ipilimumab, as only 20% of patients in the sequenced-regimen group exhibited objective responses.

Studies examining the effects of blocking PD-1 ligands in humans are scarce, although preliminary studies have been initiated and are presently ongoing. The first clinical trial with anti-PD-L1 antibodies (BMS-936559) demonstrated durable tumor regression in 6 to 17% in patients treated, and disease stabilization in 12 to 41% of patients with non-small cell lung cancer, melanoma and renal cell cancer at 24-weeks (120). An ongoing clinical trial documenting the efficacy of another PD-L1 antibody (MPDL3280A) has also yielded promising results, as 23% of non-small cell lung cancer patients had objective responses. This is particularly notable, as these patients had difficult to treat tumors as evidenced by their treatment history (investigator update http://www.roche.com/investors/ir_update/inv-update-2013-09-29.htm). Consistent with

the findings from PD-1 blockade, an association was also seen between PD-L1 tumor expression and treatment benefit.

To date, only one reagent targeting PD-L2 has been tested in humans. A recombinant PD-L2-Fc fusion protein (AMP-224) has been developed, which binds human PD-1. The mechanism of action appears to rely on the depletion of PD-1+ T cells, which would eliminate exhausted T effector cells and is hypothesized to lead to the replenishment of functional T cells with anti-tumor capabilities (http://meetinglibrary.asco.org/content/117257-132). In individuals with partial or mixed responses, AMP-224 treatment reduced PD-1hi cells and enhanced functional T cells in patients with advanced solid tumors.

2.4 Biomarkers for responsiveness to PD-1, PD-L1 & PD-L2 blockade

In the majority of patients responding to PD-1 or PD-L1 blockade, the expression of PD-L1 in the tumor was correlated with treatment responsiveness (117,118, http://www.roche.com/investors/ir_update/inv-update-2013-09-29.htm). While it may appear counterintuitive that a T cell inhibitor may predict better prognosis, the upregulation of PD-L1 is often induced by cytokines produced by effector immune cells under physiological conditions. In particular, PD-L1 can be induced by IFN-γ not only on hematopoietic cells, but also on epithelial and stromal cells to protect against immune-mediated damage (121). Evidence for this in the context of tumor growth was provided in one study that demonstrated the localization of IFN-γ to the same area where PD-L1+ tumors associated with TILs (97). Mechanistic studies in mice revealed that the induction of PD-L1 was dependent on tumor-associated CD8+ T cells which produce IFN-γ (122). Taken collectively, this implies that the expression of PD-L1 in the tumor milieu is indicative of pre-existing immune responses, and that immunosurveillance still occurs in advanced tumors. Further, a recent clinical study revealed that tumors with mismatch-repair defects predicted clinical benefit in colorectal cancer patients treated with an anti-PD-1 antibody, suggesting that aberrations in DNA repair may contribute to the availability of neoantigens and stimulate adaptive immunity, which may in turn lead to the upregulation of PD-L1 (123).

3.1 Engaging T cell co-stimulatory molecules in cancer immunotherapy: ICOS & ICOSL

The expressions of both ICOS and ICOSL have been observed in some human cancers, yet whether or not, and how, this signaling pathway contributes to the anti-tumor immune response remains unclear. As a member of the CD28 family, the inducible co-stimulatory receptor (ICOS) shares much homology with CD28. Yet, despite the ability of ICOS to stimulate downstream T cell effector functions, key differences in the structure of ICOS suggests non-redundant roles. Similar to CTLA-4, ICOS is not constitutively expressed on naïve T cells, but is induced upon T cell activation (124). During the initial priming of naïve T cells, the contribution of ICOS on T cell proliferation and IL-2 secretion appear minimal relative to that of CD28; indeed, T cells lacking ICOS show minor differences in proliferation (124-126). The cytoplasmic tail of ICOS consists of a YMFM motif that interacts with the p85 subunit of phosphoinositide 3-kinase (PI3K), and is analogous to a motif expressed by CD28 receptors. Unlike the CD28 motif, however, the YMFM ICOS sequence cannot interact with Grb2, which is essential for the secretion of IL-2 (127). Moreover, while both CD28 and ICOS can bind PI3K, engagement of ICOS has been shown to induce greater PI3K signaling than CD28 co-stimulation (128). The significance of this finding was demonstrated by our group and others in being crucial for the production IL-21 and IL-4, key cytokines involved in the differentiation and function of T follicular helper cells (Tfh) (129,130,132). In mice and humans, abrogation of ICOS signaling results in impaired germinal centre reactions, antibody class switching and affinity maturation (124,125,131). With the use of knock-in mice in which the cytoplasmic tail of ICOS has been modified to prevent binding with PI3K, we similarly observed diminished humoral responses in concordance with lower IL-4 and IL-21 levels, despite intact calcium signaling (132). This highlights the importance of ICOS-mediated PI3K signaling in the development of Tfh cells, which provides crucial signals to B cells at the onset of humoral immunity. Expression of ICOS has also been detected on memory T cells and T regulatory cells, and its expression in both populations is crucial in the homeostasis of these subsets (133,134). Additionally, while ICOS is present on both Th1 and Th2 cells, it is maintained at greater levels on Th2 cells (127,135).

The ICOS receptor is engaged by ICOSL, another member of the B7 family that exhibits some homology with B7.1 and B7.2 (136). Unlike B7.1 and B7.2, however, ICOSL expression is not restricted to hematopoietic cells, and is detected on subsets such as B cells, macrophages, dendritic cells, endothelial cells, and epithelial cells (127). Interestingly, while IFN-γ treatment has been shown to upregulate B7.1 and B7.2 on B cells and monocytes via NF-kB, the induction of ICOSL by IFN-γ is independent of NF-kB in these populations (124). The regulation of ICOSL also appears to differ in lymphoid versus non-lymphoid tissues; IFN-γ was demonstrated to inhibit ICOSL on embryonic fibroblasts, and while TNF-α could upregulate ICOSL mRNA in non-lymphoid tissues, it was unable to do so in splenocytes (137). These observations suggest that the regulation of ICOSL may depend upon tissue-specific inflammatory cues, and that the role of ICOSL as expressed on immune cells may differ when found in non-lymphoid compartments.

3.2 ICOS signaling in the cancer microenvironment

ICOS:ICOSL interactions function primarily as a co-stimulator of T cell activity, and in many cases, the down-regulation of ICOS or ICOSL is associated with poor prognosis. In the peripheral blood of colon cancer patients, genes encoding T cell co-inhibitory molecules were seen to be upregulated while co-stimulatory genes, such as ICOS and CD28, were significantly diminished. The same study also showed that the downregulation of ICOSL was related to lymph node metastasis and aggressive tumor invasion (138). In the same vein, high ICOS expression on TILs in metastatic melanoma lesions were also associated with postrecurrence survival (139). In murine models that support the anti-tumor role of ICOS signaling, the expression of ICOSL on myeloma cells enhanced the expansion of tumor-specific CTLs, and resulted in greater tumor destruction *in vivo*(140), while fibrosarcoma cells with ectopic ICOSL expression demonstrated greater rejection by CD8 T cells relative to control tumors (141).

While these findings support the co-stimulatory role of ICOS:ICOSL in facilitating the anti-tumor T cell response, other findings have revealed a pro-tumor role for ICOS signaling which may be related to its function in Treg homeostasis. In one study, acute myeloid leukemia patients exhibiting over 25% ICOSL positivity had significantly

decreased survival; ICOSL was also shown to be functional, as leukemic cells expressing B7.2 and ICOSL induced allogeneic CD4 T cells to proliferate and secrete IL-4 and IL-10 *in vitro*(142), reminiscent of the role of ICOS in Th2 and Treg maintenance. Further, in freshly isolated human melanomas, 25% of samples expressed ICOSL and 50% of metastatic samples demonstrated high ICOSL expression (143). The ICOSL present in these melanoma samples were able to engage ICOS on activated Tregs and stimulate suppressive functions, thus providing a means of tumor immune evasion through the activation of Tregs. Notably, in melanoma patients, the specific expansion of ICOS+ Tregs following the first cycle of high-dose IL-2 therapy is correlated with worse clinical outcome relative to patients with less ICOS+ Treg expansion (144). In human breast cancer, another study found that the majority of ICOS in the tumor milieu is expressed by Tregs, and that the expansion of these ICOS+ Tregs relied heavily upon pDCs that express ICOSL (145). ICOS positivity was also associated with poor prognosis in this study. Given the function of ICOS in T effector and Treg homeostasis, it is not surprising that the expression of ICOS and its ligand during tumor growth may contribute to both anti- and pro-tumorigenic responses; further studies are required to determine if the tumor milieu and its associated inflammatory signals facilitate the skewing of one arm over the other. In line with this, the contribution of ICOS signaling may differ depending on the context and presence of other T cell co-stimulatory or co-inhibitory molecules. In support of anti-tumor immunity, one study that observed the ability of tumor-associated ICOSL to co-stimulate and expand CTLs *in vivo* noted that this expansion required the expression of B7.1 and B7.2 on endogenous APCs (140). Contrasting this, ICOSL expression on metastatic melanomas was shown to promote Treg expansion, and co-expression of ICOSL and PD-L1, another T cell co-inhibitory molecule frequently associated with poor prognosis, was observed in a large proportion of samples (143). Thus, ICOS engagement may act as a facilitator, or fine tuner, of the immune response during tumor growth, whose function may rely heavily upon the presence of either T cell co-stimulatory or co-inhibitory molecules in a given tumor.

3.3 ICOS & ICOSL as biomarkers of CTLA-4 blockade efficacy

The contradictory role of ICOS signaling in tumor immunity has made it difficult to ascertain whether or not targeting this pathway would prove effective in cancer treatment. Despite this obstacle, accumulating evidence suggests that ICOS engagement may be required for optimal responses in anti-CTLA-4 therapy, acting to promote the expansion of T effector cells to reduce tumor burden. ICOS-deficient mice bearing B16 melanomas exhibited drastically diminished anti-tumor T cell responses upon CTLA-4 blockade; intriguingly, while ICOS-deficient mice also showed reduced Tregs, there was no enhanced anti-tumor immunity following anti-CTLA-4 treatment (146). Another study also demonstrated augmented efficacy of anti-CTLA-4 therapy when ICOSL-expressing tumor cell vaccines were administered in combination to treat B16/F10 melanomas (147). These data suggests that ICOS:ICOSL interactions are necessary for the optimal effects of CTLA-4 blockade, and that combinatorial therapies targeting both CTLA-4 and ICOS pathways may skew the immune response such that the net effect of this therapy would result in the enhanced co-stimulation of anti-tumor T cells. In regards to mechanism, it appears as though the capacity of ICOS to induce strong PI3K signaling is linked to the expression of T-bet, a critical regulator of Th1 anti-tumor response. In mice harboring ICOS mutants that are unable to recruit PI3K, as well as in human T cells transfected with ICOS siRNA, diminished PI3K signaling was associated with decreased T-bet expression, and subsequently reduced Th1 anti-tumor immunity (148). In clinical settings, ipilimumab treatment in patients with urothelial carcinoma of the bladder led to an increased frequency of CD4+ICOShi T cells in both the tumor and systemically (149). Likewise, in hormone-responsive advanced breast cancer patients, administration of both tremelimumab and exemestane (an aromatase inhibitor) also resulted in enhanced circulating ICOS+ T cells, and an increase in the ratio of T cells versus Tregs (150), supporting the role of ICOS as a marker for anti-CTLA-4 responsiveness. Correlation between ICOS positivity and clinical benefit has also been examined, albeit to a lesser extent. In a small cohort of metastatic melanoma patients treated with ipilimumab, retrospective analysis showed that a sustained increase in CD4+ICOShi T cells was correlated with increased likelihood of clinical benefit (149). CD4+ICOShi T cells obtained from bladder cancer patients treated with anti-CTLA-4 antibodies also showed IFN-γ production, the ability to recognize the NY-

ESO-1 tumor antigen, and an increase in the ratio of T effector cells to Tregs (151). Collectively, these findings support the role of ICOS as a biomarker of host responsiveness to CTLA-4 blockade, which may prove useful in the monitoring of patients to ameliorate treatment efficacy. While further studies are required to determine the benefits of manipulating the ICOS pathway in cancer, the present data obtained from murine studies suggests that it would be most advantageous in combination with anti-CTLA-4 therapy, where ICOS:ICOSL interactions appear to be crucial.

4.1 Newly identified B7 family ligands in cancer anti-tumor immunity: B7-H3

The observation that B7-H3 has been detected in a variety of human cancers, including prostate, pancreatic, ovarian, and others has led to the notion that this inhibitory B7 ligand may be involved in tumor immunity, and could be a potential therapeutic target. B7-H3 is a type I transmembrane protein that has been identified in recent years as belonging to the B7 family, and shares 20-27% amino acid homology with other T cell co-stimulatory and co-inhibitory molecules (152). While B7-H3 maintains 88% amino acid identity between mouse and human, structural differences between species exists (153). Murine B7-H3 contains an extracellular variable-type immunoglobulin IgV-IgC domain bound to an intracellular domain (2Ig B7-H3), in contrast to human B7-H3 which exhibits an additional isoform whereby the IgV-IgC domains are duplicated to form 4Ig B7-H3 (154). In humans, 4Ig B7-H3 appears to be the predominant isoform (155). Although the receptor for B7-H3 has yet to be identified, one candidate, the triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2), has been proposed. In one study, B7-H3 engaged murine TLT-2 expressed constitutively on CD8+ T cells and on activated CD4+ T cells, which led to enhanced proliferation and IFN-γ production; this was abrogated upon administration of blocking B7-H3 or TLT-2 antibodies (156). While these findings provide evidence for TLT-2 as the B7-H3 binding partner in certain contexts, other groups were unable to confirm this interaction in mouse and human systems (157). Adding to the complexity of B7-H3, contradictory findings regarding the co-stimulatory or co-inhibitory function of B7-H3 also exist. *In vitro*, one group found that B7-H3 induces T cell proliferation, differentiation of CTLs and IFN-γ secretion amidst strong TCR signals. In the presence

of antisense B7-H3 oligonucleotides in DCs, allogeneic T cell responses were inhibited (152). In the same line, B7-H3-deficient mice exhibit enhanced cardiac and islet allograft survival due to decreased production of cytokines and chemokines, inferring that B7-H3 promotes T cell responses (158). In contrast to these observations, other groups have shown that B7-H3 can inhibit T cell proliferation upon TCR engagement, and that *in vitro* cultures with blocking B7-H3 antibodies revealed greater T cell proliferation (159,160). To further demonstrate the co-inhibitory action of B7-H3, B7-H3 knockout mice also had earlier onset of experimental autoimmune encephalomyelitis, and exhibited more severe airway inflammation (159). Interestingly, DCs exposed to Tregs were observed to upregulate B7-H3 and downregulate MHC-peptide complexes, resulting in diminished T cell response (161).

B7-H3 is constitutively expressed on murine APCs, but must be induced on human immune cells (162). In mice, B7-H3 on DCs is upregulated by IFN-γ and downregulated by IL-4 (159). The expression of B7-H3 is not restricted to immune cells however, as is the case for B7-1 and B7-2, and is found on osteoblasts, fibroblasts, epithelial cells and other cells of non-lymphoid lineage (163).

4.2 Pre-clinical evidence for B7-H3 in tumor immunity

While *in vitro* studies and murine disease models have provided support for both the co-stimulatory and co-inhibitory capacities of B7-H3, experiments with mouse tumor models appear to support a co-stimulatory role for B7-H3 in the regulation of anti-tumor immunity. P815 tumors transfected with B7-H3 show enhanced immunogenicity, as characterized by the rapid expansion and activation of tumor-specific CTLs, resulting in tumor regression (164). Likewise, intratumoral administration of a B7-H3 expression plasmid led to the complete regression or significantly reduced EL-4 tumor burden, which was mediated by CD8 T cells and NK cells (165). Another group also reported success in reducing colon cancers and metastases upon intratumoral injection of an adenovirus expressing B7-H3, which augmented the level of IFN-γ-secreting CD8 T cells and IL-12 (166). Lastly, intratumoral treatment of hepatocellular carcinoma with B7-H3-expressing plasmids in combination with vasostatin-expressing plasmids (potent

anti-angiogenic agent) was able to completely eradicate tumors (167), further implying a potential for B7-H3 as a cancer therapeutic.

4.3 Expression of B7-H3 in human cancers & implications

Despite the evidence from murine studies implicating B7-H3 as a positive regulator of adaptive tumor immunity, expression of B7-H3 in human cancers tend to favor poor prognosis. To date, only a handful of studies have reported a co-stimulatory function for B7-H3 in cancer patients. This includes the observation that B7-H3 is abundantly expressed in pancreatic cancer, and is correlated with prolonged post-operative survival (168), as well as in gastric carcinoma, where B7-H3 positivity was related to survival time and was judged as being beneficial to patient prognosis (169). In contrast to these findings, the majority of reports demonstrate an inverse correlation between B7-H3 staining and patient outcome. In the case of non-small-cell lung cancer, tumor B7-H3 expression was associated with decreased TILs and lymph node metastasis (170); likewise, soluble B7-H3 was also detected in patients with non-small-cell lung cancer and high levels were similarly correlated with higher tumor stage, burden and metastases (171). A high frequency of ovarian cancers also express B7-H3 in the membrane and cytoplasm, and the presence of B7-H3 was linked with shorter survival and higher incidence of recurrence (172). Strong B7-H3 positivity was similarly observed in colorectal carcinomas and as circulating soluble proteins, both of which was correlated with tumor grade and decreased TILs; notably, TNF-α, an inflammatory protumorigenic molecule, could induce the shedding of soluble B7-H3 by colon cancer cell lines, suggesting another method of cancer immune evasion via B7-H3 (173). B7-H3 has also been implicated in prostate cancer, whereby B7-H3 is abundantly expressed and is associated with cancer progression following surgery (174,175).

The discrepancy between the co-stimulating and co-inhibiting capacities of B7-H3 poses an obstacle when considering this molecule as a target for cancer immunotherapeutics. Many questions must be addressed prior to wider use in the clinic; for instance, while TLT-2 has been demonstrated to bind B7-H3 in humans to co-stimulate T cell responses, alternate receptors with opposing functions may exist. Further, the expression pattern of B7-H3 may also dictate its binding to various

receptors, as in the case for B7-1 and B7-2 molecules that have a higher affinity for CTLA-4 relative to CD28. Thus, further characterization of TLT-2:B7-H3 engagement, as well as the identification of alternate B7-H3 binding partners, may provide additional clues regarding the opposing function of B7-H3. Additionally, the structure of B7-H3 in humans and mice also differ, and this may contribute to the observed differences in mediating T cell immunity. Recently, the crystal structure of murine B7-H3 was revealed, wherein the FG loop of the IgV domain was found to be crucial for the inhibitive properties of B7-H3 on T cell proliferation *in vitro*(176). Future studies comparing the FG loop in mice and humans, as well as examining the functional differences in the 2Ig versus the 4Ig isoform of B7-H3, may shed light on the B7-H3 enigma. Moreover, while B7-H3 has been diligently examined in regards to T cell responses, the role of B7-H3 in activating or inhibiting other immune cells (such as NK cells and other APCs) may further explain the contradictory findings seen in human cancer patients, as different tumor types may preferentially recruit certain immune subsets over others.

Although B7-H3's capacity to influence the immune response is a critical factor to examine, immune-independent functions of B7-H3 have also been reported, and should be considered in regards to B7-H3 as a potential drug target. In particular, one notable study demonstrated the ability of B7-H3 to regulate tumor cell migration and invasion; siRNA-mediated downregulation of B7-H3 in human melanoma and breast cancer cell lines reduced cell adhesion, migration and matrigel invasion despite no impact on cell proliferation (177). In a similar study, downregulation of B7-H3 in human breast cancer cells resulted in reduced metastases in a murine experimental metastasis model, whereby metastasis-associated proteins (MMP-2, Stat3, IL-8) were positively correlated with B7-H3 expression (178). In addition, B7-H3 has also been shown to induce chemoresistance *in vitro* and *in vivo*, as overexpression of B7-H3 in human breast cancer cell lines augmented paclitaxel resistance, and B7-H3 silencing led to enhanced sensitivity, apoptosis and strong anti-tumor immunity upon paclitaxel treatment. Mechanistically, the authors provide evidence for the involvement of the Jak2/Stat3 pathway, which is downregulated upon B7-H3 silencing, and subsequently results in the decreased expression of Mcl-1 and survivin(179). Lastly, while certain immune-related

molecules have been implicated in the regulation of B7-H3, microRNA mir29 has been reported to bind the 3' untranslated region of B7-H3, and negatively regulate the expression of B7-H3. While miR-29 is highly expressed in normal tissues (where B7-H3 is poorly expressed), the authors showed that multiple solid human tumors and human tumor cell lines have reduced miR-29 expression and augmented B7-H3 levels (180), collectively supporting a protumorigenic role for B7-H3.

5.1 New B7 ligands and potentials for clinical use

In addition to B7-H3 and B7-H4 (see below), other newly discovered B7 ligands have also shown possibilities of being exploited for cancer therapeutics. Murine studies targeting another T cell co-inhibitor B7-H5, or VISTA, showed enhanced anti-tumor T cell immunity and reduced melanoma tumor burden (181,182). In the same vein, B7-H7, or HLAA2, has been demonstrated to inhibit CD4 and CD8 proliferation and cytokine production *in vitro*(183). Evidently, blockade of VISTA and B7-H7 may help enhance T cell responses in cancer patients, and further studies regarding these pathways will offer new avenues to explore in ameliorating current immunotherapeutics. B7-H6, another B7 molecule, has been reported to bind the NK receptor NKp30 and activate anti-tumor cytotoxicity and cytokine production. Intriguingly, B7-H6 was expressed on human tumors despite its absence in non-cancerous tissues, prompting further interest to examine B7-H6 during cancer growth (184).

6.1 B7-H4 shows distinct expression patterns in human cancers: applications for immunotherapy

As another member of the B7 family, B7-H4 represents another T cell co-inhibitory molecule whose expression pattern in the tumor microenvironment has garnered significant attention. The shared homology between B7-H4 and other B7 members is approximately 25%, and analysis of murine and human amino acid sequences reveals 87% amino acid identity (185). While similar in structure, notable differences between species exist, such as the absence of GPI-linkage in human B7-H4 which occurs in mice, and the inclusion of a nuclear localization sequence, demonstrated to be required for the trafficking of human B7-H4 between the nucleus and cytoplasm, which was not detected in the sequence of murine B7-H4 (186-188).

Whereas B7-H4 positivity in the nucleus was associated with renal cell carcinoma tumor stage, TILs were not correlated with nuclear B7-H4 expression, suggesting an unknown, immune-independent role of B7-H4 in the nucleus (186).

The expression of B7-H4 transcripts is ubiquitous in healthy individuals, and B7-H4 mRNA has been found in both lymphoid and non-lymphoid organs such as the lung, liver, spleen, thymus, kidney, pancreas, and other tissues. B7-H4 protein, however, remains highly restricted in both mice and humans, indicating a tightly regulated translational mechanism (185,189). On hematopoietic cells, B7-H4 protein can be induced following *in vitro* stimulation of human T cells, B cells, monocytes, and DCs (185). Similarly, tumor-associated macrophages were observed to express B7-H4, and this upregulation was attributed to the presence of IL-6 and IL-10 in the tumor microenvironment (190).

Functionally, B7-H4 has been well-documented to inhibit T cell responses. *In vitro* studies on murine T cells using B7-H4 fusion protein revealed an inhibitory role for B7-H4 in cell cycle progression, proliferation and cytokine secretion (191); likewise, human B7-H4 was shown to reduce T cell proliferation, arrest cell cycle progression and induce apoptosis (192). In accordance, mice deficient in B7-H4 protein displayed upregulated Th1 response upon *Leishmania major* infection, but did not show enhanced hypersensitive inflammatory responses or increased CTL activity during viral infections, suggesting that the role of B7-H4 may be one of a fine tuner (193). In addition to mediating T cell activity, B7-H4 has also been found to regulate the activity of myeloid cells. B7-H4 knockout mice show enhanced CD11b+Gr-1+ neutrophils and lower *Listeria monocytogenes* burden, concordant with the observations that in vitro administration of B7-H4 fusion protein diminishes the expansion of bone marrow-derived neutrophil progenitors (194). Intriguingly, we have also observed enhanced suppressive capacities of CD11b+Gr-1+ myeloid-derived suppressor cells (MDSCs) in the absence of B7-H4, further implicating an inhibitive capacity for B7-H4 in the context of tumor growth (195). Contrasting this, in an experimental model of lung metastasis, B7-H4 was seen to enhance tumor-associated neutrophils while simultaneously suppressing T cell responses (196). Further studies will be required to determine the

precise capacity of B7-H4 to regulate myeloid lineages. Other than its role in influencing the immune response, B7-H4 has also been reported to have immune-independent functions. Notably, despite the tight control of B7-H4 protein expression in peripheral tissues, an array of human cancers overexpress B7-H4 molecules despite its restricted profile in healthy individuals (188). These findings have prompted the study of B7-H4 in the growth of cancer cells outside of their capacity to modulate immunity. Overexpression of B7-H4 on cancer cells appear to enhance cell growth, adhesion, migration and protect from apoptosis *in vitro*, and accordingly, tumor cells overexpressing B7-H4 showed enhanced growth in SCID mice (197,198).

To date, the receptor for B7-H4 has not yet been identified, and although initial studies pointed to BTLA as the B7-H4 binding partner, a subsequent study showed a lack of interaction between the two molecules (199). Based on functional studies involving B7-H4 fusion proteins, it is apparent that the receptor should be expressed on T cells, and is distinct from CD28, CTLA-4, ICOS and PD-1 (191). Notably, recent studies attributing a function for B7-H4 in influencing myeloid cell activity further suggest the presence of a B7-H4 binding partner on the surface of this subset. Indeed, neutrophils and tumor-associated neutrophils bound B7-H4 protein (194,196), suggesting that B7-H4 can act on both innate and adaptive immunity.

6.2 Role for B7-H4 in cancer and cancer therapeutics

While B7-H4 protein has limited expression in healthy individuals, numerous human cancers such as breast, ovarian, prostate, and gastric cancers exhibit aberrant B7-H4 positivity (200). In particular, B7-H4 staining was detected almost ubiquitously in the majority of ovarian and breast cancers, and is thus believed to have potential as a biomarker (198,201,202). Further, tumor B7-H4 positivity was correlated with reduced TIL in invasive ductal carcinomas, and macrophage B7-H4 in ovarian tumors was associated with worse patient outcome (203,204). In prostate cancer, high levels of B7-H4 are also detected, and appears to be associated with spread of disease and worse outcome (205); similarly, B7-H4 expression in renal cell carcinoma and gastric cancer predict worse patient outcome (206,207). In a separate study, low levels of B7-H4 in melanoma was associated with survival benefit, and overexpression of B7-H4 on

melanoma cells inhibited cytokine production in CD8 T cells *in vitro*(208). Soluble B7-H4 in ovarian cancer has also been detected, and B7-H4 found in the sera of patients was shown to be a strong predictor of time to progression and chemotherapy response (209). Taken together, these findings provide a basis for targeting B7-H4 in cancer, and several studies performed in mice have further validated this notion. In an experimental model of lung metastasis, host B7-H4 was demonstrated to reduce the level of tumor-specific T cell responses and augment the infiltration of immunosuppressive subsets into the lung. As a result, B7-H4 WT mice exhibited more lung metastases relative to B7-H4 knockout mice (196). Further support for the abrogation of B7-H4 signaling in ameliorating disease progression stem from studies involving anti-B7-H4 reagents. In a humanized murine model of ovarian cancer, administration of anti-B7-H4 single-chain fragments variable (scFv) was able to delay tumor growth; the same reagent was also able to rescue tumor-specific T cell activity from inhibition by B7-H4+ APCs *in vitro*(210). Moreover, injection of anti-B7-H4 antibodies inhibited the growth of CT26 tumors ectopically expressing B7-H4, providing evidence for the role of abrogating tumor-associated B7-H4 in the development of cancer therapeutics (211).

While manipulation of membrane-bound B7-H4 in the tumor has been demonstrated to reverse T cell inhibition in murine tumor models, accumulating evidence has revealed subcellular localization of B7-H4 in multiple cancers, some of which predominantly or exclusively contain cytosolic B7-H4 protein (186,190,208). Notably, in contrast to surface B7-H4 expression, intracellular B7-H4 protein has not been demonstrated to inhibit T cell activity (186,190). Recent data also suggests that hypoxia, through HIF-1α signaling, upregulates B7-H4 protein solely in the cytosol of cancer cell lines (212). Given the hypoxic nature of the tumor microenvironment and the tendency for B7-H4 to remain intracellular under these conditions, the impact of B7-H4 blockade in the tumor may be limited. Additional challenges to the development of anti-B7-H4 agents is the contradicting data that has recently surfaced regarding the anti-tumor capacity of B7-H4, as one study reported that host B7-H4 expression was associated with limiting tumor development in MMTV-PyMT mammary tumors and in a murine insulinoma model (213), denoting an anti-tumor role for host B7-H4. The same study also found that breast cancer patients with enhanced B7-H4 positivity

demonstrated a longer time to cancer recurrence. To date, no anti-human B7-H4 blocking antibody has been tested in the clinic, yet based on the findings in murine tumor models, the high degree of B7-H4 staining in cancers relative to non-cancerous tissue, and given that B7-H4 has shown promise as a biomarker in multiple human cancers, it is of great interest to see if abrogation of B7-H4 signaling can be used in combination with other immunotherapeutics or other chemotherapies.

7.1 Examining the function of host B7-H4 in anti-tumor immunity: rationales & objectives

Immune evasion is a hallmark of developing tumors, and therapies targeting immune checkpoints mediated by inhibitory B7 family molecules such as CTLA-4 and PD-1 have shown success in enhancing the anti-tumor T cell response and reducing tumor burden. Several groups have shown that tumors overexpressing B7-H4 have enhanced growth, and that blockade of tumor-expressed B7-H4 could rescue T cell responses and suppress tumor development. These findings implicate a role for targeting B7-H4 in human cancer patients, yet is limited to those whose tumors display B7-H4 positivity. In contrast to tumor-expressed B7-H4, the function of host B7-H4 has seldom been addressed in the context of cancer, given that immune cells also express functional B7-H4 protein in the tumor microenvironment (190, 217).

7.2 Objective 1: Address the role of host B7-H4 in the growth of a transplanted, syngeneic mammary carcinoma model, 4T1

a) *Determine the role of host B7-H4 in tumor growth.* Given that many human cancer samples display intracellular B7-H4 localization, which has been demonstrated to play no role in modulating T cell responses, this argues that blocking tumor-associated B7-H4 may have limited potency in rescuing adaptive anti-tumor immunity. Thus, examining the function of host B7-H4 independent of its expression in the tumor may provide valuable insights into the applicability and efficacy of blocking B7-H4 in cancer patients. The 4T1 mammary carcinoma cell line, derived from a spontaneously arising mammary tumour in an MMTV+ Balb/c mouse, is a well-studied model of transplantable tumour growth. Its resistance to 6-thioguanine is particularly useful, as *in vivo* passaged 4T1 cells

can be readily selected for further analysis. Furthermore, 4T1 was also found to be negative for the expression of surface B7-H4, which allowed for the focus to be solely on the host response to tumor challenge in the presence or absence of B7-H4.

b) *Clarify the pro- or anti-tumorigenic capacity of B7-H4 in 4T1 mammary carcinoma growth.* Since the role of host B7-H4 has been controversial given that data supporting both B7-H4's anti- and pro-tumorigenic have been demonstrated and previously discussed, our aim is to provide further clarity on the impact of host B7-H4 in adaptive immunity during 4T1 progression. To this end, 4T1 tumor growth was monitored in WT and B7-H4-deficient animals, and the anti-tumor T cell response was analyzed and quantitated.

Using the 4T1 model, we showed that B7-H4 KO mice displayed stronger anti-tumor cytokine profiles, yet exhibited similar growth of transplanted 4T1 tumor cells compared with WT controls. Further, we also observed that B7-H4 could also modulate the immunosuppressive function of myeloid-derived suppressors cells (MDSCs), and saw enhanced MDSC function in the absence of B7-H4. This suggests a dual role for B7-H4 in modulating both adaptive and innate immunity, both of which have been previously described by others. Further, since B7-H4 has been implicated as a fine-tuner of T cell immunity based on data obtained from knockout mice, we wondered if this lack of difference in tumor growth may be due to the low immunogenicity of the 4T1 mammary carcinoma cells.

7.3 Objective 2: Investigate the function of host B7-H4 in the development of anti-tumor T cell responses in an immunogenic tumor model

To further examine the role of host B7-H4 in the development of anti-tumor T cell responses and to enhance the efficacy of B7-H4 inhibition on tumor reduction, we used a highly immunogenic derivative of 4T1 that expresses firefly luciferase termed 4T1-12B. Since luciferase+ 4T1-12B cells are eliminated in immunocompetent hosts relative to T cell-depleted mice (214), and because MHC-I-restricted luciferase epitopes have

recently been identified (215), this implies that luciferase can give rise to strong T cell tumor rejection antigens and allows us to examine how B7-H4 modulates the anti-tumor response. To this end, 4T1-12B tumor cells were inoculated into WT or B7-H4-deficient mice, and tumor growth, luciferase activity, and tumor-associated T cell responses were quantified.

In the immunogenic 4T1-12B mammary carcinoma model, we show that host B7-H4 inhibits anti-tumor T cell responses promoting the growth of immunogenic primary mammary tumors and metastases. We also reveal that combinatorial treatment with the chemotherapeutic drug gemcitabine can lead to complete tumor rejection in B7-H4 KO mice, but not in WT hosts. These KO mice are protected from not only a subsequent re-challenge of 4T1-12B cells, but are also partially protected from a following inoculation with parental 4T1 cells. These observations suggest that blockade of host B7-H4 may improve anti-tumor T cell immunity independent of tumor-expressed B7-H4, and also indicate that combination of B7-H4 blockade and chemotherapy on highly immunogenic tumors may lead to tumor rejection and protection from recurrence.

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Chapter II

Host B7-H4 regulates anti-tumor T cell responses through inhibition of myeloid-derived suppressor cells in a 4T1 tumor transplantation model

Previous studies have shown a tumor-dependent function of B7-H4, whereby the expression of B7-H4 on cancer cells can influence their growth and survival *in vitro* and *in vivo*. To investigate the tumor-independent role of host B7-H4 on the immune response during cancerdevelopment, we studied the progression of a transplantable murine mammary carcinoma, 4T1, in the absence or presence of endogenous B7-H4. We also examine herein the anti- and protumorigenic capacity of B7-H4 to shed further light on the contribution of this co-inhibitory molecule during tumor growth.

Host B7-H4 regulates anti-tumor T cell responses through inhibition of myeloid-derived suppressor cells in a 4T1 tumor transplantation model

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1. Abstract

B7-H4, a member of the B7 family of T cell immunomodulatory proteins, has been shown to inhibit T cell responses and neutrophil expansion during bacterial infections. However, the role of B7-H4 in the immune response during tumor growth has been unclear. Here, we examined the host immune responses in B7-H4-deficient (KO) or sufficient (WT) Balb/cJ mice upon transplantation of murine 4T1 carcinoma cells that had little B7-H4 expression. We reveal that host B7-H4 not only dampens the anti-tumor Th1 responses, but that it also inhibits the pro-tumor function of myeloid-derived suppressor cells (MDSC). We observed increased expression of both anti-tumor immune effectors and pro-tumor MDSC-associated transcripts in 4T1 tumors grown in B7-H4 KO mice compared to those grown in WT hosts. Consistently, MDSCs derived from B7-H4 KO mice suppressed T cell proliferation more potently than their WT counterparts. Although the primary growth of 4T1 tumors in B7-H4 KO hosts was similar to that in WT mice, tumors that had grown in B7-H4 KO hosts grew much slower than those from WT mice when subsequently transplanted into WT hosts. Importantly, this differential tumor growth during the secondary transplantation was abrogated when recipient mice lacked T cells, indicating that the immune environment in B7-H4 KO hosts allowed outgrowth of 4T1 tumors with reduced immune-evasive capacities against T cells. Thus, B7-H4 can inhibit both anti-tumor T cells and pro-tumor MDSCs, influencing the immune-evasive character of the outgrowing tumors. These factors should be considered if B7-H4 blockade is to be used for cancer immunotherapy.

2. Introduction

The B7 family of T cell co-stimulatory and co-inhibitory molecules plays a pivotal role in the regulation of adaptive immune responses. As such, B7-H4 (also known as B7S1 or B7x) was identified as a co-inhibitory member of the B7 family (1-3). In mice, B7-H4 transcripts are found ubiquitously in lymphoid and nonlymphoid organs, yet protein expression is limited to B cells and antigen presenting cells (1-3). In humans, B7-H4 mRNA is also widely distributed with little protein expression, but can be induced in monocytes, macrophages, and dendritic cells upon IL-6 and IL-10 stimulation (4-6). Functionally, engagement of the putative, unidentified B7-H4 receptor on T cells with recombinant B7-H4-Fc proteins *in vitro* reduced CD4 and CD8 T cell proliferation and cytokine production (1-3,7). Consistently, B7-H4 KO mice displayed elevated Th1 responses against *Leishmania major* (8) and enhanced Th1 and Th17 responses to experimental autoimmune diseases (9). Interestingly, B7-H4 has also been shown to negatively regulate neutrophil-mediated innate immune responses. Upon *Listeria monocytogenes* infection, more neutrophils were recruited into peripheral lymphoid organs in B7-H4 KO mice, conferring greater resistance to the pathogen relative to wild-type mice (10). The same study showed that B7-H4-Fc proteins suppressed the expansion of bone marrow-derived CD11b+Gr-1+ neutrophil progenitors *in vitro*.

Apart from the immune system, B7-H4 transcripts and proteins are overexpressed in breast cancer (11-13), ovarian cancer (6,13-16), lung cancer (6,17,18), and many other solid tumors (19-25) to varying extents. In some cancers, the proportion of B7-H4 positive tumor area and the intensity of B7-H4 immunostaining have been positively correlated with the invasiveness of the tumors, poor prognosis and mortality (20-25). Overall, these studies suggest that B7-H4 promotes tumor progression; as such, B7-H4 blockade has been proposed as a cancer therapeutic option (26). However, the mechanism by which B7-H4 regulates anti-tumor immunity is yet to be clarified.

Accumulating evidence shows that myeloid cells play important roles in shaping both the progression of cancer, as well as the host anti-tumor immunity. Notably, myeloid-derived suppressor cells (MDSCs), a heterogeneous population of immature myeloid cells and myeloid progenitors, accumulate during pathological conditions in response to

various cytokines, particularly those produced during tumorigenesis (27,28). One of their most significant contributions during tumor progression stems from their ability to effectively suppress the adaptive and innate immune response by producing immunosuppressive factors including arginase, inducible nitric oxide synthase, and ROS (28). Accordingly, multiple studies have demonstrated that the inhibition of MDSCs via drug treatment in various mouse models can effectively reduce the accumulation of MDSCs, and ultimately, hinder tumor development (27,29,30).

In this study, we examine the anti-tumor and pro-tumor factors in tumors growing in the presence or absence of host B7-H4 using the 4T1 tumor transplantation model. We confirm that B7-H4 has a negative regulatory role for Th1-mediated anti-tumor immunity, yet we also reveal, for the first time, that B7-H4 can inhibit myeloid-derived suppressor cells. Collectively, we provide evidence that the dual inhibitory roles of B7-H4 on anti- and pro-tumor immune cells have a potential to alter not only the growth of tumors, but the immune-evasive capacities of outgrowing tumor cells. Thus, we uncovered the opposing immune-regulatory functions of B7-H4, which should be considered in the application of B7-H4 blockade in cancer treatment.

3. Results

3.1 Absence of B7-H4 in host does not change the growth of 4T1 tumors

The 4T1 mammary carcinoma cell line, derived from a spontaneous mammary tumor in a MMTV+ BALB/c mouse, is a well-studied model of transplantable tumor growth (33). Because 4T1 cells are resistant to 6-thioguanine, cells that have grown as tumors can be isolated by culturing *in vitro* in the presence of 6-thioguanine for further analyses. The 4T1 cells were also found to be negative for the expression of surface B7-H4 compared with the human breast cancer cell line SKBR3 and murine mammary epithelial cells ectopically expressing B7-H4, NMuMG-B7H4 (Supplemental Fig. 1A). We also confirmed that B7-H4 is not induced on the surface of 4T1 cells by IFN-γ treatment, whereas MHC class II and PD-L1 (also known as B7-1H) can be easily detected under the same conditions (Supplemental Fig. 1B). To determine the role of B7-H4 in the host immune system, 4T1 cells were injected s.c. into the flanks of either

WT or B7-H4 KO mice, and tumor growth kinetics and host immune reactions were analyzed.

Within the 21 d of growth, 4T1 tumor volume in B7-H4 KO mice was comparable to that of tumors from WT hosts (Fig. 1A, left). Consistently, final tumor weights were also similar (Fig. 1A, right). To further examine the impact of B7-H4 on primary tumor growth, single-cell suspensions of ex vivo 4T1 tumors were analyzed by flow cytometry. In accordance with tumor growth, the tumor microenvironment of mice deficient or sufficient for B7-H4 had similar percentages of T cells, macrophages, dendritic cells, and NK cells over the total CD45+ host hematopoietic cells (Fig. 1B). Moreover, the level of MDSCs, which accumulate during tumor growth and reflect the degree of tumor burden (28), was also similar in the tumors of WT and B7-H4 KO mice (Fig. 1B). Peripheral lymphoid organs, including the spleen and draining lymph nodes, also displayed comparable percentages of macrophages, T cells, and MDSCs (Supplemental Fig. 2).

Despite similarities in tumor growth, 4T1 tumors from B7-H4 KO host displayed a modest, yet significant increase in the percentage of total tumor-infiltrating immune cells (CD45+ live cells over total live cells) relative to tumors grown in WT hosts (Fig. 1B, total immune cells). However, there were no significant changes in the percentages of immune cell subpopulations, including T cell subsets and MDSC subsets comprising CD11b+Ly6G+ granulocytic MDSCs and CD11b+Ly6C+ monocytic MDSCs (Fig. 1B).

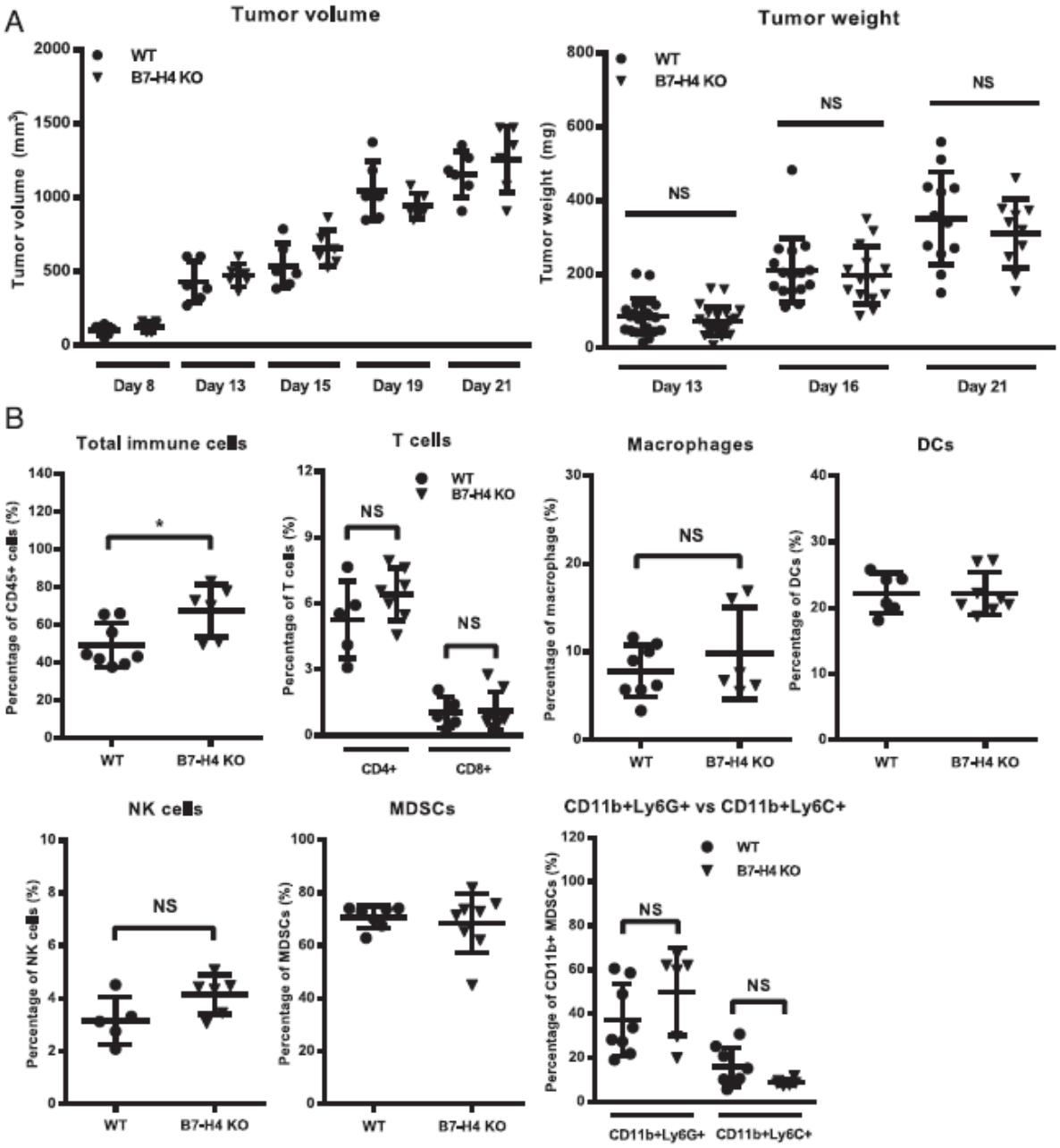

Figure 1.4T1 tumors grew at equal rates in B7-H4 KO and WT mice. (A) Kinetics of 4T1 tumor growth (Left) and the final tumor weights (Right, day 13) grown in WT and B7-H4 KO hosts. The size of tumors was measured and converted into volume as described in *Materials and Methods*. Data depict one of three independent experiments with similar results. Each data point represents one tumor. (B) Tumor-infiltrating immune cells were analyzed by FACS analysis at day 13 post-injection. The percentage of the total immune cells (7AAD-, CD45+) over the total live cells (7AAD-) or the percentages of T cells (CD4+/CD8+), macrophages (CD11b+F4/80+), dendritic (CD11b+CD11c+) cells, NK cells (CD45+CD3-DX5+), MDSCs (CD11b+Gr-1+), and granulocytic MDSCs (CD11b+Ly6G+) over the total live immune cells infiltrates (7AAD-CD45+) are depicted. Data represent one of three independent experiments with similar results. Each data point represents one tumor.

3.2 Host B7-H4 influences the cytokine expression profiles within 4T1 tumors

The 4T1 tumors grow more aggressively in IFN-γ–deficient mice (34), and, in the context of a *L. major* infection, T cells from B7-H4 KO mice showed augmented Th1 responses evidenced by elevated T-bet expression and IFN-γ production (8). Thus, we analyzed IFN-γ expression in tumors taken from WT or B7-H4 KO mice. Intracellular staining of IFN-γ in tumor-infiltrating T cells did not generate meaningful results due to the paucity of IFN-γ–producing cells (data not shown). However, it has been shown that upregulation of MHC class II and PD-L1 on the surface of 4T1 tumors in vivo reliably reflects biologically active IFN-γ in the tumor microenvironment (34). Thus, we examined the expression of these two proteins in 4T1 tumors grown in WT versus B7-H4 KO hosts. Tumors derived from B7-H4 KO mice had elevated expression of MHC class II both in the percentages and in the expression levels (Fig. 2A). Likewise, IFN-γ mRNA levels in the total tumor lysates showed a consistent difference (Fig. 2B). Therefore, our data suggest that 4T1 tumors grown in the absence of B7-H4 experienced elevated levels of IFN-γ compared with those grown in WT hosts.

Despite higher levels of IFN-γ in tumors grown in the absence of B7-H4, the overall tumor growth rate was unaltered. Thus, we hypothesized that the presence of antitumor and protumor immune factors counterbalanced each other in tumors grown in B7-H4 KO mice. We tested this idea by a comprehensive examination of the cytokine expression patterns in the 4T1 tumors by qPCR.

Consistent with the known negative regulatory role of B7-H4 for Th1 responses in bacterial infection and keeping in line with the elevation of IFN-γ in 4T1 tumors grown in B7-H4 KO hosts, we saw that multiple Th1-associated factors were upregulated in tumors derived from B7-H4 KO mice, as follows: T-bet, IL-2, and IL-12 subunits (Fig. 2B). In contrast, the expression pattern of cytokines associated with cytotoxic immune cells (CTL and NK) was similar between tumors grown in WT versus B7-H4 KO hosts, as follows: granzyme B, granzyme A, perforin, IL-15, and NKG2D (data not depicted).

Examination of immune-suppressive factors yielded more insightful results. The levels of transcripts encoding iNOS were >4-fold higher in tumors harvested from B7-H4 KO mice compared with those from WT control (Fig. 2C). Similar differences were found in both monocytic and granulocytic MDSC subsets (Fig. 2C). We also noticed a marginal increase of arginase-1 mRNA (Fig. 2C). Because iNOS and arginase-1 are the hallmark effector proteins used by MDSCs (27), these data strongly suggest that B7-H4 normally acts as a negative regulator of MDSC as well as Th1 responses.

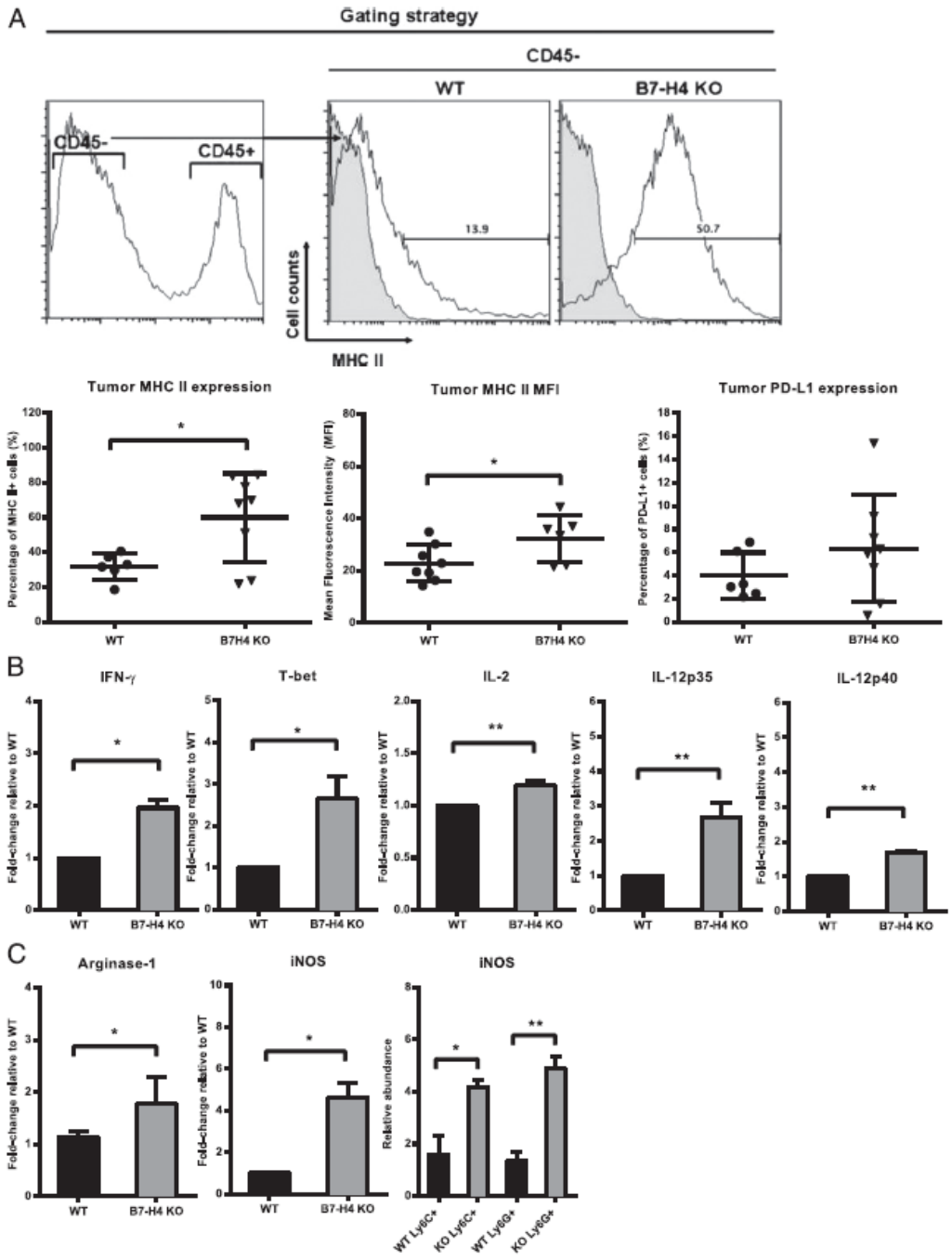

Figure 2.Differential expression of anti- and pro-tumorigenic genes in 4T1 tumors grown in B7-H4 KO vs WT hosts(A)Surface expression levels of MHC II and PD-L1 on 4T1 tumor cells. Live tumor cells (7AAD-CD45-) were gated to measure the levels of MHC class II and PD-L1. Data depict one of three independent experiments with similar results. Each data point represents one tumor.(B and C) Levels of immune effector transcripts in ex vivo 4T1 tumors. Tumor lysates from WT (n = 6 tumors) and KO (n = 8 tumors) were analyzed via quantitative PCR for the indicated transcripts, and the relative abundance or the fold change relative to WT was calculated, as described in Materials and Methods. Subsets of tumor MDSC were sorted, as described in Materials and Methods, prior to qPCR analysis. All qPCR data were mean ± SEM and show one of two independent experiments with similar results. *p < 0.05, **p < 0.01.

3.3 B7-H4 affects the immunosuppressive activity of MDSCs

Next, we determined whether MDSCs derived from B7-H4 KO mice display differences in T cell-suppressive activity relative to WT MDSCs *in vitro.* To this end, bone marrow cells harvested from WT and B7-H4 KO mice were cultured in the presence of GM-CSF and IL-6 to induce MDSC differentiation (31). No difference was seen between genotypes in the generation of MDSC populations based on the expression patterns of Gr-1 and CD11b (Fig. 3A). Bone marrow–derived MDSCs from WT or B7-H4 KO mice were cocultured with WT splenocytes in the presence of plate-bound CD3 and CD28 Abs, and T cell proliferation was measured by a thymidine incorporation assay. Remarkably, MDSCs from B7-H4 KO mice were more suppressive on T cell proliferation than MDSCs from WT mice (Fig. 3B).

Because MDSCs are known to suppress the cytotoxicity of NK cells (35–37), we tested whether B7-H4 deficiency affects MDSC-mediated suppression of NK killing of tumor cells. We measured NK cell cytotoxicity against 51Cr-labeled YAC-1 target cells in the presence or absence of bone marrow–derived MDSCs. We found that MDSCs effectively suppressed NK cell killing, but no difference was seen between MDSCs from WT or B7-H4 KO mice (Fig. 3C). Therefore, these *in vitro* results indicate that, in the absence of B7-H4, MDSCs have a stronger ability to inhibit T cell proliferation, but an equal capacity to inhibit NK cells compared with WT MDSCs.

We next compared the immunosuppressive capacities of splenic MDSCs isolated from WT or B7-H4 KO mice bearing 4T1 tumors. Consistent with results from bone marrow–derived MDSCs, CD11b+ splenic cells (>85% MDSCs by FACS analysis) from B7-H4 KO tumor-bearing mice had much more potent suppressive activities against T cell proliferation compared with their WT counterparts (Fig. 3D). Importantly, B7-H4 KO MDSCs had higher levels of iNOS expression, but similar levels of gp91 (a key component of NOX2, the major reactive oxygen species–generating enzyme in MDSCs (38)) (Fig. 3E) and undetectable levels of arginase 1 (data not depicted). Using bone marrow–derived MDSC, we confirmed that the iNOS inhibitor, L-NMMA, abrogated the ability of MDSC to suppress T cell proliferation (Fig. 3F). Taken together, these data

indicate that MDSCs generated in the absence of B7-H4 have heightened immunosuppressive function that relies on the elevated expression of iNOS.

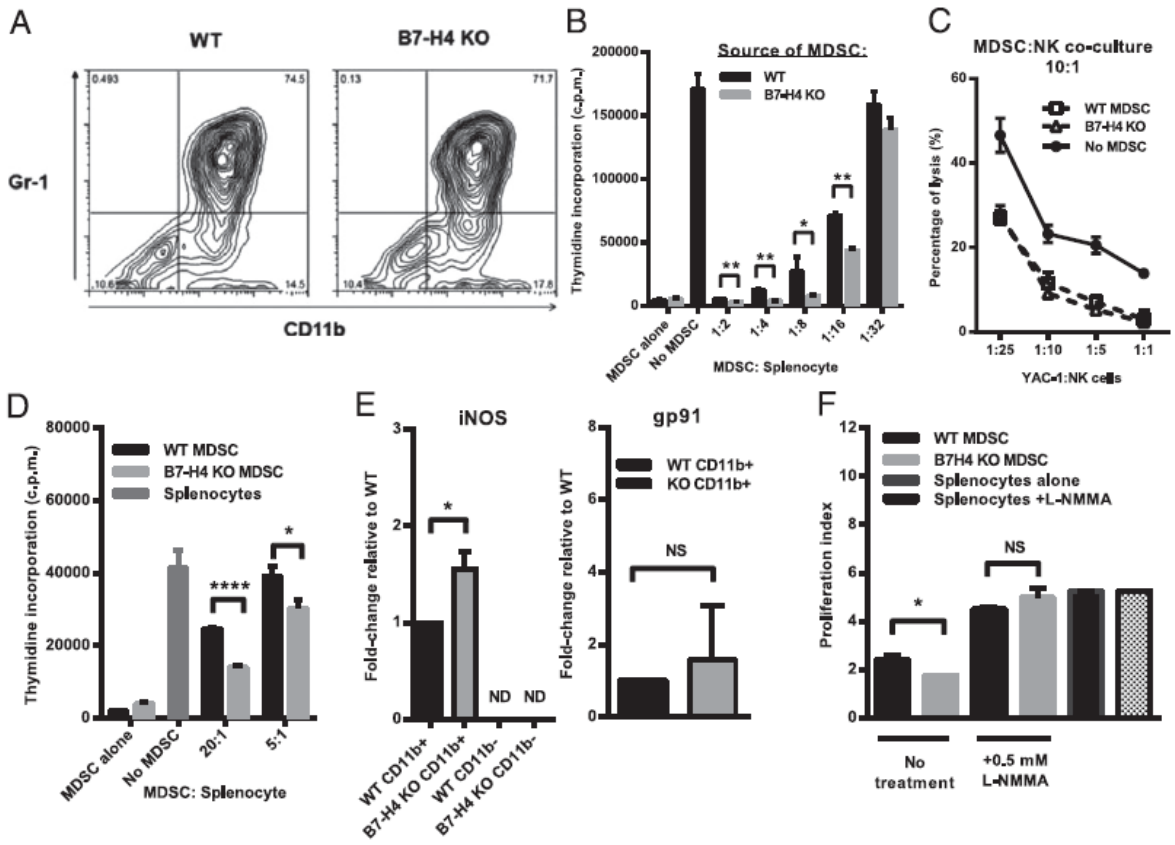

Figure 3. MDSCs fromB7-H4 KO mice suppress T cell proliferation more potently.(A) In vitro cultured bone marrow–derived MDSCs from WT or B7-H4 KO mice show no differences in CD11b or Gr-1 expression. MDSCs were derived from bone marrow cells, as described in Materials and Methods, and differentiation was confirmed by flow cytometry prior to further experimentation. Data depict one of three independent experiments. (B) MDSC-mediated inhibition of T cell proliferation. MDSCs were derived from bone marrow cells from WT or KO mice, as described in Materials and Methods. T cell proliferation in the presence of MDSCs was measured by thymidine incorporation assays on day 2. Mean ± SEM of triplicate wells from one representative experiment of two is shown. (C) MDSC-mediated suppression of NK cell cytotoxicity. NK cells were preincubated with BM-MDSCs for 4 h at the indicated ratio before the addition of YAC-1 target cells for a subsequent 4-h killing assay. Mean ± SEM of triplicate wells from one representative experiment of three is shown. (D) Inhibition of T cell proliferation by MDSCs from 4T1-bearing mice. CD11b+ splenic MDSCs were isolated, as described in Materials and Methods. T cell proliferation was measured by thymidine incorporation assays on day 1. Mean ± SEM of duplicate wells from one representative experiment of three is shown. (E) Enhanced iNOS expression in B7-H4 KO MDSCs. Quantitative PCR analysis of iNOS and gp91 transcripts was performed on CD11b+ splenic MDSCs and CD11b− spleen fractions from WT (n = 6) and KO (n = 6) mice, and fold change relative to WT was calculated, as described in Materials and Methods. qPCR data were mean ± SEM and show one of two independent experiments with similar results. (F) iNOS inhibitor abrogates suppressive function of BM-MDSC. BM-MDSCs were cocultured with CFSE-labeled T cells and treated with 0.5 mM iNOS inhibitor L-NMMA, as described in Materials and Methods. Cells were harvested on day 3 of the coculture and stained with anti-CD4 or anti-CD8. Data represent CD4 T cells, whereas CD8 T cells showed similar results. Mean ± SEM of triplicate wells from one representative experiment of two is shown. *p < 0.05, **p < 0.01, ****p < 0.0001.

3.4 Gemcitabine treatment differentiates tumor growth rates

Our model predicts that B7-H4's dual inhibitory effects on T cells and MDSCs counterbalance each other, leading to equal tumor growth rates in WT and B7-H4 KO mice. We reasoned that inhibition of MDSCs would accentuate the impact of elevated antitumor T cell responses and consequently change tumor growth patterns. To test this idea, we treated 4T1 tumor-bearing mice with gemcitabine, a chemotherapeutic drug that preferentially inhibits MDSCs in addition to its tumoricidal effects (39). As expected, 4T1 tumors grew slower in mice that were treated with gemcitabine compared with untreated mice, with concomitant reduction in iNOS expression (Fig. 4A, 4B). Remarkably, we noticed that, upon gemcitabine treatment, 4T1 tumor growth was more drastically reduced in B7-H4 KO hosts compared with that in WT hosts. The difference in tumor growth rates was well correlated with signs of elevated IFN-γ expression (Fig. 4C). These data strengthen our view that MDSCs negate the enhanced antitumor T cell activities in B7-H4 KO hosts, and that a combination of gemcitabine and B7-H4 blockade may be a good therapeutic option.

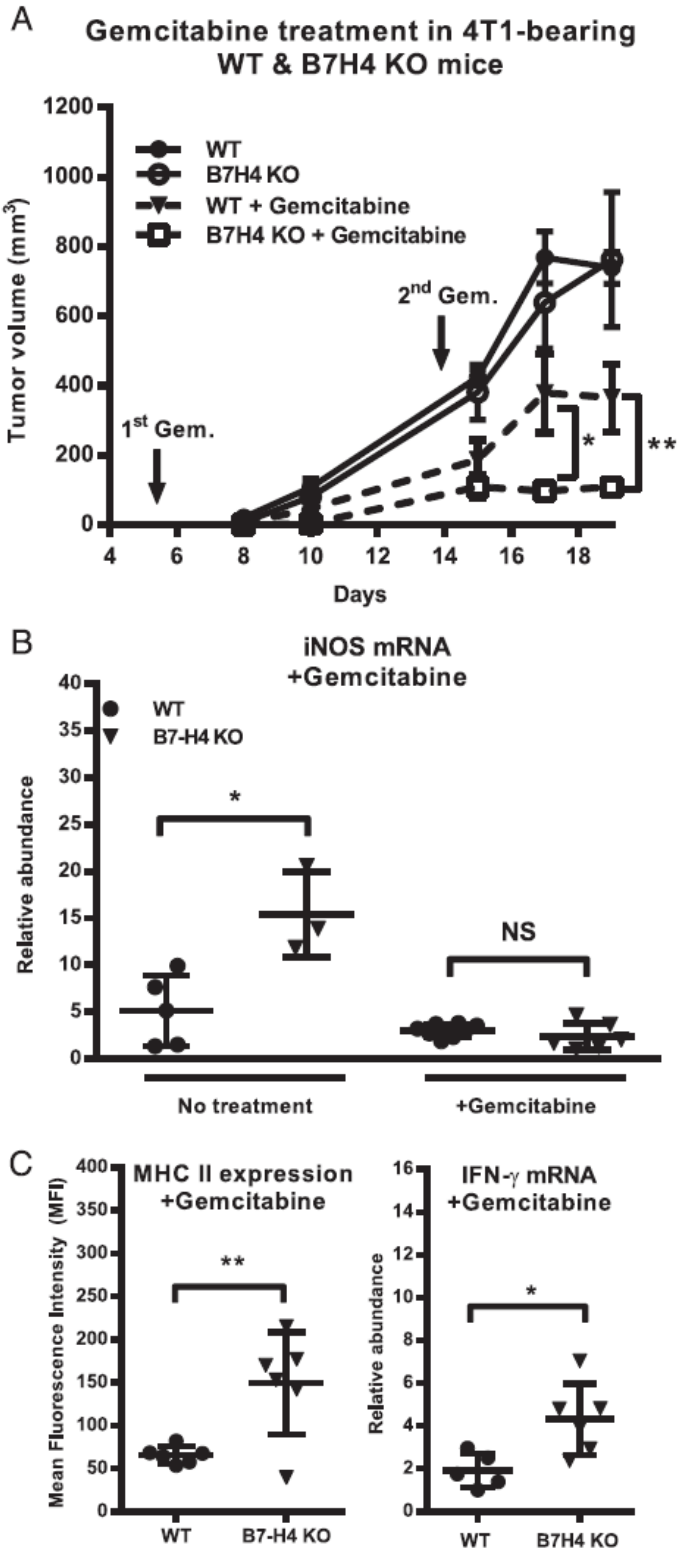

Figure 4. Differential tumor growth in gemcitabine-treated mice.(A) Kinetics of 4T1 tumor growth in WT and B7-H4 KO hosts treated with gemcitabine. Mice were treated with gemcitabine on days 5 and 14 post-4T1 injection. The size of tumors was measured and converted into volume, as described in Materials and Methods. Mean tumor volume ± SEM is shown. (B) Gemcitabine treatment equalizes iNOS expression in WT and KO mice. Tumor lysates from WT and KO mice treated with or without gemcitabine were analyzed via quantitative PCR for iNOS transcripts, and the relative abundance was calculated, as described in Materials and Methods. (C) Enhanced T cell response is intact in gemcitabine-treated B7-H4 KO mice. Surface expression level of MHC II on 4T1 tumor cells was determined by gating on viable tumor cells (7AAD−CD45−) via flow cytometry. Tumor lysates were also taken from WT and KO mice treated with gemcitabine and analyzed via qPCR for IFN-γ transcripts. Relative abundance was calculated, as described in Materials and Methods. All the data depict one of two independent experiments with similar results. Each data point represents one tumor. *$p < 0.05$, **$p < 0.01$.

3.5 Host B7-H4 influences the immune-evasive capacity of 4T1 tumors

Accumulating evidence indicates that there is ongoing immune–tumor interactions during tumor progression that shape the immune-evasive nature of tumors (immunoediting processes) (40), such that tumors developed in Rag KO mice grow slower than those developed in WT mice when reinjected into immune-competent WT hosts (40, 41). Furthermore, an intact T cell compartment has been shown to be important in preventing occult tumor cells from outgrowing (42). We reasoned that if augmented immunosuppressive components prevail over antitumor immune components in B7-H4 KO hosts, 4T1 tumor cells that have grown in B7-H4 hosts should grow slower when reinjected into immune-competent WT hosts compared with 4T1 tumors that have grown in the presence of B7-H4

To test this possibility, 4T1 primary tumors were extracted and selected in media containing 6-thioguanine. Pure populations of 4T1 cells that had grown in either B7-H4 KO or WT mice were reinjected into WT mice. Remarkably, tumors that had grown in the absence of B7-H4 (immunoedited in B7-H4 KO, I.E. KO) grew substantially slower than tumors that had grown in B7-H4–sufficient host (I.E. WT hereafter) (Fig. 5A, left). Consistent with this, when mice were sacrificed after 13 d, the weights of I.E. KO tumors were >3 times less than I.E. WT tumors (Fig. 5A, right). To rule out the possibility that I.E. KO tumors intrinsically proliferate at a slower rate than I.E. WT, we performed an *in vitro* proliferation assay. Notably, tumors from both groups had comparable rates of proliferation (Supplemental Fig. 3), indicating that the discrepancies in tumor growth *in vivo* were most likely due to differential immune-evasive capacities stemming from differences in immunoediting during the primary 4T1 growth in either WT or B7-H4 KO hosts.

Next, we tested whether the diminished tumor growth of I.E. KO cells after reinjection into WT hosts correlated with their greater immunogenicity (as a consequence of reduced immune-evasive capacity) by examining the host response toward I.E. KO versus I.E. WT tumors. Indeed, we observed a substantially higher number of CD4 T cells infiltrating into I.E. KO tumors and a marginal increase of T cells in the spleen (Fig. 5B). Consistent with this, higher levels of transcripts for immune

factors involved in antitumor responses were detected in I.E. KO tumors, as follows: IL-2, IL-12, T-bet, granzyme B, and perforin (Fig. 5C). Taken together, these data suggest that the enhanced immunosuppression observed in the primary 4T1 tumor growth in B7-H4 KO mice allowed for the expansion of 4T1 cells with diminished immune-evasion capacities and higher immunogenicity, which led to slower growth when reinjected into immune-competent hosts.

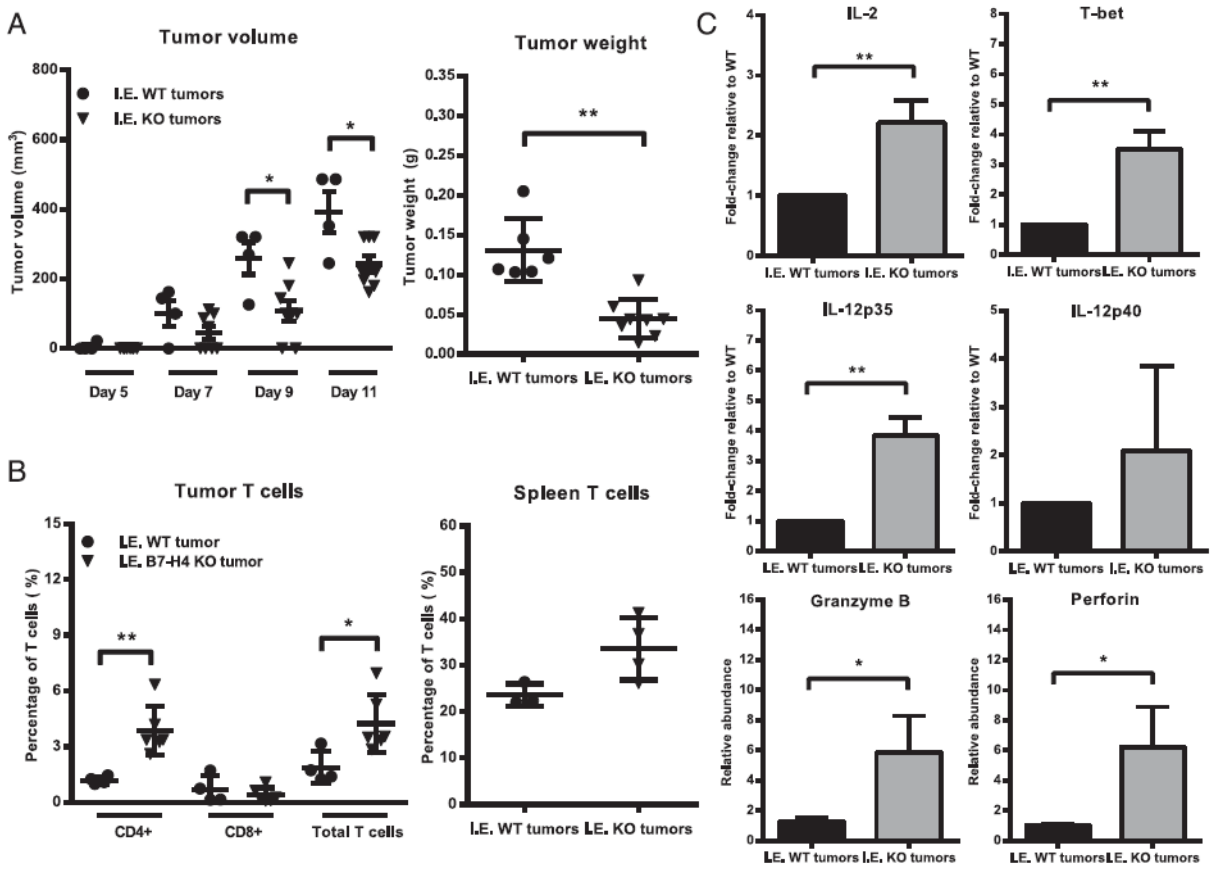

Figure 5.Host B7-H4 influences the immune-evasive capacity of 4T1 tumors.(A) 'Immunoedited' tumors from B7-H4 KO mice (I.E. KO tumors) have smaller volume (Left) and diminished final tumor weight (Right, day 13) compared to immunoedited tumors from WT mice (I.E. WT) during secondary growth in WT hosts. Data depict one representative experiment of three independent experiments with similar results. Each data point represents one tumor. (B) I.E. KO tumors re-injected into WT hosts recruit more T cells into the tumor milieu and spleen. The percentage of T cells among the live immune cells (7AAD-CD45+) was calculated. Data depicts one of three independent experiments with similar results. Each data point represents one tumor. (C) I.E. KO tumors are more immunogenic compared to I.E. WT tumors, as indicated by increased transcripts of anti-tumorigenic cytokines and factors in the tumor microenvironment. Tumor lysates from WT (n = 4 tumors) and KO (n = 5 tumors) were analyzed via quantitative PCR for IL-2, T-bet, IL-12p35, IL-12p40, granzyme B, and perforin transcripts. Data were mean ± SEM and depicts one of two independent experiments with similar results. * $p< 0.05$, ** $p< 0.01$.

3.6B7-H4-mediated immunosuppression primarily targets T cells *in vivo*

To determine the immune components that were responsible for the slow growth of I.E. KO 4T1 tumors compared with I.E. WT counterparts, I.E. KO and I.E. WT 4T1 cells were injected s.c. into the flanks of NOD-scid IL-2Rγnull (NSG) mice that lack T cells and NK cells, two main antitumor immune cell subsets. Strikingly, I.E. KO and I.E. WT tumors displayed similar growth kinetics and comparable final weights (Fig. 6A). Consistently, tumor immune cell infiltration (CD45+) was also similar, as was the percentage of MDSCs within the tumor milieu (Fig. 6A). These results demonstrate that it is the immune pressure that differentiates the secondary growth of I.E. KO versus I.E. WT 4T1 tumors. More importantly, based on the phenotype observed in NSG mice, the immune cell components responsible for the immune–tumor interactions are most likely T and/or NK cells as opposed to myeloid cells.

To further distinguish the contributions of T cells and NK cells, we repeated the experiment in BALB/c nude mice, which lack only T cells, but have an intact NK cell compartment (43, 44). Akin to our previous observations with NSG mice, I.E. KO and I.E. WT 4T1 tumors grew at similar rates, and also had comparable final tumor weights (Fig. 6B). Furthermore, little differences were observed in the total immune cell population as well as MDSCs and NK cells infiltrating into the tumor microenvironment (Fig. 6B). We further confirmed that the growth rates of I.E. WT and I.E. KO 4T1 cells were equalized in host BALB/c mice that were depleted of CD4 or CD8 T cells by Ab treatments. In contrast, depletion of NK cells did not abrogate the difference (Fig. 6C). Thus, these data strongly implicate a role for T cells, but not NK cells, in mediating the differential growth of immunoedited 4T1 tumors in immune-competent WT hosts. This is consistent with our view that, during the primary 4T1 tumor growth, MDSC-mediated suppression of antitumor T cell activities in the tumor microenvironment of B7-H4 KO mice facilitates an outgrowth of 4T1 cells that have diminished immune-evasive capabilities against T cells. Also congruent to this finding is the pronounced inhibitory effect of MDSCs from B7-H4 KO mice toward T cells as opposed to NK cells *in vitro*.

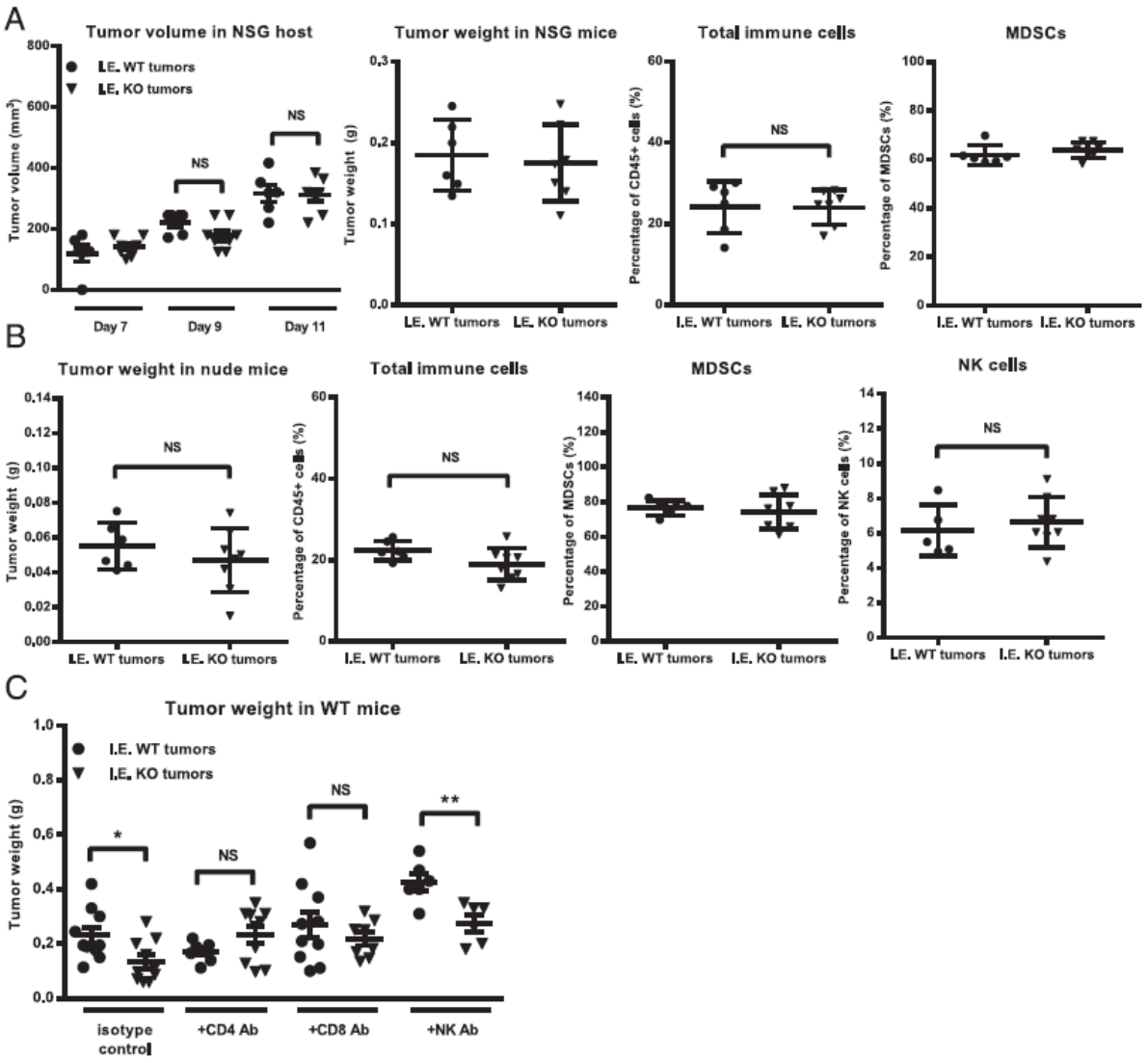

Figure 6. I.E. WT and I.E. KO tumors grow equally in the absence of T cells.(A) I.E. KO and I.E. WT tumors grew at similar rates when grown in NSG hosts. In these tumors, similar levels of total immune cells (CD45+) and MDSCs (CD11b+Gr-1+) are found. Data represent six I.E. WT tumors from three mice and eight I.E. KO tumors from four mice. Each data point represents one tumor. (B) No differences in final tumor weights (day 12) were observed when I.E. KO and I.E. WT tumors grew in BALB/c nude mice. Within these tumors, similar levels of total immune cells (CD45+), MDSCs (CD11b+Gr-1+), and NK cells (CD3−DX5+) were recruited. Data represent six I.E. WT tumors from three mice and eight I.E. KO tumors from four mice. Each data point represents one tumor. (C) Selection pressure on I.E. KO tumors in WT host is primarily mediated by T cells. Final tumor weight is depicted on day 12 in WT mice depleted of CD4, CD8, or NK cells. Data show one representative of two independent experiments. Each data point represents one tumor. *$p < 0.05$, **$p < 0.01$.

4. Discussion

To date, the elevated expression of B7-H4 protein has been well documented in human cancers, yet little is known as to how B7-H4 functions to influence tumor growth. Our study provides new insight into the role for B7-H4 in shaping the tumor microenvironment. In addition to confirming that B7-H4 has a negative regulatory role in T cell responses during antitumor immunity we showed in this work that B7-H4 also has the capacity to hinder the function of MDSCs. This is supported by the data that 4T1 tumors in B7-H4–deficient mice have increased T cell– and MDSC-associated transcripts. Furthermore, treatment with the chemotherapeutic drug gemcitabine, which is known to suppress MDSCs in addition to its antitumor effects, accentuated elevated IFN-γ responses in B7-H4 KO mice and concomitantly delayed tumor growth in B7-H4 KO hosts to a greater extent than it did in WT hosts.

Although the opposing effects of antitumor T cells and protumor MDSCs led to no changes in the growth of primary 4T1 tumors, they significantly influenced the immunoediting process. This was evident, as a secondary injection of 4T1 cells that have grown in B7-H4 KO mice into WT hosts resulted in slower tumor growth and greater immunogenicity relative to 4T1 tumors grown in WT mice. Differences in tumor growth were abrogated when 4T1 cells from either group were injected into T cell–deficient or T cell–depleted mice, supporting the notion that 4T1 tumors that had grown in the absence of host B7-H4 developed reduced resistance to T cell–mediated immune attack. This indicates that the enhanced MDSC suppression in B7-H4 KO mice is mainly targeted toward T cells in vivo, consistent with results from in vitro MDSC suppression assays.

To date, the putative B7-H4 receptor has been detected at a low level on the surface of activated T cells by staining with B7-H4-Fc (2, 7), but its identity remains elusive. In addition, the expression pattern of B7-H4 varies in myeloid cells. For example, B7-H4 is highly expressed on the surface of tumor-associated macrophages in human ovarian cancer and plays a key role in inhibition of antitumor T cell responses (4). However, neither B7-H4 nor the putative B7-H4 receptor was visualized in murine neutrophils

despite a clear negative role for B7-H4 in the expansion of neutrophils during Listeria infection (10). Similarly, we had difficulties in detecting B7-H4 or the putative B7-H4 receptor on the surface of MDSCs. Presumably, these proteins are expressed at low levels in most conditions. Identification of the B7-H4 receptor and elucidating its signaling mechanisms should facilitate better understanding of B7-H4 function.

The relative importance of B7-H4 expression in tumor cells versus host immune cells has also been speculative. In the setting of antitumor immunity, B7-H4 overexpression on tumor cells was thought to play a dominant role. It is possible that abundant B7-H4 proteins on the surface of tumor cells can impair the effector functions of tumor-infiltrating lymphocytes akin to a molecular shield model proposed for PD-L1 (45, 46). In support of this concept, it has been shown that the quantity of B7-H4 on the surface of pancreatic β cells positively correlates with their resistance to T cell attack in murine models of type I diabetes (9). However, our data have shown that, regardless of tumor B7-H4 expression, host B7-H4 still contributes to differences in both pro- and antitumor immune components, which drives the differences observed in immunoediting between WT and B7-H4 KO mice. In keeping with this, B7-H4 deficiency or blockade also led to augmented T cell responses during experimental autoimmune encephalomyelitis (2, 9) and anti-*Leishmania* responses (8), situations in which B7-H4's role should be pronounced during T cell priming. Given that host B7-H4 affects the antitumor T cell immunity with little B7-H4 in tumors in our 4T1 model, B7-H4–mediated immunotherapies need to be considered for patients even without B7-H4 overexpression.

There is some evidence that B7-H4 may play immune-independent, tumor-intrinsic roles in tumorigenesis. An ectopic overexpression of B7-H4 in human ovarian cancer cells led to enhanced tumor growth in SCID mice (13). In the same study, knockdown of B7-H4 in human breast cancer cells rendered them more susceptible to anoikis *in vitro*, although the mechanism was not clear (13). We also found that ectopic overexpression of B7-H4 in immortalized murine mammary epithelial NMuMG cells partially protected them from anoikis (J. Leung and W.-K. Suh, unpublished observations). In contrast, Ab-

mediated ligation of surface B7-H4 in EBV-transformed human B cells or B cell lymphoma cell lines induced apoptosis or cell cycle arrest, respectively (47, 48). Ligation of B7-H4 overexpressed in NMuMG cells, however, did not cause apoptosis or cell cycle arrest (J. Leung and W.-K. Suh, unpublished observations). Therefore, these apparently conflicting data suggest that B7-H4 may regulate cell death or proliferation in certain types of cells. In an attempt to address the role of B7-H4 expressed in tumor cells in our model, we sought to overexpress B7-H4 in 4T1 cells by transfection, but failed to obtain 4T1 clones overexpressing B7-H4 despite an extensive effort. Obviously, further investigation is required to establish the tumor-intrinsic and -extrinsic roles of B7-H4 during in vivo tumor progression. We are currently developing mouse models to address these questions.

Many types of solid tumors display abundant expression of B7-H4 to various extents, and the majority of studies have found a link between B7-H4 overexpression and poor prognosis as judged by invasiveness, metastasis, recurrence, and mortality (15, 18–25). Given that another inhibitory B7 protein, PD-L1, has been known to be overexpressed in multiple cancers and blockade of PD-1/PD-L1 pathway is currently in clinical trials (49), comparisons between B7-H4 and PD-L1 can provide insight as to how B7-H4 overexpression may influence tumor progression and immunotherapeutic outcomes. Notably, a recent study revealed that PD-L1 is preferentially expressed in human melanoma subtypes driven by BRAF mutations and PD-L1 expression is geographically colocalized with CD8 tumor-infiltrating lymphocytes (TILs) and IFN-γ (50). Therefore, at least in melanoma patients, PL-L1 overexpression appears to be induced as an adaptive mechanism in response to immune attack, but not driven by oncogenic processes such as loss of PTEN that is known to upregulate PD-L1 expression in gliomas (51). Paradoxically, this study also showed that among the patients with metastatic melanoma who received immunotherapies, PD-L1 overexpression is positively correlated with overall patient survival, contrasting previous studies that showed no difference or a negative correlation (52, 53). This is consistent with a newly emerging notion that a pre-existing T cell–inflamed tumor microenvironment (which correlates with upregulation of PD-L1 and possibly other immunosuppressive markers)

predicts better responsiveness to immunotherapies (54). Unlike PD-L1, however, several pieces of evidence suggest that B7-H4 overexpression can be largely driven by oncogenic processes rather than antitumor immunity. First, in contrast to the expression patterns of PD-L1, a small study on melanoma patients found that a high level of B7-H4 was not associated with the degree of CD8 T cell infiltration (24). In fact, several reports documented that B7-H4 expression in other types of tumors negatively correlated with the number of TILs (12, 18, 22). Relevant to this, we found in this study that B7-H4 is not highly induced in 4T1 tumor cells under conditions in which PD-L1 and MHC class II were abundantly expressed, presumably in response to IFN-γ–producing T cells. Second, expression of B7-H4 in human breast cancer cell lines has been shown to be dependent on phosphoinositide 3-kinase/mTOR/S6kinase signaling, a pathway frequently altered in cancer (55). Third, human B cells express a high level of B7-H4 upon EBV-mediated transformation in vitro without apparent immune attack (47). Taken together, these findings suggest that overexpression of B7-H4 in cancer cells could be largely an outcome of oncogenic processes and may be associated with the low immunogenic nature of the developing tumor. Therefore, more detailed studies are required to validate B7-H4's value as a predictive biomarker and to optimize therapeutic strategies targeting B7-H4, as overexpression of negative immune modulators may not always predict poor prognosis and immunotherapeutic outcome. Based on what is now known for PD-L1, it is important to examine B7-H4 expression patterns with regard to the tumor subtypes, geographical distributions of TILs/inflammatory cytokines, and the medical history of the patients. In addition, our current study suggests that examination of the relative locations of B7-H4–overexpressing tumor cells, TILs, and MDSCs in the tumor microenvironment may provide additional insights as to how B7-H4 works in human cancer.

Most studies to date have implicated B7-H4 as a biomarker of tumors with poor prognosis (13, 15, 18–25). As such, B7-H4 blockade has been suggested in the treatment of cancers, especially those with elevated B7-H4. Our data show that, in addition to inhibiting T cell responses, B7-H4 also negatively regulates MDSCs, and thus, inhibition of B7-H4 may result in immunosuppression as we have seen in our 4T1

model. Importantly, these differences are driven by a differential expression of B7-H4 in the host immune cells. We also analyzed anti- and protumor immune responses in WT and B7-H4 KO hosts (C57BL/6 background) using a B16F10 murine melanoma (B7-H4–negative) transplantation model. Consistent with our 4T1 model, we observed increased IFN-γ expression in B7-H4 KO hosts (data not depicted). However, the level of MDSC infiltration and iNOS expression was lower in the B16F10 model compared with the 4T1 model (~28% of CD45+ cells in B16F10 versus ~70% of CD45+ cells in 4T1 model), and there was no significant increase of MDSC/iNOS expression in B7-H4 KO mice. One possibility to explain this is that the dual inhibitory effects of host B7-H4 become pronounced for tumors that induce strong MDSC responses. Therefore, our results suggest that, for tumors that elicit robust MDSC responses, beneficial effects of B7-H4 blockade could be maximized when it is delivered in conjunction with treatments that inhibit MDSCs (e.g., gemcitabine). Also, tumors without B7-H4 overexpression may still respond to such treatments.

5. Materials & Methods

Mice

Six-to-ten week old wild-type BALB/cJ (The Jackson Laboratory) and B7-H4 knockout mice maintained in BALB/cJ backgrounds (N10) were used for all *in vitro* and *in vivo* experiments. Generation of B7-H4 knockout mice has been described previously (8). Eight week old nude mice in Balb/c background (Taconic) and NOD-scid IL-2Rγ^{null} (NSG) mice (The Jackson Laboratory) were used for secondary 4T1 tumor injection experiments. All the animal experimentations were performed based on the animal use protocols approved by the Animal Care Committee of the IRCM.

Cell culture

The murine 4T1 mammary carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). YAC-1 thymoma cells were a generous gift from Dr. André Veillette (IRCM, Montreal). All the cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated FBS (Hyclone), L-glutamine, penicillin/streptomycin, β-mercaptoethanol, and HEPES. Cells were maintained in CO_2

incubator at 37°C in humidified air with 5% CO_2. 4T1 cells were harvested with 0.05% Trypsin-EDTA and viability of cells was determined by trypan blue dye exclusion.

Preparation of MDSC and NK cells

We generated MDSCs from mouse bone marrow cells as previously described (31). Briefly, tibias and femurs of BALB/cJ or B7-H4 knockout mice were extracted and bone marrow was obtained. Red blood cells were lysed with hypotonic solution. Cells were then cultured in complete RPMI medium supplemented with 5 % culture supernatant of GM-CSF-secreting X63Ag8 cells (a gift from Dr. André Veillette) and 40 ng/ml of murine recombinant IL-6 (eBioscience) for 7 days. To obtain NK cells, splenic NK cells enriched by EasySep Negative Selection kit (StemCell Technologies) were expanded for 5 days in RPMI media supplemented with murine IL-2 (1,000 U/ml, Peprotech).

In vivo 4T1 tumor experiments

Early passages of 4T1 tumor cells were harvested from culture and were washed twice with sterile PBS. Fifty thousand viable 4T1 cells in 200 µl PBS were then injected into each flank of female BALB/cJ or B7-H4 KO mice. In some cases, mice were injected with gemcitabine (1.5 mg per mouse, i.p.) on days 5 and 14 following 4T1 injection. To isolate 4T1 cells from tumors grown in mice, tumors were excised and a single-cell suspension was cultured in complete RPMI 1640 medium containing 60 µM 6-thioguanine (Sigma-Aldrich) for 7 d. These selected 4T1 cells were then reinjected into BALB/cJ mice without or with lymphocyte depletion, nude, or NSG mice. Tumor growth was determined by measuring the length and width of growing tumors, and volume was calculated by the following formula: volume = (length × width2)/2. Typically, the final tumors were taken at day 11–21 postinjection for analysis. For T cell depletion, WT mice were injected i.p. with 100 µg anti-CD4 (clone GK1.5; Bio X Cell) or anti-CD8 (clone 2.43; Bio X Cell) on days −2, −1, +2, +6, and +9 prior to or after 4T1 injection (day 0) to deplete CD4 or CD8 T cells. For NK depletion, 20 µl anti-mouse asialo GM1 Abs (Wako) were i.p. injected into mice on days −5, −1, +3, and +9 prior to or after 4T1 injection. Depletion of targeted cell populations was confirmed by FACS analysis using peripheral blood and splenocytes. CD4 and CD8 T cell depletion was >99% effective,

and NK cell depletion was ~70% effective. Injection of isotype control Ab (rat IgG2b) or normal rabbit serum had no effect on tumor growth.

Preparation of single cell suspensions

4T1 tumors were carefully extracted and minced with scissors in 2 ml of Hank's balanced salt solution (GIBCO). Cells and tumor fragments were digested with filter-sterilized collagenase type I (GIBCO, 10 mg/ml) at 37 °C for 1 hour on a platform rocker. Subsequently, cells were washed twice with PBS and were prepared for further analysis. Spleen and draining (inguinal) lymph nodes were excised and made into single cell suspensions by passing the organs through a 70 μM nylon cell strainer. Splenocytes were treated with hypotonic solution to lyse red blood cells.

Flow cytometry

Single cell suspensions prepared as above were washed and resuspended in FACS buffer (1% bovine serum albumin & 0.05% sodium azide in PBS). After treating with Fc-block (5 min on ice), cells were stained with primary antibodies followed by secondary antibodies (20 min at 4°C each). Cells were washed twice with FACS buffer after each staining. Stained cell suspensions were briefly incubated with 7-Aminoactinomycin D (7AAD, BD Pharmingen) and subsequently analyzed using Beckman Coulter Cyan ADP Analyzer.Raw flow cytometry data were analyzed with FlowJo software (Tree Star). Cell populations that are 7AAD- CD45+ were gated as live host hematopoietic cells and 7AAD- CD45- populations were defined as 4T1 tumor cells. Anti-CD45 (30-F11), anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), anti-F4/80 (BM8), anti-CD11c (N418), anti-CD3 (145-2C11), anti-CD49b (DX5), anti-MHC II (M5/114.15.2), anti-PD-L1 (MIH5), anti-CD8 (53-6.7), anti-CD4 (GK1.5) and secondary antibodies were purchased from eBioscience. Anti-Ly6G (1A8) antibody was obtained from Biolegend. Anti-mouse B7-H4 antibody, isotype control and secondary antibody were from R&D Systems.

Quantitative PCR

Total RNA was isolated from tumor single-cell suspensions using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. The names of genes and their

primer sequences are listed in Supplemental Table 1. All reactions were performed as follows: 5 min at 45°C, 3 min at 95°C, 15 s at 95°C, and 1 min at 60°C for 40 cycles. The amount of a given transcript was normalized against the amount of hypoxanthine phosphoribosyltransferase in the same sample. The relative abundance was determined against the least abundant sample (set to a unit) among the group of samples analyzed in the same batch of PCRs. Fold change relative to WT samples was calculated by determining the mean relative abundance of each group, and then dividing values from B7-H4 KO samples by respective WT values.

In vitro NK cell killing assay

To analyze NK cell cytotoxicity in the presence of MDSCs, NK cells were obtained from BALB/cJmice as described above and co-cultured with MDSCsderived from WT or B7-H4 KO bone marrow cells for 4 hours at a ratio of 2:1 or 10:1 (MDSC to NK). Afterwards, ^{51}Cr labeled YAC-1 target cells were added (3 x 10^3cells per well) at varying effector:target ratios. After an additional 4-hour incubation, the supernatants were taken to measure ^{51}Crrelease using a γ-counter (Wizard 1470, Perkin Elmer). Percentage of lysis was calculated over complete lysis of the target cells with 5% Triton X-100 (Sigma-Aldrich).

In vitro MDSC suppression assay

To measure suppressive activities of MDSCs on T cell proliferation, we followed a protocol established by others (32). Briefly, bone marrow–derived MDSCs in complete RPMI 1640 media were added to 96-well plates (at 2-fold serial dilutions starting from 3 × 105 cells/well) that were coated with anti-CD3 (3 μg/ml) and anti-CD28 (2 μg/ml). Afterward, RBC-lysed splenocytes were added to the Ab-coated, MDSC-containing wells (6 × 105 cells/well). Subsequently, [3H]thymidine was added (1 μCi/well) to the wells for the last 8 h of 1- or 2-d culture periods. DNA was harvested onto 96-well filter plates after lysing the cells with water using Filtermate harvester (Packard), and [3H]thymidine incorporation was measured with a microplate scintillation counter (Packard). For iNOS inhibition experiments, WT splenocytes were labeled with CFSE (Invitrogen) and used as responders in cocultures without or with 0.5 mM L-NG-monomethyl-arginine (L-NMMA; Calbiochem). After a 3- or 4-d culture period, cells were

harvested and stained with anti-CD4, anti-CD8, and 7AAD. Viable cells were then gated based on CD4+ or CD8+ expression, and CFSE was analyzed via flow cytometry. The proliferation index was calculated using ModFit software. In some experiments, spleens from tumor-bearing mice were mechanically disrupted, and splenic CD11b+ cells were purified by CD11b microbeads (Miltenyi Biotec). These cells were typically >85% CD11b+Gr1+ MDSCs by FACS analysis. MDSC–splenocyte cocultures were set up as above, and [3H]thymidine was added for the last 8 h of day 1 culture period. Remaining CD11b+ and CD11b− splenocytes were used for quantitative PCR (qPCR) analysis.

In vitro 4T1 proliferation assay

For proliferation assays, 4T1 tumors were made into single-cell suspensions, as described, and selected in 6-thioguanine–containing medium. After selection, equal numbers of 4T1 cells derived from WT or B7-H4 KO hosts were plated in 96-well flat-bottom plates in triplicates and left overnight in culture. On days 1 and 2, [3H]thymidine was added for the last 8 h of incubation periods to measure the proliferation. For IFN-γ stimulation experiments, 1 × 105 4T1 cells were plated in a 96-well plate in duplicates and stimulated with 10 ng/ml murine rIFN-γ (PeproTech) for 24 h. Afterward, 4T1 cells were harvested and stained for MHC II, PD-L1, and B7-H4, and then analyzed via flow cytometry.

Statistical analyses

Prism software was used to determine statistical significance by unpaired Student’s t-tests (two-tailed).

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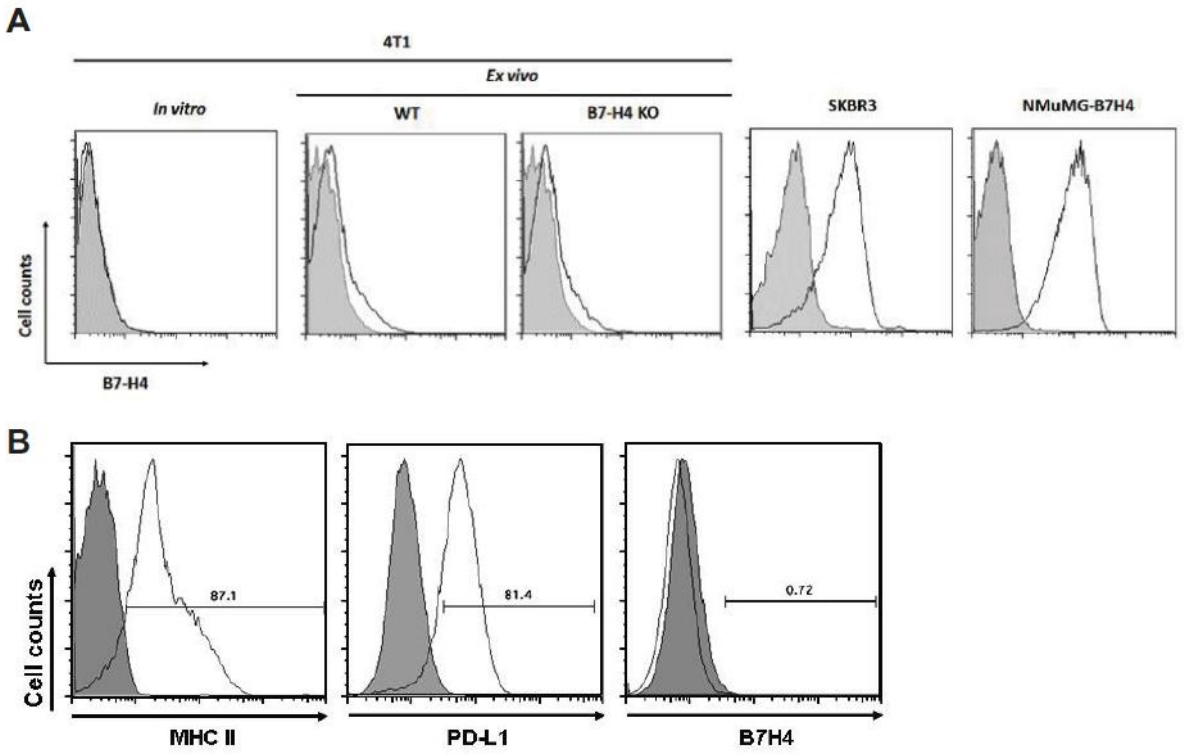
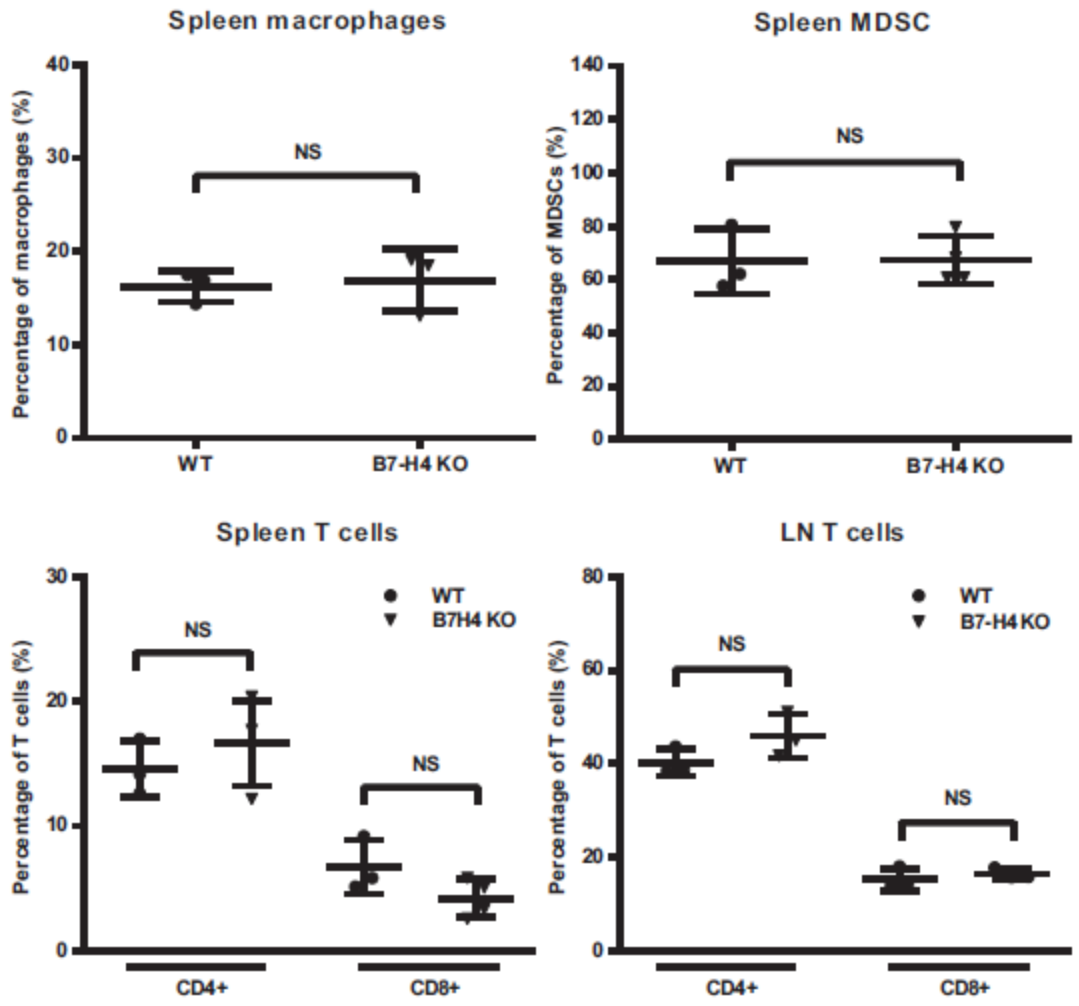
8. Supplementary Figures

Supplementary table 1: List of primers used for quantitative PCR (Tm = 60°C)

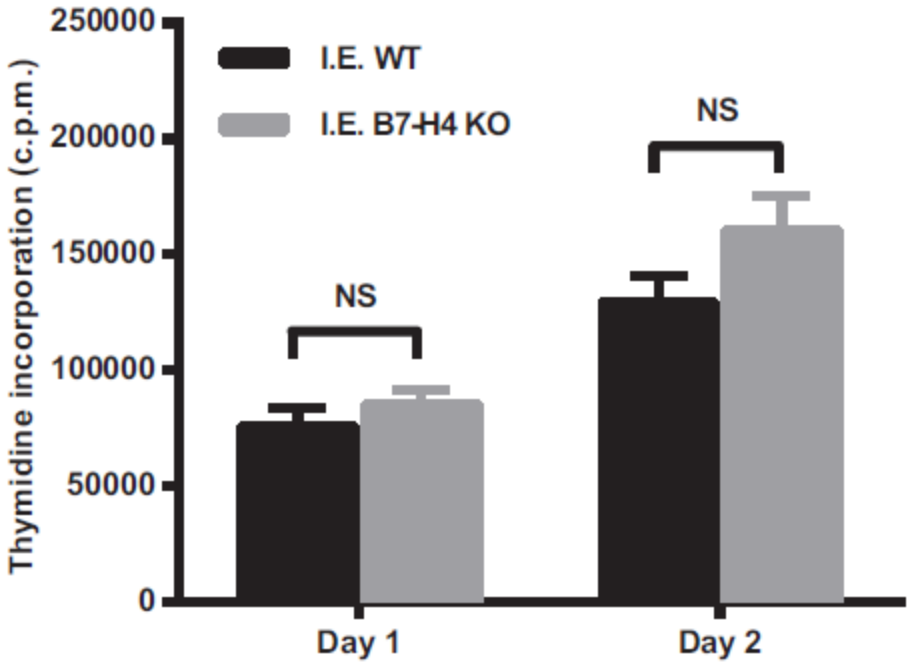
Gene	Primer Sequence
IFN-γ	Forward 5’-AAG GCG AAA AAG GAT GCA TTC-3’ Reverse 5’-CTG GAC CTG TGG GTT GTT GAC-3’
IL-2	Forward 5’-TCA AAT TTT ACT TGC CCA AGC A-3’ Reverse 5’-CCA AGT TCA TCT TCT AGG CAC TGA-3’
HPRT	Forward 5’-CCG AGG ATT TGG AAA AAG TGT T-3’ Reverse 5’-CCT TCA TGA CAT CTC GAG CAA GT-3’
IL-12p35	Forward 5’-AAA TGA AGC TCT GCA TCC TGC-3’ Reverse 5’-TCA CCC TGT TGA TGG TCA CG-3’
IL-12p40	Forward 5’-GGA AGC ACG GCA GCA GAA TA-3’ Reverse 5’-AAC TTG AGG GAG AAG TAG GAA TGG-3’
IL-15	Forward 5’-CAT CCA TCT CGT GCT ACT TGT GTT-3’ Reverse 5’-CAT CTA TCC AGT TGG CCT CTG TTT-3’
T-bet	Forward 5’-GTT CCC ATT CCT GTC CTT C-3’ Reverse 5’-CCT TGT TGT TGG TGA GCT T-3’
Arginase-1	Forward 5’-GAT TGG CAA GGT GAT GGA AG-3’ Reverse 5’-TCA GTC CCT GGC TTA TGG TT-3’
iNOS	Forward 5’-CCA CCT CTA TCA GGA AGA AA-3’ Reverse 5’-CTG CAC CGA AGA TAT CTT CA-3’
Granzyme B	Forward 5’-ATC AAG GAT CAG CAG CCT GA-3’ Reverse 5’-TGA TGT CAT TGG AGA ATG TCT-3’
Granzyme A	Forward 5’-AGA CCG TAT ATG GCT CTA CT-3’ Reverse 5’-CCC TCA CGT GTA TAT TCA TC-3’
Perforin	Forward 5’-GAT GTG AAC CCT AGG CCA GA-3’ Reverse 5’-AAA GAG GTG GCC ATT TTG TG-3’
NKG2D	Forward 5’-ACG TTT CAG CCA GTA TTG TGC-3’ Reverse 5’-GGA AGC TTG GCT CTG GTT C-3’
gp91	Forward 5’-AGC TAT GAG GTG GTG ATG TTA GTG G-3’ Reverse 5’-CAC AAT ATT TGT ACC AGA CAG ACT TGA G-3’



Supplemental Figure 1. 4T1 cells do not express surface B7-H4. (A) 4T1 cells maintained *in vitro* or extracted from WT or B7-H4 KO mice *ex vivo*, SKBR3 cells, or NMuMG-B7H4 cellswere stained with anti-B7-H4 (thick line) or isotype control Ab (shaded) and analyzed by FACS.Cells were gated on 7AAD- populations. (B) 4T1 cells express MHC II, PD-L1 but not B7-H4upon IFN- treatment. 4T1 cells were stimulated with IFN-y for 24 hours (thick line) or notreatment (shaded). After 24 hours, 4T1 cells were harvested and stained with anti-MHC II, PDL1and B7-H4 Ab, and analyzed by FACS. Results shown are representative of two independentexperiments.



Supplemental Figure 2. WT or B7-H4 KO mice bearing 4T1 tumors have similar levels of macrophages (CD11b+F4/80+), MDSCs (CD11b+Gr-1+) and T cells (CD4+/CD8+) in peripheral lymphoid organs. Viable immune cells were analyzed by FACS as described in *Materials and Methods*. Data depict one of three independent experiments. Each data point represents one mouse.



Supplemental Figure 3. Tumors grown in WT or B7-H4 KO mice proliferate at similar rates *in vitro*. Equal numbers of I.E. WT or I.E. KO 4T1 cells were plated in a 96-well plate in triplicates,and [3H]-thymidine was added for the last 7 hours on days 1 and 2. Data show mean ± SEM ofsix I.E. WT and eight I.E. KO 4T1 cell lines and depict one of two independent experiments withsimilar results.

Chapter III

Host B7-H4 restricts anti-tumor T cell immunity to allow the growth of an immunogenic murine mammary carcinoma

Using the poorly immunogenic 4T1 model, I have shown that host B7-H4 suppresses anti-tumor adaptive immunity and inhibits the immunosuppressive capacity of myeloid-derived suppressor cells. While this may explain the lack of difference in tumor growth between B7-H4 KO mice and wild-type controls, B7-H4 has also been demonstrated to be a fine-tuner of T cell immunity, and the poorly immunogenic property of 4T1 tumors may not have induced a strong enough T cell response to manifest the subtle impact of B7-H4. Thus, a highly immunogenic derivative of 4T1, capable of eliciting robust T cell responses, was used to tease out the function of B7-H4 during cancer progression.

Host B7-H4 restricts anti-tumor T cell immunity to allow the growth of an immunogenic murine mammary carcinoma

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1. Abstract

B7-H4, a B7 family inhibitor of T cell activity, is expressed in human cancers and correlates with decreased infiltrating lymphocytes and poor prognosis. In murine models, tumor-expressed B7-H4 enhances tumor growth and reduces T cell immunity, and blockade of tumor-B7-H4 rescues T cell activity and lowers tumor burden. This implicates B7-H4 as a target for cancer immunotherapy, yet limits the efficacy of B7-H4 blockade exclusively to patients with B7-H4+ tumors. We have previously shown that mice lacking host B7-H4 have enhanced anti-tumor profiles, yet similar 4T1 tumor growth relative to control. Given the fine-tuning capacity of B7-H4 and the poorly immunogenic capacity of 4T1 cells, we further investigated the function of host B7-H4 in the growth of an immunogenic derivative of 4T1, 4T1-12B, which expresses firefly luciferase. Notably, B7-H4 KO hosts not only mounted greater tumor-associated anti-tumor T cell responses, but also displayed significantly reduced tumor loads. Additionally, B7-H4-deficiency synergized with gemcitabine to further inhibit tumor development – in some cases, combinatorial therapy led to tumor eradication and the generation of long-term immunological memory. These findings imply that inhibition of host B7-H4 can enhance anti-tumor T cell immunity in immunogenic cancers, and can be combined with other anti-cancer therapies to further reduce tumor burden regardless of B7-H4 tumor positivity.

2. Introduction

Immune evasion is a hallmark of cancer, and therapies targeting immune checkpoints mediated by T cell inhibitory B7 family molecules have shown clinical efficacy in enhancing adaptive anti-tumor responses and reducing tumor burden (1,2). B7-H4, an inhibitory B7 molecule, negatively regulates T cell activity and is expressed on APCs and numerous murine and human tumors (3,4). Several groups have shown that tumors overexpressing B7-H4 have enhanced growth, and that blockade of tumor-expressed B7-H4 could rescue T cell responses and suppress tumor development (3,5,6). These findings implicate a role for targeting B7-H4 in human cancer patients, yet is limited to those whose tumors display B7-H4 positivity. Further, while manipulation of membrane-bound B7-H4 in the tumor may help to reverse T cell inhibition, accumulating evidence has revealed subcellular localization of B7-H4 in multiple cancers, some of which predominantly or exclusively contain cytosolic B7-H4 protein (7-9). Notably, in contrast to surface B7-H4 expression, intracellular B7-H4 protein has not been demonstrated to inhibit T cell activity (7,8), and would not likely represent a target for immunotherapy. Recent data also suggests that hypoxia, through HIF-1alpha signaling, upregulates B7-H4 protein solely in the cytosol of cancer cell lines (10). Given the hypoxic nature of the tumor microenvironment and the tendency for B7-H4 to remain intracellular under these conditions, the impact of B7-H4 blockade in the tumor may be limited, and the detection of tumor-associated B7-H4 protein may prove useful primarily as a diagnostic biomarker.

In contrast to tumor-expressed B7-H4, the function of host B7-H4 has seldom been addressed in the context of cancer, given that immune cells also express functional B7-H4 protein as evidenced by studies with B7-H4-deficient mice (11-13). To date, contradicting data has surfaced regarding the anti- or pro-tumor capacity of host B7-H4. On one hand, B7-H4 expression in non-immune and/or in the tumor was associated with limiting tumor development in MMTV-PyMT mammary tumors and in a murine insulinoma model (14), denoting an anti-tumor role for host B7-H4. The same study also found that breast cancer patients with enhanced B7-H4 positivity demonstrated a longer

time to cancer recurrence. However, consistent with its capacity to negatively regulate T cell immunity, other studies have demonstrated a pro-tumor role for host B7-H4, including one report showing that B7-H4-deficient mice had less lung nodules and greater survival relative to WT mice in an experimental model of metastasis (15). Multiple studies also correlate the expression of B7-H4 in human cancer patients with decreased tumor infiltrating lymphocytes (TILs) and poor prognosis (16-20). Thus, a greater understanding of the function of host B7-H4 during tumor growth will no doubt be invaluable when considering the merit of blocking B7-H4 in human cancer patients.

We previously showed that B7-H4 KO mice displayed stronger anti-tumor cytokine profiles, yet exhibited similar growth of transplanted 4T1 tumor cells compared with WT controls. Since B7-H4 has been implicated as a fine-tuner of T cell immunity based on data obtained from knockout mice, we wondered if this lack of difference in tumor growth may be due to the low immunogenicity of the 4T1 mammary carcinoma cells, which has been previously characterized to (33, 34). To further investigate the function of host B7-H4 in the development of anti-tumor T cell responses and to enhance the efficacy of B7-H4 inhibition on tumor reduction, we used a highly immunogenic derivative of 4T1 that expresses firefly luciferase termed 4T1-12B, which is negative for B7-H4. Since luciferase+ 4T1-12B cells are eliminated in immunocompetent hosts relative to T cell-depleted mice, and because MHC-I-restricted luciferase epitopes have recently been identified, this implies that luciferase can give rise to strong T cell tumor rejection antigens and allows us to examine how B7-H4 modulates the anti-tumor response.

Using the immunogenic 4T1-12B mammary carcinoma model, we show for the first time that host B7-H4 inhibits tumor-associated T cell responses to promote the growth of primary mammary tumors and metastases. Additionally, we also reveal that combinatorial treatment with the chemotherapeutic drug gemcitabine can lead to complete tumor rejection in B7-H4 KO mice, but not in WT hosts. These KO mice are also protected from not only a subsequent re-challenge of 4T1-12B cells, but are also partially protected from a following inoculation with the poorly immunogenic parental 4T1 cells, suggesting a role for B7-H4 in the development of long-term immunological

memory against broad tumor-associated antigens. Taken collectively, these observations suggest that blockade of host B7-H4 may improve anti-tumor T cell immunity independent of tumor expressed B7-H4, and also indicate that combination of B7-H4 blockade and chemotherapy on highly immunogenic tumors may facilitate tumor rejection and protection from recurrence.

3. Results

3.1 4T1-12B tumors have delayed growth and reduced luciferase activity in the absence of host B7-H4

To study the role of B7-H4 in mediating the host immune response during tumor growth, we injected 4T1-12B cells into WT and B7-H4 KO mice and monitored tumor growth and luciferase activity. Strikingly, B7-H4-deficient mice showed markedly reduced 4T1-12B growth kinetics and lower luciferase activity relative to WT mice throughout the course of the experiment (Fig 1A, B & C). Consistently, the final weight of 4T1-12B tumors grown in B7-H4 KO hosts was approximately two-folds less than that of tumors from WT hosts (Fig 1D). In addition, whereas WT mice were observed to have extensive metastases in the liver and spleen, B7-H4 KO mice had significantly less metastatic burden in peripheral organs (Fig 1E, F). Taken together, these observations imply a pro-tumor role for host B7-H4, and also suggest that the impact of host B7-H4 is more pronounced in the presence of a highly immunogenic antigen, as parental 4T1 tumors were previously observed to grow similarly with or without B7-H4.

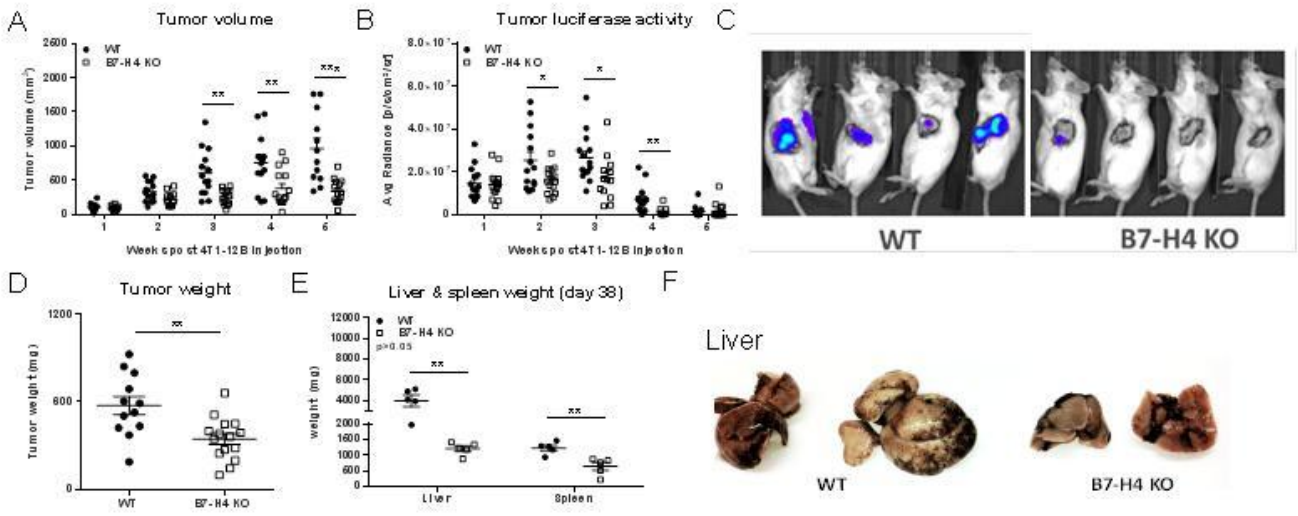

Figure 1. B7-H4 KO mice exhibit slower 4T1-12B tumor growth relative to WT mice. WT & B7-H4 KO mice were injected with $1x10^6$ 4T1-12B cells and tumor growth (A) and luciferase activity (B) were measured weekly. (C) Luciferase activity measured on week 4. Tumor contours were shaded in black to denote tumor area. (D) Final tumor weight was measured after 5 weeks. (E) Liver and spleens were weighed after 5 weeks. (F) A picture of WT and KO livers placed side by side was taken using the iPhone camera, and a photo bleach filter was applied to enhance contrast and clarity. Mean values ± standard error of the mean. All data were analyzed using unpaired two-tailed Student *t* tests. Data were pooled from 2 to 3 experiments, or a representative of 2 or more independent experiments. * *P*< .05; ** *P*< .01; ****P*< .001.

3.2 B7-H4-deficient mice show signs of enhanced anti-tumor immunity

Since B7-H4-deficient mice were seen to have reduced tumor burden relative to WT mice, we next sought to examine the mechanism behind these observations by characterizing the host anti-tumor response. B7-H4 KO mice exhibited enhanced CD8+ T cells in the tumor, and elevated CD4+/CD8+ T cell infiltration in the spleen (Fig 2A & B). Moreover, *ex vivo* 4T1-12B cells from KO mice also revealed a greater proportion and relative expression of MHC I, MHC II and B7-H1 protein compared to controls (Fig 2C & D); since 4T1 and 4T1-12B cells have been previously demonstrated to induce the upregulation of these markers in response to IFN-γ (12,21),figure not shown), it suggests increased levels of biologically active IFN-γ in the tumors of B7-H4 KO mice. Accordingly, qPCR analysis confirmed the 2-fold enhancement of IFN-γ transcripts in *ex vivo* 4T1-12B tumors coming from B7-H4-deficient hosts relative to WT hosts (Fig 2E). This predicts a role for host B7-H4 in the regulation of IFN-γ-producing T cells during tumor growth, and links 4T1-12B tumor regression with enhanced levels of IFN-γ secreting T cells.

In addition to modulating T cell activity, B7-H4 has also been demonstrated to regulate myeloid cells (12,13,15). Using the 4T1 tumor model, we have previously shown that B7-H4 can inhibit the immunosuppressive capacity of MSDCs, and others have also revealed an inhibitory role for B7-H4 in mediating neutrophil expansion(12,13). To test these findings in the 4T1-12B model, we analyzed MDSC infiltration and found similar levels in the tumor milieu of B7-H4-deficient and sufficient mice (Fig 2F). Since the proportion of splenic MDSCs is indicative of tumor burden, MDSC infiltration was significantly decreased in the spleens of B7-H4 KO animals (Fig 2F). To investigate the function of these cells, we next co-cultured *ex vivo* splenic MDSCs from WT and B7-H4 KO tumor-bearing mice with naive splenocytes stimulated with anti-CD3 and anti-CD28 antibodies. We observed no differences in the ability of either MDSC subsets to suppress T cell proliferation (Fig 2G). This may imply the limited influence of B7-H4 in regulating the function of MDSCs in the presence of an anti-tumor response induced by a strong T cell antigen.

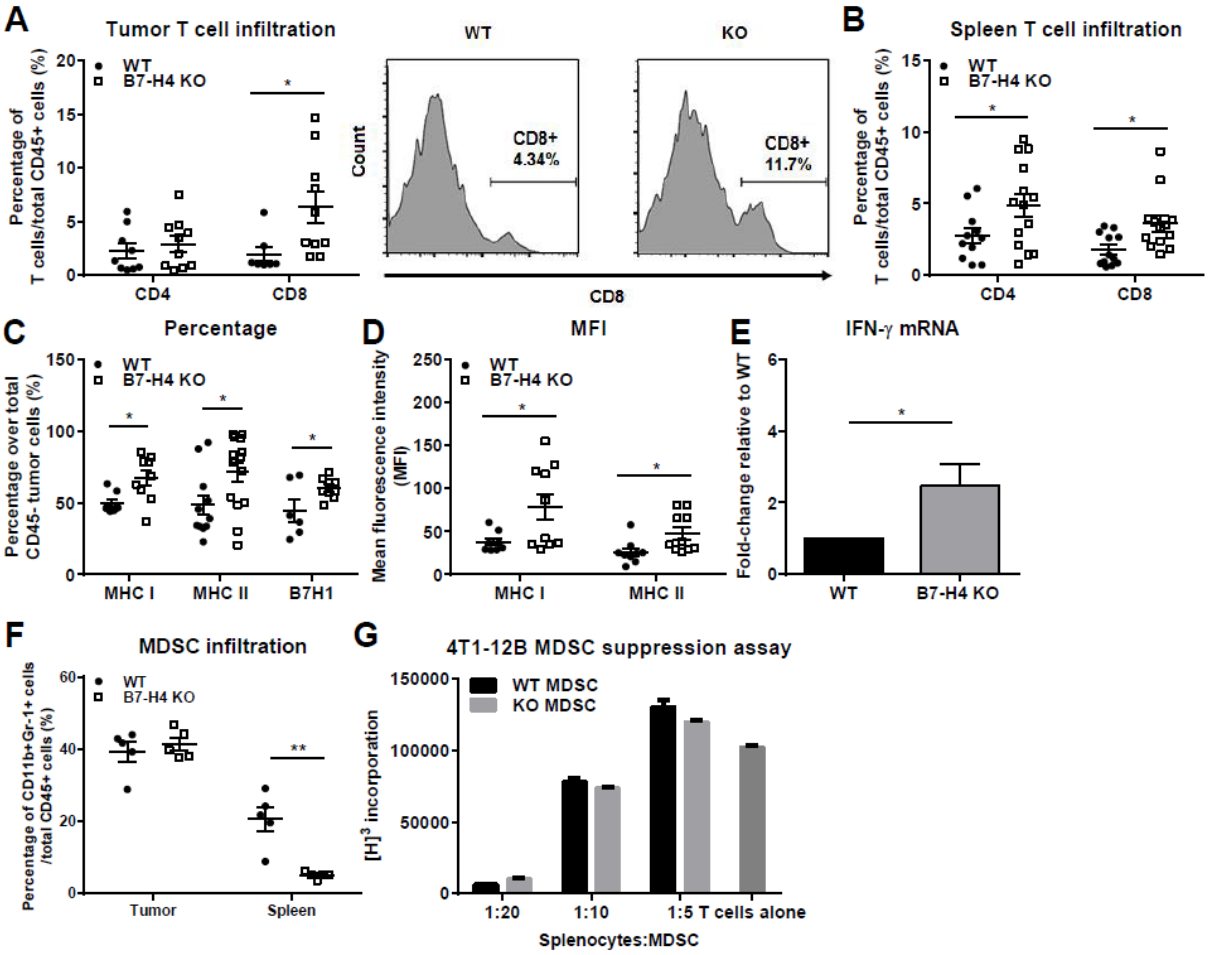

Figure 2. B7-H4 KO mice display enhanced anti-tumor T cell responses relative to WT mice. After 5 weeks, tumor cells (A) and splenocytes (B) from WT or B7-H4 KO mice were harvested, stained and analyzed via flow cytometry for CD4+ and CD8+ T cells, gated on live, CD45+ cells. (C) Percentage of tumor cells positive for MHC I, MHC II & B7-H1, gated on live, CD45- cells. (D) Mean fluorescence intensity of MHC I & MHC II on tumor cells. (E) mRNA was extracted from WT & KO tumors, and qPCR was performed to quantitate the abundance of IFN-γ transcripts in the tumor microenvironment. (F) Percentage of CD11b+Gr-11+ MDSCs gated over total CD45+ cells from tumor and spleen. (G) Ex vivo myeloid-derived suppressors were isolated from splenocytes of WT and B7-H4 KO tumor-bearing mice, and co-cultured with CD3 & CD28-stimulated naïve splenocytes. 3[H] incorporation was used to measure T cell proliferation and MDSC suppression after 1-2 days. Mean values ± standard error. All data were analyzed using unpaired two-tailed Student's t tests. Data were pooled from 2 to 3 experiments, or a representative of 2 or more independent experiments. * P < .05; ** P < .01; ***P < .001.

3.3 B7-H4 KO hosts exhibit increased tumor-associated T cell responses compared to WT hosts

In cancer therapy, the existence of prior or ongoing immune responses predicts better survival and patient outcome. In particular, patients exhibiting tumor-associated T cell activity tend to respond better to CTLA-4 blockade, implying the significance of antigen-specific T cell immunity (2,22). To further examine the capacity of B7-H4 to mediate the anti-tumor response induced by 4T1-12B, we assessed the tumor-associated T cell response elicited in WT and B7-H4 KO hosts. 4T1 & 4T1-12B tumors express AH1, an MHC I-restricted tumor-associated antigen from gp70 of an endogenous murine leukemia virus (23). As AH1-tetramers detect T cells specific to a tumor-associated antigen, we proceeded to stain *ex vivo* tumor cells. Notably, B7-H4 KO tumors were found to contain a slightly greaterpercentage of AH1-specific CD8+ T cells compared with WT samples (Fig 3A & B). We next sought to determine the functional capacity of AH1-specific T cells coming from mice deficient or sufficient for B7-H4. To this end, we acutely stimulated lymphocytes from tumor-bearing mice with AH1 peptides and quantitated the proportion of cytokine-producing T cells. In line with the previous finding, we also observed an increase in the percentage of IFN-γ-secreting CD8+ T cells from B7-H4 KO hosts (Fig 3C). To further confirm the T cell inhibitory role of B7-H4, splenocytes from tumor-bearing WT and KO mice were cultured for approximately one week *in vitro* with IL-2 and AH1 peptides; consistent with the notion that B7-H4 dampens T cell activity, KO splenocytes were enriched with a greater percentage of AH1-specific T cells relative to WT (Fig 3D).

Since 4T1-12B cells express firefly luciferase, we also sought to determine if B7-H4 could influence the generation of T cells recognizing luciferase epitopes. So far, one dominant and two minor T cell epitopes, restricted to H2-K^d, have been identified in Balb/c mice (24). Using synthetic peptides representing these epitopes, we stimulated *ex vivo* splenocytes harvested from tumor-bearing WT and KO mice, and assessed the percentage of IFN-γ+CD8+ T cells recognizing luciferase epitopes on 4T1-12B tumors. Importantly, splenocytes coming from B7-H4-deficient hosts had significantly higher percentages of IFN-γ+CD8+ T cells responding to the dominant luciferase epitope (Fig

3E). Likewise, a similar trend was observed in KO splenocytes responding to the minor luciferase epitopes (Fig 3E). Taken together, these observations imply an inhibitory role for B7-H4 in the generation of 4T1-12B tumor-specific T cell immunity, and suggests that B7-H4 blockade may be effective in reducing cancer burden regardless of tumor B7-H4 positivity, since despite the absence of B7-H4 on 4T1-12B cells, B7-H4 KO animals were nonetheless able to show augmented tumor-associated T cell responses.

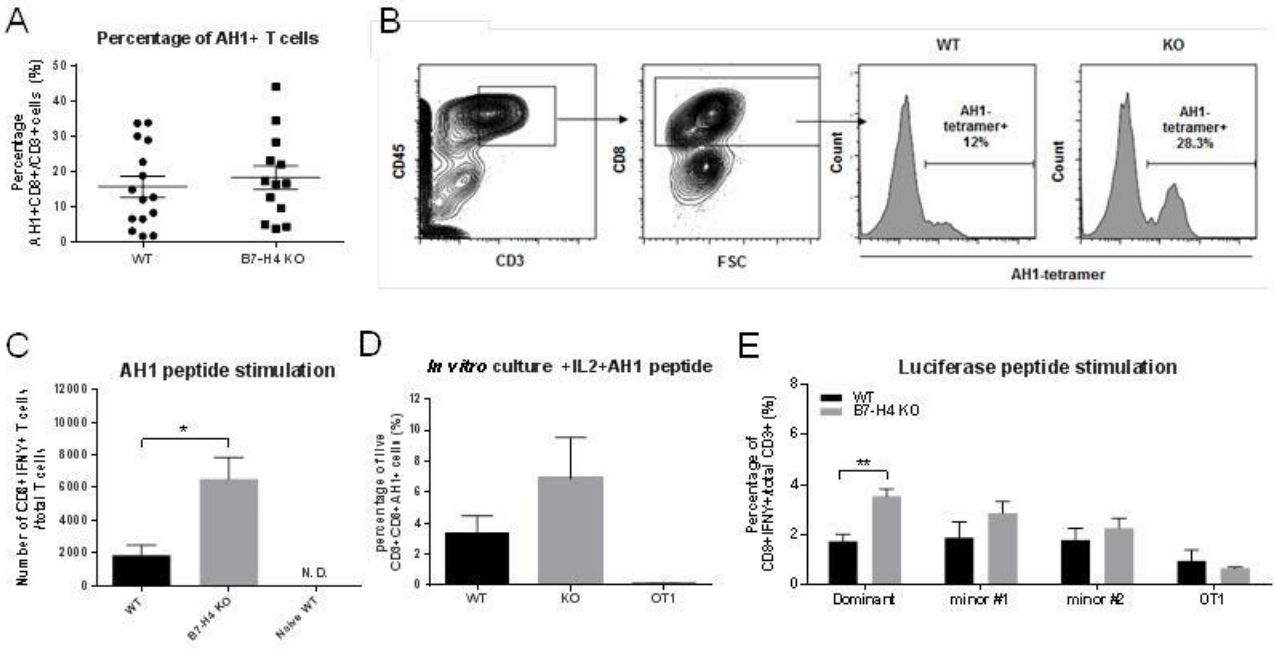

Figure 3. B7-H4 deficiency augments tumor-associated T cell immunity. (A) *Ex vivo* tumors were stained with AH1-loaded H2-Kd tetramers to determine percentage of AH1-specific T cells. (B) Gating strategy for detection of AH1-tetramer+CD8+ T cells in the tumor. (C) *Ex vivo* cells from the draining lymph node of tumor-bearing WT, KO or naïve mice were acutely stimulated in vitro for 5 hours with 10 µg/ml of AH1 peptide in the presence of Golgiplug (BD). Cells were then collected and stained to determine the absolute number of CD8+IFN-γ+ T cells responding to AH1. (D) Splenocytes from WT and KO tumor-bearing mice were cultured for 7 days with IL-2 and AH1 (or OT1 control) peptides *in vitro* prior to staining with AH1-tetramers. (E) Whole spleens were enzymatically dissociated and acutely stimulated *in vitro* for 5 hours with 10 µg/ml of luciferase peptides corresponding to the dominant or minor epitopes (#1 & 2), or with irrelevant control peptide (OT1) in the presence of Golgiplug. Cells were then stained to quantitate the percentage of CD8+IFN-γ+ T cells responding to luciferase epitopes. Mean values ± standard error of the mean. All data were analyzed using unpaired two-tailed Student *t* tests. Data were pooled from 2 to 3 experiments, or representative of 2 or more independent experiments. * *P*< .05; ** *P*< .01; ****P*< .001.

3.4 B7-H4-deficiency synergizes with gemcitabine treatment to further reduce 4T1-12B growth

In light of increasing clinical studies reporting the success of combinatorial immunotherapies in reducing tumor load, we wondered if B7-H4 blockade could also be combined with other chemotherapeutic agents to further enhance tumor regression. To address this question, we treated 4T1-12B tumor-bearing WT and KO mice with gemcitabine, a nucleoside analog currently in use as a chemotherapeutic drug for breast cancer, and which has also been shown to delay 4T1 growth in mice (25). While gemcitabine treatment was able to delay 4T1-12B growth in both WT and B7-H4-deficient hosts, the absence of B7-H4 resulted in a significantly pronounced reduction of tumor burden relative to all other groups (Fig 4A). In addition, we and others have found that gemcitabine treatment was effective primarily during the early stages of tumor development, since late-stage injections did not appear to have as great an effect on reducing tumor growth (Fig 4A, (25).

In some cases, B7-H4 KO mice were able to completely eradicate or contain 4T1-12B tumors; in several experiments, around 20% of KO mice showed no signs of tumor or maintained stable tumor size indefinitely following removal from gemcitabine treatment (figure not shown). To further enhance the impact of B7-H4 deficiency alongside gemcitabine treatment, we repeated the experiment by injecting only a half-dose of 4T1-12B cells into WT or KO recipients. While the difference in tumor growth between KO and WT hosts was similar to previous experiments (Fig 4B), ultimately two-thirds of KO mice administered gemcitabine were able to reject 4T1-12B tumors, which did not occur in WT mice (Fig 4B, C). This strongly suggests that B7-H4 blockade may synergize with chemotherapies, and that the effectiveness of these therapies may be maximized when administered during early phases of tumor growth.

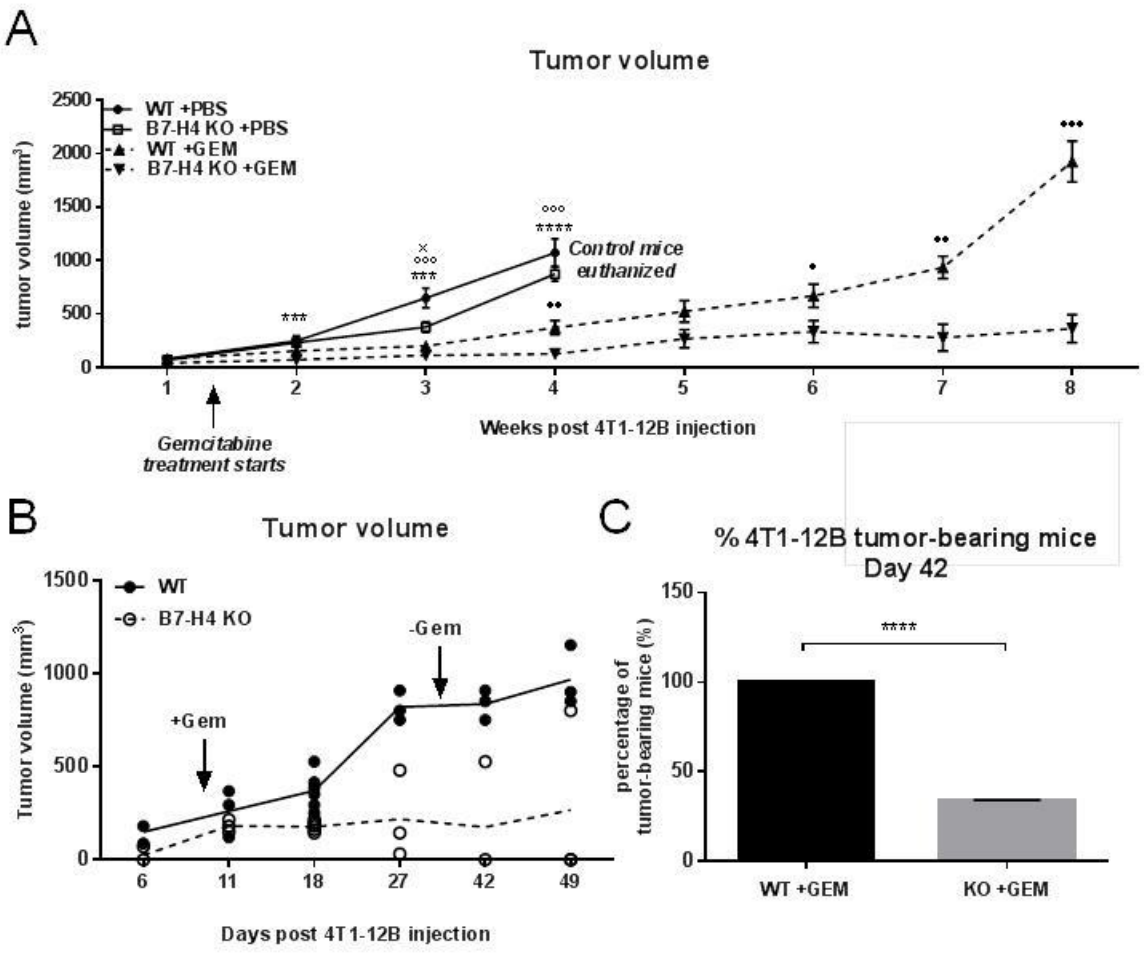

Figure 4. B7-H4 deficiency synergizes with gemcitabine to further reduce 4T1-12B tumor burden. (A) WT and KO mice were injected with 4T1-12B as described. In some groups, gemcitabine (1.5 mg/mouse, intraperitoneal) was administered one week after tumor inoculation. Treatment was repeated every 3-4 days. Tumor volume was measured weekly and statistical significance was evaluated pairwise: x = WT+PBS vs KO+PBS, º = WT+PBS vs WT+gem, * = KO+PBS vs KO+gem, • = WT+gem vs KO+gem. (B) WT and B7-H4 KO mice were injected with $0.5x10^6$ 4T1-12B cells, and treated with gemcitabine (+Gem)between one to six weeks (-Gem). Each circle represents one mouse. (C) After 6 weeks, the percentage of tumor-bearing mice was quantitated. Mean values ± standard error of the mean. All data were analyzed using unpaired two-tailed Student *t* tests. Data were pooled from 2 to 3 experiments, or representative of 2 or more independent experiments. * *P*< .05; ** *P*< .01; ****P*< .001.

3.5 Combinatorial therapy elicits protective anti-tumor immunity against subsequent tumor re-challenge

Since B7-H4 deficiency and gemcitabine treatment led to complete 4T1-12B tumor rejection in some of the mice, we predicted that the adaptive immune response elicited could also protect against a subsequent 4T1-12B re-inoculation. To test this, KO mice that had previously eliminated 4T1-12B tumors were re-challenged with the same tumors. Consistent with the features of adaptive immunity, previously challenged KO mice were refractory to 4T1-12B growth relative to naïve KO hosts (Fig 5A, B), indicating the development of long-term immunological memory. To confirm the role of T cells in 4T1-12B tumor rejection, a third inoculation of 4T1-12B cells was performed following CD8+ T cell depletion in KO mice that had previously rejected 4T1-12B cells (Fig 5C). In the absence of CD8+ T cells, 4T1-12B tumors grew undeterred relative to T cell sufficient KO mice (Fig 5D), indicating that CD8+ T cells are a necessary component of protective anti-tumor immunity in our model.

While 4T1-12B tumors are highly immunogenic and induce a robust T cell response, parental 4T1 cells are poorly immunogenic and do not elicit strong T cell responses. Since 4T1 and 4T1-12B tumors share common antigens, we wondered if the recognition of highly immunogenic antigens on 4T1-12B could provoke and enhance the recognition of poorly immunogenic antigens expressed on parental 4T1 cells, and stimulate immunological protection against poorly immunogenic tumor cells. Thus, following gemcitabine administration and 4T1-12B tumor rejection, B7-H4 KO mice were re-challenged with 4T1 cells. Consistent with the notion of epitope spreading, which frequently occurs in autoimmune diseases, KO mice were partially protected against 4T1 tumor growth relative to naïve KO hosts (Fig 6A, B). Moreover, previously challenged KO mice revealed a higher proportion of tumor-specific T cells in the blood and lymphoid organs (Fig 6C, D), and also demonstrated a greater percentage of tumor-specific T cells following long-term *in vitro* culture with IL-2 and AH1 peptides (Fig 6E). Taken collectively, these data indicate the usefulness of B7-H4 blockade in combination with other chemotherapies to induce protective anti-tumor T cell immunity.

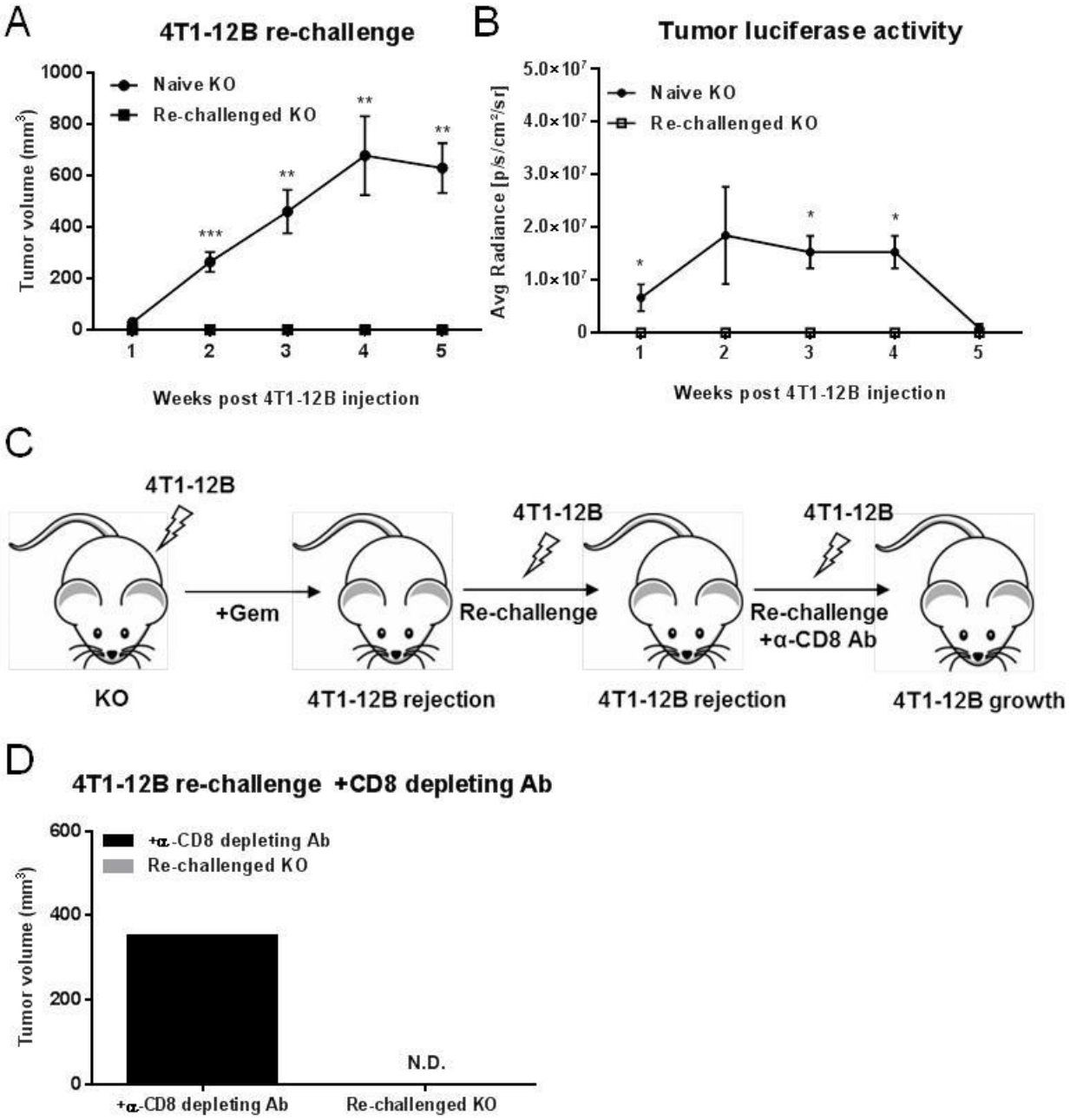

Figure 5. B7-H4 KO mice generate long-lived immunological memory against 4T1-12B cells following gemcitabine treatment.B7-H4 KO mice that had previously eliminated or maintained stable tumor size (less than 3 mm in diameter) were re-challenged with $1x10^6$ 4T1-12B cells on the opposite mammary gland. Tumor volume (A) and luciferase activity (B) were measured every week post re-challenge. (C) Diagram depicting series of tumor inoculation and CD8 T cell depletion. Only in the absence of CD8 T cells can 4T1-12B tumors grow undeterred in previously challenged KO mice. (D) Final tumor volume of CD8 T cell-depleted KO mice re-challenged with 4T1-12B. Mean values ± standard error of the mean. All data were analyzed using unpaired two-tailed Student *t* tests. Data were pooled from 2 to 3 experiments, or representative of 2 or more independent experiments. * *P*< .05; ** *P*< .01; ***P*< .001.

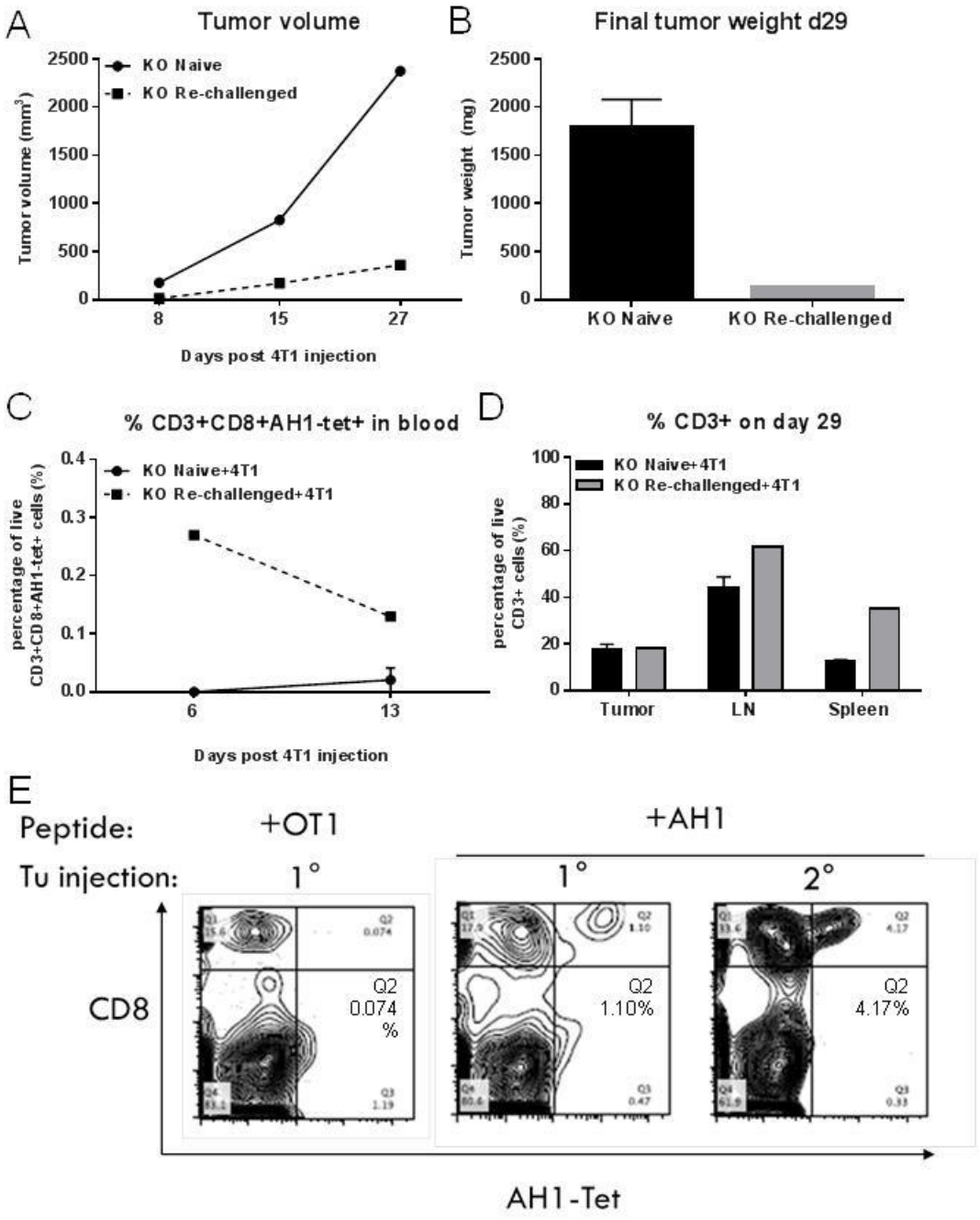

Figure 6. Eradication of 4T1-12B tumors upon gemcitabine treatment in KO mice provides partial protection against 4T1 tumor growth. (A) KO mice that had previously cleared 4T1-12B tumors upon gemcitabine treatment and naïve KO mice controls were challenged with $5x10^4$ 4T1 cells. Tumor volume was measured weekly, and final tumor weight (B) was quantitated after 4 weeks.Percentage of tumor-associated CD8+AH1-tet+ T cells in the blood (C), and percentage of T cells in the tumor and peripheral lymphoid organs (D). (E) Splenocytes from KO naïve or re-challenged mice were cultured for 7 days with IL-2 and AH1 peptides *in vitro* prior to staining with AH1-tetramers. Mean values ± standard error of the mean. All data were analyzed using unpaired two-tailed Student *t* tests. Data were pooled from 2 to 3 experiments, or representative of 2 or more independent experiments. * *P*< .05; ** *P*< .01; ***P*< .001.

4. Discussion

While there is evidence to support both the anti- and pro-tumor role of host B7-H4 in cancer development, our results indicate that in an immunogenic transplantable mammary carcinoma model, B7-H4 inhibits T cell anti-tumor immunity. Consistent with its known ability to negatively regulate adaptive immune responses, we detected increased infiltration of T cells and a greater capacity to respond to 4T1-12B antigens in B7-H4-deficient animals, resulting in diminished tumor growth. These observations are particularly striking, as previous experiments with the parental, poorly immunogenic 4T1 cells revealed similar tumor growth despite signs of enhanced anti-tumor immunity in the tumor microenvironment of B7-H4 KO mice (12). This further supports the notion of B7-H4 as a fine-tuner of T cell activity, as other groups have also observed similar discrepancies: while B7-H4 has been shown to inhibit CD3-stimulated T cell activity *in vitro* (26), and whereas Balb/c B7-H4 KO mice showed enhanced Th1 responses in a *Leishmania major* model, B7-H4-deficient mice in mixed genetic backgrounds elicited normal T cell responses during viral infections and contact hypersensitivity (11). Altogether, these data suggests that the impact of B7-H4 blockade can be most readily observed in circumstances where a robust T cell response can be elicited, such as in murine models involving strong T cell antigens, or in cancer patients with high mutation loads. These findings are reminiscent of studies demonstrating enhanced responsiveness to anti-CTLA-4 therapy in patients showing ongoing immune responses, such as those displaying tumor-specific T cells, sustained ICOS expression and the upregulation of T cell activation markers (27-29). Further, a recent whole-exome sequencing of melanoma patients treated with anti-CTLA-4 antibodies also demonstrated an association between mutational load and the degree of clinical benefit, implicating the significance of neoantigen availability and patient response to checkpoint blockades (30). These observations imply that responsiveness to B7-H4 blockade may also depend upon these factors. Nevertheless, given that the absence of host B7-H4 has been shown to enhance adaptive anti-tumor immunity in both 4T1-12B and 4T1 models, our results suggest that B7-H4 blockade may benefit patients regardless of tumor-expressed B7-H4.

In this study, we also revealed a synergistic effect of combining B7-H4 abrogation with gemcitabine treatment in the reduction of 4T1-12B growth, and showed that this combinatorial therapy could induce complete tumor rejection in B7-H4 KO mice. Since gemcitabine has been established to reduce 4T1 growth and have proven activity in advanced and metastatic breast cancer patients (31), our study argues that B7-H4 blockade may help to reduce tumor load in patients being administered gemcitabine. Notably, considering that B7-H4 acts as a co-inhibitor of T cell response, we wonder if B7-H4 deficiency could also synergize with other immune checkpoint blockades to maximize the rescue of adaptive anti-tumor immunity. The striking clinical efficacy of dual anti-CTLA-4 and anti-PD-1 therapy in advanced melanoma (32) suggests that targeting both early- and late-phase immune checkpoints is advantageous, and supports the notion that abrogating multiple T cell inhibitory pathways can provide beneficial patient outcome. While studies documenting the effects of B7-H4 deficiency with other immune checkpoint blockades in cancer growth have not yet surfaced, one report has shown a synergistic effect of activating both B7-H4 and CTLA-4 in enhancing islet allograft survival (33). This study revealed the non-redundant roles of CTLA-4 and B7-H4, the former whose activity was attributed to the restriction of T cell priming in the lymph nodes, and the latter whose function was in dampening Th1-associated T cell responses. We and others have also observed the enhancement of Th1 activity, in particular the upregulation of IFN-γ in B7-H4 KO mice (11,12); we also provide further evidence for combinatorial regimes involving CTLA-4, B7-H4, and possibly other T cell inhibitors such as PD-1, as targeting multiple and distinct inhibitory pathways have so far shown promise in murine and human systems. Additionally, B7-H4-deficient mice co-treated with gemcitabine that were capable of complete tumor rejection were also afforded protection against secondary 4T1-12B or 4T1 re-challenge. This indicates the development of long-term immunological memory mediated by CD8+ T cells, and may prove beneficial in preventing tumor recurrence in human patients.

While we showed that lack of host B7-H4 can augment T cell anti-tumor immunity, the source of B7-H4 in our 4T1-12B model remains unclear. In normal individuals, B7-H4 mRNA is ubiquitously expressed in non-hematopoietic tissues and protein expression is restricted to immune cells (3); however, it is conceivable that

during cancer development, aberrations in the regulation of B7-H4 expression may occur such that peripheral tissues may upregulate B7-H4 protein and further promote cancer immune evasion. Indeed, it has been demonstrated that IL-6 and IL-10 can induce the expression of B7-H4 on antigen presenting cells (8), and more recently, HIF-1α has also been shown to enhance B7-H4 translation in cancer cell lines, albeit this expression was restricted to the cytoplasm (10). Since IL-6 and HIF-1α are crucial mediators of inflammation, it is plausible that long-term exposure to these factors could promote the translation of B7-H4 transcripts in stromal cells, and facilitate tumor development wherein chronic inflammation is a well-characterized hallmark (1). Overall, our results indicate that in cancer patients with robust, pre-existing immune responses, B7-H4 blockade may prove beneficial regardless of the level of tumor-expressed B7-H4, and can be combined with chemotherapy or other immunotherapies to improve its efficacy in eliciting tumor clearance and long-term protection against tumor recurrence.

5. Materials & Methods

Mice

Six- to 10-wk-old BALB/cJ (The Jackson Laboratory) and B7-H4 KO mice in BALB/cJ backgrounds (N10) were used for all experiments. Generation of B7-H4 KO mice has been described previously (11). All the animal experimentations were performed based on the animal use protocols approved by the Animal Care Committee of the Institut de Recherches Cliniques de Montréal.

Cell culture

Murine 4T1-12B mammary carcinoma cells were a gift from Dr. Gary Sahagian (Tufts University, Boston, USA). Murine 4T1 mammary carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). All cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, L-glutamine, penicillin/streptomycin, 2-ME, and HEPES (Life Technologies). Cells were maintained in CO_2 incubator at 37°C in humidified air with 5% CO2. Cells were harvested with 0.05% trypsin-EDTA, and viability of cells was determined by trypan blue dye exclusion.

In vivo tumor experiments

Early passages of 4T1-12B tumor cells were harvested from culture and were washed twice with sterile PBS. $1x10^6$ viable 4T1-12B cells were subcutaneously injected into the third mammary gland of female BALB/cJ or B7-H4 KO mice. Luciferase activity was measured using the Xenogen IVIS 200 weekly. In some experiments, mice were treated with gemcitabine (1.5 mg/mouse, Sigma Aldrich) intraperitoneally starting on week 1, and treatment was administered every 3-4 days until endpoint. For 4T1 experiments, $5x10^4$ viable 4T1 cells were injected into the third mammary gland of female BALB/cJ or B7-H4 KO mice. Tumor growth was determined by measuring the length and width of growing tumors, and volume was calculated by the following formula: volume = (length × $width^2$)/2. For T cell depletion, WT mice were injected i.p. with 100 µg anti-CD4 (clone GK1.5; Bio X Cell) or anti-CD8 (clone 2.43; Bio X Cell) on days −2, −1, +2, +6, and +9 prior to or after 4T1-12B injection (day 0). Depletion of targeted cell populations was confirmed by FACS analysis using peripheral blood. CD4 and CD8 T cell depletion was >95% effective. Injection of isotype control Ab (rat IgG2b) had no effect on tumor growth (not shown).

Preparation of single-cell suspensions & flow cytometry

Tumors were carefully extracted and minced with scissors in filter-sterilized collagenase cocktail containing 0.05 mg/ml collagenase type I (Life Technologies), 0.025 mg/ml hyaluronidase (Sigma Aldrich), 0.01 mg/ml DNAse I (Sigma Aldrich), 400 U/ml collagenase type IV (Sigma Aldrich), and were digested at 37°C for 45 minutes on a platform rocker. Cells were washed twice with PBS for further analysis. Spleen and draining lymph nodes were excised and made into single-cell suspensions by passing organs through a 70 µM nylon cell strainer. Splenocytes were treated with hypotonic solution to lyse RBCs. For flow cytometry experiments, single-cell suspensions were resuspended in FACS buffer (1% BSA in PBS). After treating with Fc-block (5 min on ice), cells were stained with antibodies (20 min at 4°C). Cells were washed with FACS buffer after each staining. Stained cell suspensions were briefly incubated with 7-aminoactinomycin D (7AAD; BD Pharmingen) and subsequently analyzed using BD LSR Fortessa. For intracellular cytokine staining, fixable viability dye was used to

exclude dead cells, and cells were fixed and permeated with BD Cytofix/Cytoperm according to the manufacturer's instructions (BD). Raw flow cytometry data were analyzed with FlowJo software (Tree Star). Cell populations that are 7AAD− CD45+ were gated as live host hematopoietic cells, and 7AAD− CD45− populations were defined as tumor cells. Anti-CD45 (30-F11), anti-CD11b (M1/70), anti–Gr-1 (RB6-8C5), anti-CD3 (145-2C11), anti-MHC I (34-1-2S), anti-MHC II (M5/114.15.2), anti–PD-L1 (MIH5), anti-CD8 (53-6.7), anti-CD4 (GK1.5), anti-IFN-γ (XMG1.2), Fixable Viability Dye eFluor 450 were purchased from eBioscience. AH1-H-2Ld tetramers were generated by NIH Tetramer Core Facility.

Quantitative PCR

Total RNA was isolated from tumor single-cell suspensions using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. All reactions were performed as follows: 5 min at 45°C, 3 min at 95°C, 15 s at 95°C, and 1 min at 60°C for 40 cycles. The amount of a given transcript was normalized against the amount of hypoxanthine phosphoribosyltransferase in the same sample. Primer sequence for IFN- γ: (forward) 5'-AAG GCG AAA AAG GAT GCA TTC-3'; (reverse) 5'-CTG GAC CTG TGG GTT GTT GAC-3', HPRT: (forward) 5'-CCG AGG ATT TGG AAA AAG TGT T-3'; (reverse) 5'-CCT TCA TGA CAT CTC GAG CAA GT-3'.

Ex vivo peptide stimulation & in vitro cultures

For *ex vivo* peptide stimulation, collagenase-digested cells were cultured with peptides (10 μM) for 5 hours in the presence of GolgiPlug (BD). Cells were then collected and processed for intracellular cytokine staining. For long-term *in vitro* cultures, $1x10^6$ collagenase-digested splenocytes were plated in 1 ml of complete media supplemented with 100 U of IL-2 (Peprotech) and 10 μM of peptide. After one week in culture, cells were harvested and processed for intracellular cytokine staining. AH1 peptides (SPSYVYHQF) were purchased from AnaSpec, and dominant & minor luciferase peptides (GFQSMYTFV and VPFHHGFGM, VALPHRTAC, respectively) (24) were custom synthesized (Peptron Inc., Daejeon, Republic of Korea).

In vitro MDSC suppression assay

Spleens from tumor-bearing mice were mechanically disrupted, and splenic CD11b+ cells were purified by CD11b microbeads (MiltenyiBiotec), typically >85% CD11b+Gr1+ MDSCs by FACS analysis. MDSCs were added to 96-well plates coated with anti-CD3 (3 µg/ml) and anti-CD28 (2 µg/ml). Subsequently, RBC-lysed splenocytes from naïve WT mice were added to the Ab-coated, MDSC-containing wells. [^{3}H]thymidine was added (1 µCi/well) to the wells for the last 8 h of 1- or 2-d culture periods. DNA was harvested onto 96-well filter plates using the Filtermate harvester (Packard), and [3H]thymidine incorporation was measured with a microplate scintillation counter (Packard).

Statistical analyses

Prism software was used to determine statistical significance by unpaired Student's *t* tests (two-tailed).

6. Acknowledgements

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Chapter IV

Discussion

1. Summary: B7-H4 blockade in human patients

Since its initial discovery in 2003, substantial efforts have been made to characterize the function of B7-H4 over the past decade, the majority of which has been focused on its role in tumor growth and its potential as a target for cancer immunotherapy. Consistent with *in vitro* studies and results obtained from knockout mouse models, multiple groups have identified B7-H4 as a negative fine-tuner of T cell immunity during cancer development, and have shown that its abrogation could facilitate the rescue of adaptive immunity and promote tumor regression (1-6). In our hands, we have also observed similar findings, as anti-tumor T cell factors were upregulated in the absence of host B7-H4 in both the poorly and highly immunogenic 4T1 and 4T1-12B models, respectively. However, whereas B7-H4 deficiency significantly reduced 4T1-12B burden, lack of host B7-H4 had minimal impact on the growth of 4T1 tumors, further implicating B7-H4 as a subtle mediator of T cell response relative to other T cell co-inhibitors. Indeed, other groups have also reported modest or no difference in the ability of endogenous B7-H4 to influence the development of poorly immunogenic cancers: in the MMTV-PyMT model, while B7-H4 KO mice displayed differences in tumor size, there was ultimately no difference in the number of tumors between KO and WT mice (7), and in the TRAMP+ model of human prostate cancer, ablation of B7-H4 did not affect tumor progression (8). Our group has also observed comparable tumor growth in a MMTV-NeuT transgenic mammary carcinoma model between WT and B7-H4-deficient hosts (unpublished data). Presumably then, B7-H4 blockade in human cancers could be foreseen to have limited effectiveness, as it is well-established that cancer cells utilize numerous mechanisms to evade immune detection and downregulate tumor-associated antigens (9). Despite this, the efficacy of B7-H4 blockade may be revealed under circumstances where strong T cell responses are observed, such as in the presence of a strong T cell rejection antigen, or perhaps following immunotherapies that elicit anti-tumor T cell immunity such as those seen after CTLA-4 blockade in advanced melanoma patients (refer to previous chapters). It is worth noting that recent studies show an association between the degree of clinical benefit and mutation load in melanoma patients treated with anti-CTLA-4 antibodies (10), and similarly, mismatch-repair defects in colorectal cancer patients treated with

anti-PD-1 reagents also correlated with clinical benefit (11). This points to the notion that patients with high mutation loads, and thus a greater availability of neo antigens, would induce a more robust adaptive immune response and respond better to immune checkpoint blockade. B7-H4 abrogation in these cases may serve to further strengthen and propagate a pre-existing adaptive response to facilitate tumor regression. Therefore, given B7-H4's capacity as a fine-tuner of T cell activity, it is highly likely that the application of a B7-H4 blockade will be best when combined with other cancer therapies.

2. Combinatorial therapy with gemcitabine& current immunotherapies

Indeed, we have demonstrated the effectiveness of B7-H4 deficiency and gemcitabine in reducing both 4T1 and 4T1-12B burden. Since gemcitabine has been demonstrated to have direct tumor cytotoxicity (12), it can be speculated that the killing of tumor cells by gemcitabine would increase the abundance and availability of T cell rejection antigens, and enhance anti-tumor T cell immunity. Consistently, one study showed that the anti-tumor effects of gemcitabine were abrogated in nude mice, suggesting the ability of gemcitabine to modulate the immune response; more importantly, this study also revealed that the efficacy of gemcitabine was dependent on the immunogenicity of the tumor (13). This may provide a mechanism for how gemcitabine may contribute to the enhanced T cell-mediated anti-tumor immunity in B7-H4-deficient mice, and why gemcitabine treatment was more effective in reducing highly immunogenic 4T1-12B tumors relative to poorly immunogenic 4T1 cancers. Further, given that gemcitabine has also been shown to inhibit the expansion of pro-tumorigenic MDSCs in the 4T1 model (14), this may provide yet another mechanism as to how the administration of gemcitabine and B7-H4 blockade may work synergistically. Therefore, it can be hypothesized that this combinatorial regime may prove beneficial particularly for cancer patients with high levels of MDSCs.

In addition to gemcitabine, B7-H4 blockade may also be combined with other immunotherapies to further enhance the anti-tumor immune response. Indeed, one report has shown the non-redundant and synergistic capacity of CTLA-4 and B7-H4 in the survival of islet allografts (15). While studies targeting B7-H4 and other immune

checkpoints during cancer growth have not yet been published, it is conceivable that B7-H4 abrogation would further rescue T cell immunity, particularly in patients with pre-existing immune responses, or with high mutation loads, as previously discussed. In all likelihood, the contribution of B7-H4 blockade would include both its tumor-dependent and tumor-independent functions, since evidence to support an anti-tumor role for targeting B7-H4 in both capacities exists and have been previously discussed. In particular, since we and others have detected a role for B7-H4 blockade in enhancing host anti-tumor immunity in cancer models that do not involve tumor-expressed B7-H4 (5,6), evidently the therapeutic benefits of targeting B7-H4 may not be exclusive to patients whose cancers express B7-H4.

3. Consideration of the anti- and pro-tumorigenic functions of B7-H4 in cancer

Additional challenges to take into consideration when proposing B7-H4 as a potential cancer immunotherapy include the observations that B7-H4 may act as a promoter of anti-tumor immunity; one notable study demonstrated that the absence of B7-H4 limited the anti-tumor response in a murine model of insulinoma, and also showed that B7-H4 deficiency did not affect the LCMV vaccine-induced anti-tumor immunity in an MMTV-PyMT tumor model (7). Mechanistically, the authors also observed a correlation between B7-H4 and the expression of MHC I and granzyme B, suggesting that B7-H4 may regulate the CD8+ T cell response through the induction of these factors. Consistently, analysis of breast cancer tissues revealed improved recurrence-free survival in patients whose tumors exhibited high B7-H4 expression (7), in contrast to the majority of studies that demonstrate a negative association. These data provide a striking contrast to the T cell inhibitory role of B7-H4 as previously reported, and may indicate that the opposing function of B7-H4 may depend on context and tumor models. Further, as the receptor for B7-H4 has yet to be discovered, these contradictory findings may point to multiple receptors expressed on T cells which have the potential to inhibit or activate adaptive immunity depending on context. Since neutrophils have been reported to bind B7-H4-Ig protein to modulate their functions (5,16), the possibility of multiple B7-H4 binding partners may explain the diverse and contrasting capacity of this molecule.

4. Current challenges: development of anti-B7-H4 reagents to target membrane & cytosolic protein

While no human trials involving the blockade of B7-H4 have yet been initiated, evidence in murine tumor models have provided valuable insight as to its efficacy in reversing T cell inhibition and in deterring tumor growth, as mentioned in previous chapters. Based on these reports, it can be speculated that much of the challenge in translating the data from murine models to clinical application stems from the lack of reagents that can sufficiently neutralize human B7-H4. So far, one group has discovered an scFv capable of binding and blocking human B7-H4 in a humanized ovarian cancer model (3), and recently, another study conducted by Genentech has unveiled a novel antibody-drug conjugate capable of targeting human B7-H4 to induce stable tumor regression in xenograft models of B7-H4+ breast cancers (17). To date, B7-H4 remains a prognostic marker in multiple cancers, where in most cases the level of B7-H4 in the tumor site is inversely correlated with the degree of TILs and is associated with worse prognosis. However, in several cancer types including ovarian and renal cell carcinoma, the distribution of B7-H4 consists of not only membrane-bound B7-H4, but also the intracellular localization of B7-H4 in the cytosol and nucleus (18,19). Since intracellular B7-H4 has been shown to be incapable of suppressing T cell responses (18,19), a distinct function of B7-H4 independent of its role in immune evasion may explain its subcellular detainment. Indeed, numerous groups have found an immune-independent capacity for B7-H4 in promoting cancer growth and survival; siRNA knockdown of B7-H4 in breast cancer cells led to enhanced caspase activity and apoptosis, and human ovarian cancer cells transfected with B7-H4 showed protein localization in the cytoplasm that was associated with improved cell adhesion, migration, invasion *in vitro* and greater growth in a xenograft model *in vivo* (20,21). One study also revealed that B7-H4 overexpression in HEK293 cells induced proliferation and promoted G1/S phase transition, and that a point mutation in the nuclear localization signal of B7-H4 abrogated this phenotype, implying that the nuclear distribution of B7-H4 is important for B7-H4-induced cell growth (18). Interestingly, HIF-1α, a transcription factor expressed in hypoxic tumor microenvironments, was shown to preferentially upregulate cytoplasmic B7-H4 which was positively associated with genes

involved in cell proliferation in patients with multiple myeloma (22). Collectively, this provides a rationale as to why subcellular B7-H4 expression is detected in numerous cancer patients, and suggests that the presence of cytosolic B7-H4 may be a result of oncogenic stress and/or a means for cancer cells to cope with stressful conditions such as hypoxia.

While targeting surface B7-H4 may prove ineffective in cases where intracellular B7-H4 is detected within cancer cells, tumor-associated macrophages have also been demonstrated to upregulate B7-H4 on their surface, and targeted depletion of B7-H4+ myeloid subsetsmay represent a novel strategy to elevate anti-tumor responses. In a model of ovarian cancer, abundant IL-6 and IL-10 in the tumor microenvironment was shown to induce the expression of B7-H4 on macrophages, which were found to inhibit tumor-specific T cell immunity (19); in human lung cancer patients, one study also demonstrated a correlation between circulating B7-H4-positive macrophages and clinical grade (24). Notably, exposure to tumor-associated macrophages was observed to upregulate B7-H4 on the surface of lung cancer cells, which was further demonstrated to inhibit T cell responses *in vitro* (25). These data suggest an additional function of targeting B7-H4 in the tumor, as depletion of B7-H4-expressing tumor-associated macrophages with anti-B7-H4 reagents would presumably enhance anti-tumor immunity, regardless of tumor-B7-H4 positivity.

5. Future objectives & concluding remarks

Overall, while accumulating data supports the targeting of B7-H4 in multiple murine and xenograft tumor models, several aspects must be considered prior to the administration of blocking B7-H4 reagents in human patients; firstly, a greater understanding regarding the role of B7-H4 in cancer growth should be ascertained at the very least in murine systems, since both anti- and pro-tumorigenic functions have been reported and previously discussed. The resolution of this controversy may stem from the discovery of the B7-H4 binding partner(s), as multiple inhibitory or activating B7-H4 receptors could explain the opposing pro- and anti-tumor functions of B7-H4. Secondly, a realistic assessment of the impact of B7-H4 blockade in various tumor models suggests that B7-H4 abrogation is most effective only under certain

circumstances; notably, when B7-H4 is over-expressed on the surface of tumors, and in cases where a robust T cell response has been induced by a strong T cell antigen. Although B7-H4 staining has been abundantly detected in numerous human cancers, it must be emphasized that in many cases, B7-H4 is retained intracellularly and displays no inhibitory T cell functions. Intracellular B7-H4 has also been shown to enhance cancer cell survival and growth independent of its immune function. It can then be argued that the administration of anti-B7-H4 reagents would have limited efficacy in patients with low or cytosolic B7-H4 positivity. However, since we and others have shown that host B7-H4 can modulate the anti-tumor response regardless of B7-H4 expression in the tumor, it indicates that B7-H4 may still remain a viable target for cancer immunotherapy. Interestingly, one way to target both surface and intracellular B7-H4 may be available at the genomic level, as one group demonstrated that a B7-H4-specific morpholino that could inhibit B7-H4 expression in macrophages led to enhanced T cell activity and reduced tumor growth (19). Moreover, B7-H4 siRNA was revealed to inhibit the proliferation, invasion and migration of colorectal cancer cells (23). Lastly, given the subtle impact of B7-H4 on T cell activity, the success of anti-B7-H4 monotherapy also appears to depend on cancer immunogenicity; thus the benefits of B7-H4 blockade should be studied in combination with other cancer therapies, particularly those that can elicit strong T cell response such as anti-CTLA-4 therapy, and especially in patients with high mutation loads, as mentioned above. Considering that B7-H4 KO mice do not show signs of spontaneous autoimmunity and display largely normal T cell responses (2), it is plausible that therapies targeting B7-H4 will not induce immune-related adverse side effects, similar to therapies targeting PD-1, and thus reveal B7-H4 as a relatively safe molecule to target in cancer immunotherapy. Taken collectively, the evidence to target B7-H4 in cancer therapy has been demonstrated in multiple murine tumor models, yet controversies regarding its anti-tumor capacity and the subtle phenotypes observed in some disease models beg for further research in order to truly place B7-H4 as a candidate for human cancer immunotherapy, and not merely as a prognostic biomarker.

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Now, if it selectively kills cancer cells in a petri dish, you can be sure it's at least a great breakthrough for everyone suffering from petri dish cancer.

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