INDUCTION OF IMMUNE RESPONSES TO WILMS TUMOR 1 (WT1), A SELF-ANTIGEN, IN MICE

By

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Abstract

WT1, Wilms tumor 1, protein is overexpressed in a wide variety of cancers, including hematopoietic malignancies and solid tumors. WT1 is an attractive tumor-associated antigen to target for cancer immunotherapy as it is not normally expressed in adult human tissues, except in a few restricted locations, including $CD34^+$ cells of the bone marrow, glomerular podocytes of the kidney, Sertoli cells of the testis, and granulosa cells of the ovary. However, because of its critical role in embryonic development and its expression in hematopoietic stem cells (HSCs), WT1 is a self-antigen. High affinity T cells are deleted from the repertoire in the thymus, leaving only low avidity T cells. Activating and expanding T cells that can recognize and kill WT1-expressing cancers has been difficult because the frequencies of WT1-specific T cells are very low. Moreover, these T cells may be anergic or suppressed by regulatory T cells. To obtain WT1-specific T cells from mice, which also express WT1 in the spleen stroma, we used a spectrum of immunization strategies, including vaccination with cDNA, protein, adjuvants, *Listeria* vaccines, WT1-expressing BMDCs, and WT1 overexpressing tumor cell lines. One approach to eliciting functional T cells that detect self-antigens that has been successful is the use of xenogeneic DNA vaccines. This approach works because the antigen is similar enough that cross-reactive epitopes may be utilized, but different enough to circumvent tolerance barriers. We created a consensus sequence DNA vaccine, based on homologous WT1 genes from ten different species. This maintained WT1 exons conserved across species, but was different enough at various amino acid positions to circumvent tolerance. The consensus sequence is 91% identical to the wild type murine

WT1 sequence. Lymphocytes from mice vaccinated with the consensus WT1 DNA vaccine respond to a wild type murine WT1 peptide library. Our findings suggest that using a consensus sequence DNA vaccine is an effective method to produce an immune response to a self-antigen.

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Chapter I: Introduction

Cancer Immunotherapy

The American Cancer Society estimates that there will be 1.6 million new cancer cases this year, and the overall lifetime probability of developing cancer is over 43% in men, and 38% in women[1,2]. Given the estimated prevalence of cancer, it is vitally important to develop therapies to treat cancer. While surgery, radiation and chemotherapy have had success in treating a variety of cancers, they also have deleterious side effects, may miss micro-metastases and risk relapse. The potential of cancer immunotherapy is that it uses the body's own defense system to identify and precisely attack cancer cells, and maintain surveillance to prevent future occurances[3]. In the late 1800's, William Coley observed a patient's tumor disappear following an infection. He then injected other cancer patients with a mixture of bacteria, Coley's toxins, which was possibly the first widely used cancer immunotherapy[4,5]. Today immunotherapies may include adjuvants, cytokine, antibodies, adoptive cell transfer, and vaccination which attempt to activate the innate and adaptive immune systems.

The adaptive immune system is exquisitely suited to targeting a diseased cell while sparing normal neighboring cells, but it is the innate system that typically recognizes pathogens or stressed cells and starts the immune response[6]. The innate immune system is not specific for any individual pathogen and it does not lead to lasting immunity, but it can immediately combat a wide range of pathogens. Initially, antimicrobial proteins target the cell walls and membranes of bacteria, complement can lyse pathogens or mark them for phagocytosis by innate immune cells, and natural killer

cells can kill infected cells and tumor cells and produce interferon-γ (IFNγ), which activates many different cells of the immune system. Phagocytic white blood cells of the innate immune system, such as neutrophils, macrophages, and dendritic cells (DCs), can ingest and kill microbes and dying cells. DCs (and under certain conditions macrophages as well) secrete proinflammatory cytokines and present antigens derived from those microbes and dying cells to the cells of adaptive immune system, thereby activating them.

The adaptive immune system is composed of B lymphocytes and T lymphocytes. Each cell has antigen receptors specific for one antigen and take a few days after pathogen exposure to activate, proliferate and fully develop effector functions. Briefly, B cells become plasma cells that secrete antibody (humoral immunity); $CD8^+$ T cells become cytotoxic T cells (CTL) that directly kill cells expressing the antigen to which their receptors bind; $CD4^+$ T cells may become helper T cells (T_H) that support B cells and $CD8^+$ T cells to become fully functional, and develop memory cells; or $CD4^+$ T cells may become regulatory T cells (T_{reg}) that suppress the activity of other lytphocytes.[6] The innate immune system identifies features that discriminate infected, stressed cells or abnormal cells and microenvironments[7]. Features that trigger the innate immune system can be pathogen-associated molecular patterns (PAMPs), danger (or damage)-associated molecular patterns (DAMPs), inflammation or the presence or absence of certain cell surface markers[6]. PAMPs such as bacterial cell walls, and DAMPs that are released by tissue damage or necrotic cell death, bind and activate DCs through Toll-like receptors (TLRs) and other pattern recognition receptors (PRR). This causes DCs to upregulate antigen presentation, costimulatory markers and the secretion of cytokines[8].

Inflammation recruits cells to the site, and enables trafficking of cells to back local lymph nodes, where antigen is presented to lymphocytes[6]. Activation of antigen presenting cells (APCs) by PAMPs, may be why Coley's toxins was effective in some of his cancer patients. Activated DCs present antigen to T cells (signal 1) with costimulation (signal 2) and in the presence of proinflammatory cytokines (signal 3)[7,9]. The innate and the adaptive immune systems, bridged by DCs, work together to defend the body[10].

Therapeutic cancer vaccines attempt to take advantage of the multiple ways that the immune system can be educated and prodded to act by targeting both innate and adaptive immune cells. Ideally, the immune system has a cancer immunosurveillance function, in that it can specifically identify tumor cells on the basis of their expression of tumor-specific antigens (TSAs) and eliminate them before they can establish malignancy[11]. Some cancer cells evade immunosurveillance because they exhibit low-level expression foreign, viral or tumor-specific antigens. They may secrete tumor-derived factors (transforming growth factor-β (TGF-β), interleukin-10 (IL-10) to dampen the response in their microenvironment. Other known mechanisms of suppression include tumor expression of PD-L1 which binds PD-1 on T cells, thereby downregulating proliferation and function of effector cells[12]. In the absence of TSAs, tumor-associated antigens (TAAs) can be used to target tumor cells. However, TAAs are self-antigens. One example is melanoma antigen E (MAGE). While melanoma patients may have MAGE-specific T cells[13], these lymphocytes are often ignorant of their antigen, few in number, and/or be tolerized or supressed[14]. Cancer vaccines endeavor to instruct and activate the immune system, by presenting a tumor specific antigen (TSA) or a tumor-associated antigen (TAA) in the context of danger. While some cancers, such as B cell lymphomas

can be eradicated by treatment with antibodies[15], cellular immunity, mediated by CTL and T_H cells, which also produce IFNγ, can directly kill cancer cells, activate other cells and develop memory cells to prevent relapse[3].

Wilms Tumor 1, Tumor-Associated Antigen

Wilms Tumor 1 (WT1) is ranked at the top of the list of cancer vaccine target antigens in a report by the National Cancer Institutes (NIH) that prioritized antigens by:

> "(a) therapeutic function, (b) immunogenicity, (c) role of the antigen in oncogenicity, (d) specificity, (e) expression level and percent of antigen-positive cells, (f) stem cell expression, (g) number of patients with antigen-positive cancers, (h) number of antigenic epitopes, and (i) cellular location of antigen expression"[16].

WT1 protein is overexpressed in at least 36 different human cancers. WT1 expression is correlated to progressive disease and poor prognosis[17–24]. For example, high levels of WT1 expression post-treatment of acute myeloid leukemia (AML) correlate with poor prognosis[20], though its significance at the time of diagnosis of AML and needs to be clarified[25,26]. WT1 was originally described as a tumor suppressor gene associated with Wilms' tumors, as there are mutations in ~20% of these pediatric neoplasms[27–29]. Subsequently, it was reclassified as an oncogene because the overexpression of wild type WT1 is found in numerous cancers[30]. WT1 is overexpressed in hematopoietic malignancies such as AML, chronic myelogenous leukemia (CML), and myelodysplastic syndromes (MDS). Solid tumors such as ovarian cancer, prostate cancer, colorectal adenocarcinoma, breast cancer, glioblastoma and lung cancer also overexpress WT1[18,28,31]. WT1 expression is necessary for the survival of breast cancer cell line MCF-7[32]. In glioblastomas, WT1-shRNA knockdown of WT1 significantly reduces

proliferation and increases apoptosis as compared to glioblastoma cells transduced with control shRNA[33]. Suppression or loss of WT1 leads to decreased tumor burden in Kras-driven lung cancer in mouse models, and is prognostic of survival in patients with Kras-activated lung cancers[34]. Knockdown of WT1, by siRNA, in lung cancer cells induces cell cycle arrest at the G1 phase, reduces the expression of antiapoptotic genes, and enhances the expression of proapoptotic genes, thereby making the cells less resistent to chemotherapy [30,35].

WT1 is a zinc finger transcription factor, is involved in proliferation, cell differentiation and apoptosis and is critical in embryonic development. In human adult tissues WT1 is not expressed except for bone marrow where it has various functions in hematopoiesis[36–38] and in the glomerular podocytes of the kidney, Sertoli cells of the testis, and granulosa cells of the ovary[17,39]. In embryonic development, it is expressed in tissues that arise from the mesoderm; it has an essential role in the normal development of the urogenital system[40], the spleen[41] and the epicardial epithelial to mesenchymal transition (EMT)[42]. Deletion of WT1 in the embryo results in death at mid-gestation due to defective coronary vasculature, even before the lack of kidney, gonad and spleen affect survival[43,44]. As WT1 is a self-antigen, developing an immune response sufficient to combat WT1-expressing cancers is difficult due to immune tolerance. High avidity T cells specific for WT1 are deleted in the thymus. The frequency of WT1-specific T cells in the remainder of the repertoire is reduced. The low avidity WT1 T cells that escape from the thymus contend with tolerance in the periphery, where they may become anergized or suppressed[45,46].

Vaccination against WT1

Current immunotherapies such as peptide or DC-based vaccines show potential in clinical trials. 45.8% of patients with solid tumors and 63.5% of patients with hematological malignancies have a clinical response (a few complete or partial remissions, but mainly stable disease or a reduction in WT1 transcripts); 35.4% of patients with solid tumors and 67.5% of patient with hematological malignancies have an immunological response (WT1 tetramer+, or IFNγ+ T cells)[18]. Most reported immunotherapies to treat WT1-expressing cancers are peptide-based. This restricts therapy to a subset of patients with compatible major histocompatibility complexes (MHC), and targets a few epitopes. In 2012, Doubrovina et al. reported finding 41 new previously unreported human epitopes for WT1 (36 presented by class I, and 5 presented by class II); except for $WT1_{126\text{-}134}$, none of the previously described 20 epitopes, used in various clinical trials, produced a significant response in their study[47]. ($WT1_{126\text{-}134}$, RMFPNAPYL, is considered the dominant epitope in humans and mice.) Finding the right epitope or peptide-MHC combination is quite a task. Vaccines which use autologous DC, either electroporated with WT1 mRNA or peptide pulsed, also show promise; however, they are by definition patient-specific, costly and require lengthy preparation in the laboratory[18]. The ideal vaccine would be safe, inexpensive, and could encode as much of the antigen as possible to provide multiple epitopes, for both CD8 and CD4 T cells, without restriction by MHC.

In this study, we investigated immunogenicity to WT1 in wild type mice. Unlike humans, mice also express WT1 in the splenic stroma[48]. To induce a robust WT1-specific T cells response in mice, bordering on autoimmunity, we used a series of immunization

strategies including vaccinating with cDNA, protein, adjuvants, *Listeria* vaccines, WT1-expressing BMDCs, and WT1 overexpressing tumor cell lines. Focusing on cellular immunity, mediated by CTL and T_H1 T cells, we evaluated immune responses by IFNγ expression in ELISA (enzyme-linked immunosorbent assay), and ELISpot (enzyme-linked immunosorbent spot) assays, and by ICS (intracellular cytokine staining). We also used tetramer staining to quantify WT1-specific T cells. Each vaccine strategy was designed to test approaches to breaking tolerance. These included supplementing the antigen with mechanical stress (electroporation) or molecular danger signals (PAMPs such as LPS, and poly(I:C)); by using *Listeria monocytogenes* to target antigen to APCs; activating APCs with cytokine, PAMPS or agonist antibody; and using cell lines that express WT1 nearly 100-fold greater than spleen.

Xenogeneic DNA Vaccines

One approach that successfully elicited an immune response to wild type antigen utilized a xenogeneic DNA vaccine. In mouse models of breast cancer, in which human Her-2 (human ErbB-2) or rat neu (rat ErbB-2) transgenic mice are electrovaccinated with self-antigen, the immune response is weak. Electrovaccination with heterologous (xenogeneic) DNA or a combination of both heterologous and self DNA, induced a more robust immune response, as human and rat ErbB-2 proteins are 88.1% identical[49]. Another example of overcoming T cell tolerance, has been reported in dogs vaccinated with either human or murine tyrosinase to treat canine melanoma[50,51]. In rat experimental autoimmune encephalomyelitis, which is induced by guinea pig myelin basic protein, the shared epitope differs by one amino acid and better facilitates recognition by low-affinity

T cells[46,52]. Similar to the tolerance promoted by the expression of self-antigen Her-2/neu in transgenic murine brain and mammary glands, the expression of WT1 in the spleen is greatly tolerogenic[48,53]. However, unlike ErbB-2, WT1 is more highly conserved; pair wise alignment scores indicate a 97% identity between human and murine protein sequences. Since the human and the murine sequences are so similar, vaccinating mice with a human DNA vaccine does not work as well as it does in the Her-2/neu model. It is not xenogeneic enough.

To create a more xenogeneic vaccine, we generated a consensus sequence from ten WT1 homologs. Homologous proteins from *D. rerio, G. gallus, M. musculus, R. norvegicus, C. lupus, B. taurus, H. sapiens, P. troglodytes,* and *A. mississippiensis* were aligned to define critically conserved domains, and to identify positions of amino acid variability. Amino acids, from sequences other than *M. musculus*, substituted at those sites. Additionally, the zinc finger region and nuclear localization signal was removed as these are typically the least immunogenic portions of a protein and sequester the protein in the nucleus. Kozak and IgE leader sequences were added to facilitate protein expression. The new protein consensus sequence is 91% identical to wild type murine WT1, not including the IgE leader sequence or the deleted portions. Lymphocytes from mice electrovaccinated with the consensus readily respond to a peptide library based on the wild type murine WT1 sequence, whereas the immune response to vaccination with the wild type murine WT1 sequence is marginal. Therefore, an increase in xenogenecity of the WT1 DNA vaccine leads to robust a WT1-specific cellular immune response in the highly tolerogenic mouse model.

In this dissertation, I will discuss our vaccine strategies, the results of each strategy, and potential approaches to circumvent tolerance in future experiments.

Chapter 2: Vaccination with wild type murine WT1 does not induce a significant immune response in mice.

Introduction

A successful immune response to an antigen requires expression of the antigen in a fashion or environment that alerts the immune system, presentation of the antigen to immune cells, and in the case of cancer, an effector T cell response[6]. Even though humoral immune responses should not be discounted[15], persistent memory T cells that remain for surveillance and prevention of relapse would be ideal. When dealing with foreign antigens, the cells of the immune system have a variety of pattern recognition receptors (PRRs) to identify characteristics of non-self in a broad assortment of pathogens. In addition, the adaptive immune system has an immensely diverse selection of lymphocyte antigen receptors, each uniquely targeted to one specific antigen. However, there may not be a "foreign" antigen that the immune system can distinguish, to indicate a rogue cancer cell. Tumor-associated antigens (TAAs), some of which can be viral oncogenes, or mutated or altered self-proteins, can also be overexpressed non-mutated self-antigens, such as WT1[54–56]. In spite of many TAAs being self-antigens, it is not uncommon to find T cells specific to such TAAs in patients with cancer, indicating that immune system can recognize these TAAs. It bolsters the hope that immunotherapy can be the means to treat cancer[13,57].

Immunotherapy targeting WT1, a TAA found in a wide range of cancers, requires designing a vaccine that can initiate a successful immune response and overcome an obstacle inherent in using a self-antigen as a target, namely tolerance to that antigen.

Central tolerance limits the number and quality of WT1-specific T cells that escape the thymus, as high avidity self-reactive T cells are deleted. In the periphery, these cells may be anergized or suppressed. In humans, WT1 expression is limited to HSC, a few cells of the kidney, testis and ovary. In mice, WT1 is also expressed in the stroma of the spleen, thereby creating an environment in which it is more difficult to make an immune response to WT1. Gaiger et al. and several others show that it can be done, mainly using peptides[45,58–61]. Most clinical trials, vaccinating against WT1, have used defined, and limited peptide vaccines, but with underwhelming results[18].

To study responses to the full length WT1 antigen, rather than a limited number of peptide epitopes, we vaccinated mice with full length wild type murine WT1 using a variety of methods, types of antigen, routes of immunization, and adjuvants in an effort to induce a robust immune response. Each of the methods was designed to take advantage of various aspects necessary for successful immune response. Route of vaccination, adjuvants, and vectors presented the antigen in several ways to trigger danger signals, and to exploit aspects of the innate and adaptive immune systems such as PRRs, transient inflammation, and antigen presenting cells (APCs), in order activate Wt1-specific T cells. We used tetramer staining and *in vitro* assays for IFNγ production to identify and quantitate cell responses in splenocytes isolated from vaccinated mice. Splenocytes or isolated T cells were stained with WT1 Db126 tetramer (RMFPNAPYL/H-2D^{b}) and/or were restimulated *in vitro* by WT1 mRNA transfected B cells, using B6BL#1153 B lymphoma, an easily manipulated antigen presenting cell line, in IFNγ ELISA, ICS, and ELISpot assays.

Materials and Methods

Mice

Female or Male C57BL/6 mice between 6 to 12 weeks old were used in this experiment. Mice were obtained from the National Cancer Institute (Frederick, MD). Mouse care and experimental procedures were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) of Johns Hopkins University under an approved protocol. Female BALB/c mice between 6 to 8 weeks old were used in this experiment. Mice were obtained from the Jackson Laboratory. The mice were housed and maintained by the University Laboratory Animal Resources at the University of Pennsylvania in observance with the policies of the National Institutes of Health and the University of Pennsylvania IACUC. Naïve mice served as negative control.

Cell lines

B6BL#1153 is a C57BL/6 B lymphoma cell line (a kind gift of Dr. Rongfu Wang, Methodist Hospital Research Institute, Houston, TX). Cells were cultured in complete RPMI media: RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, nonessential amino acids, 10 mM HEPES buffer, and 100 µM 2-mercaptoethanol and grown at 37°C in 5% CO_2. B1153-WT1 cell lines were generated by electroporation-mediated plasmid transfection of the construct pWT1, which encodes the wild type murine WT1, into B6BL#1153. Transfection was performed using the Nucleofector™ system (Kit V, Program L-013, (Lonza)) according to the manufacturer's instructions. Transfectants were selected in 400 µg/ml of the neomycin analogue, G418. Drug-resistant clones were

selected after limiting dilution, and WT1 expression was confirmed by qRT-PCR and western blot. B1153-WT1 cells were maintained in complete RPMI media supplemented with 400 μg/ml of G418. B78H1-GM[62], a C57BL/6, GM-CSF secreting cell line utilized in bystander immunotherapy regimens, was maintained in complete RPMI supplemented with high dose hygromycin (1,200 μg/mL) to assure high levels of GM-CSF expression, which averaged 1 μg GM-CSF/10^6 cells/24 h. The C57BL/6 prostate adenocarcinoma cell line, TRAMP-C2 (ATCC) was cultured in TRAMP media: Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 5% fetal bovine serum, 5% Nu-Serum IV (BD), 0.005 mg/ml insulin, 10 nM *trans*-dehydroandrosterone and 100 U/mL penicillin, 100 μg/mL streptomycin. For TRAMP-C2/Gvax vaccination, 1x10^6 TRAMP-C2 cells were mixed with 5x10^4 B78H1-GM cells and irradiated (50 Gy). After three washes in PBS, cells were resuspended in a total of 200 μl of PBS and administered by subcutaneous injection (subq) of 100 μl into each hind limb. SF.TRAMP-GM (TRAMP-C2 modified to express GM-CSF via retroviral transduction with MFG muGM-CSF[63], which averaged 20 ng GM-CSF/10^6 cells/24 h) was conditioned to grow in serum free media (Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 5% Ham's F12, and 0.1% MITO+ Serum Extender (BD), 0.005 mg/ml insulin, 10 nM *trans*-dehydroandrosterone, and 100 U/mL penicillin, 100 μg/mL streptomycin). Cells used for vaccination were collected by trypsinization, and irradiated (50 Gy). After three washes in PBS, cells were resuspended in a total of 200 μl of PBS and administered by subcutaneous injection of 100 μl into each hind limb.

Relative WT1 Expression by qRT-PCR

Quantitative PCR was performed on an Applied Biosystems 7500 real time PCR system. Pre-made Taqman primer/probe gene expression assays (Mouse Gapdh: Mm99999915_g1, Mouse Wt1: Mm01337053_m1 and Mm01337052_m1) were purchased from Applied Biosystems, and QuantiTect Probe RT-PCR Kit was purchased from Qiagen for the remainder of the reagents. Each triplicate reaction, in a total of 25 µl, contained 12.5 µl of the 2X master mix, 0.25 µL RT mix, 1.25 µl of the 20X Gapdh primer/probe, 1.25 µl of the 20X Wt1 primer/probe, DEPC treated water, and RNA isolated using an RNeasy kit (Qiagen). Data were analyzed using SDS 1.5 software (Applied Biosystems). Gapdh was used as the endogenous control and relative changes in gene expression were calculated using the ΔΔCt method.

Constructs for DNA vaccination

Wild type murine WT1 cDNA, pKS+mWT1++, was a kind gift from Dr. J. Kreidberg (Harvard Medical School, Boston, MA). For the generation of pcDNA3.1 WT1 (used for DNA vaccination and creation of a stably transfected WT1-expressing B cell lines), wild type murine WT1 was first amplified by PCR using pKS+mWT1++ as the template and a set of primers, 5'-TGTAGATCTACCATGGGTTCCGACGTGCGG-3'and 5'-TTTGGATCCTGTCAAAGCGCCAGCTGGAGTTT-3'. The amplified product was then cloned into the BamHI sites of pcDNA3.1(-) vector (Invitrogen) and was named pWT1. An alternative WT1 construct, only used for DNA vaccination, was created by adding an IgE leader sequence, after which codon optimization and RNA optimization was performed by using GeneOptimizer® (GeneArt®, Life Technologies). The IgEL murine WT1 sequence was synthesized and sequence verified by GeneArt®, and cloned

into the expression vector pVAX (Invitrogen) and named WT1-pVAX, but will be referred to as pIgEL-WT1 in this manuscript. pcDNA3.1-Ova (pOva) contains ovalbumin fused to the transmembrane domain of transferrin. The TfR/OVA fragment was isolated by digestion with HindIII from the pBlueRIP-TfR/OVA plasmid, which was initially generated by Dr. F. Carbone (Walter & Eliza Hall Institute, Melbourne, Australia) and kindly provided to us by Dr. E. Sotomayer (H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL).

DNA vaccination by intramuscular injection only

Each vaccinated C57BL/6 mouse received four doses of DNA plasmid on days 0, 3, 6, and 14. Mice received 100 μg of DNA per vaccination. The DNA constructs, in 50 μL of PBS were administered via intramuscular (IM) injection of the right or left quadriceps muscle.

DNA vaccination by intramuscular injection followed by electroporation

Each vaccinated BALB/c mouse received three doses of DNA plasmid at 14 day intervals. Mice received 30 μg of DNA per vaccination. Electroporation procedure was performed as described previously[64]. Briefly, DNA constructs were administered via intramuscular injection of the right quadriceps muscle, followed by square-wave pulses generated by the CELLECTRA® constant current electroporation device (Inovio Pharmaceuticals). The device was configured to deliver two 0.1 Amp pulses of 52 ms pulse width spaced apart by a 1 sec delay.

Constructs for *in vitro* transcription and transfection of B6BL#1153 B cells

For *in vitro* transcription of mRNAs that are used to transfect B6BL#1153 B cells, all antigens are inserted into pGEM4Z/A64[65]. pGEM4Z/GFP/A64[66] which contains GFP between the HindIII/EcoRI sites of pGEM4Z/A64 was kindly provided to us by Dr. I. Strobel (University of Erlangen, Germany). Wild type murine WT1 was amplified with a set of primers, 5'-TATAAAGCTTGCCACCATGGGTTCCGACGTGCGG-3' and 5'-TTCTGAATTCTCAAAGCGCCAGCTGGAGTT -3', and cloned into the HindIII/EcoRI sites of pGEM4Z/A64, after excising GFP. Ovalbumin (Ova) was amplified with a set of primers, 5'-GGCCAAGCTTACCATGATGGATCAAGCTAGATCAGC-3' and 5'-GGGGGAATTCTTAAGGGGAAACACATCTGCCAA -3', and cloned into the HindIII/EcoRI sites of pGEM4Z/A64, after excising GFP. Plasmids were linearized using restriction endonuclease SpeI and *in vitro* transcribed using the (T7) mMESSAGE mMACHINE high yield capped RNA transcription kit (Ambion) according to the manufacturer's instructions. After digestion of the DNA template with DNase I, mRNA was purified with an RNeasy kit (Qiagen).

Transfection was performed using the Nucleofector™ system (Kit V, Program L-013, (Lonza)) according to the manufacturer's instructions, with a few modifications. Briefly, each transfection consisted of 10 μg mRNA/1x10^7 cells in 100 μL of Nucleofector™ Solution V. B6BL#1153 B cells were harvested, and washed twice with room temperature PBS, counted, aliquoted, and pelleted. The supernatant was decanted and the remaining PBS aspirated. The pellet was broken with a tap of the tube, mRNA was added to the cells, and 100 μL of Nucleofector™ Solution V was added to resuspend cells and mRNA, which was then transferred to a cuvette. The cells were shocked using

program L-013 on a Nucleofector™ II device, rescued with pre-warmed media, and transferred to a 100 mm dish containing 10 ml of pre-warmed complete RPMI media. Cells were rested at least four hours in an incubator before use in assays. B6BL#1153 B cells that are simply shocked without nucleic acids were denoted as "B cells"; those that were transfected with wild type murine WT1 mRNA are denoted as "WT1 B cells"; those that were transfected with GFP or Ova mRNA are denoted as "GFP B cells" and "Ova B cells" respectively.

WT1 protein expression in transfected cells

To confirm WT1 protein expression from constructs transfected into B6-BL#1153 B cells, $1x10^7$ cells were lysed using 1ml of NP-40 cell lysis buffer and vortexed. Lysates were cleared by centrifugation. Proteins were resolved and blotted using the Bio-Rad Mini-Protean® TGX™ Precast Gel and Mini-Trans Blot® system. Cell lysates were mixed with 2X Laemmli sample buffer, supplemented with 2-mercaptoethanol and boiled for 5 min. Lysates were loaded onto a 10% Mini-Protean® TGX™ Gel and run at 200V for ~35 min. Protein was transferred onto nitrocellulose membranes in transfer buffer (IX Tris/Glycine/20% methanol) at 30V overnight at 4ºC. Membranes were blocked with 5% nonfat dry milk diluted in Tris-buffered saline + 0.1% Tween-20 (TBST), for 60 min, then rinsed and incubated overnight with a 1:5000 dilution of rabbit anti-WT1 monoclonal antibody (Abcam, ab89901) TBST + 5% BSA at 4ºC. Membranes were washed once with TBST for 15 min, then three times with TBST for 5 min each, then probed with a 1:25,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, sc-2054) for 60 min at room temperature. Membranes were washed once with TBST for 15 min, then three times with TBST for 5

min each, then visualized using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).

Recombinant WT1 protein and immunization with protein

For expression of GST fusion proteins, fragments of wild type murine WT1 were cloned into pGEX-6P-1 (GE Healthcare). The n-terminus of WT1 (GST-N-mWT1, aa1-193) was amplified with primers, 5'- TTTTGAATTCATGGGTTCCGACGTGCGG -3' and 5'-TATTCTCGAGGTACTGCTGCTCGAACAG -3', and cloned into the EcoRI/XhoI sites of pGEX-6P-1. The middle section of WT1 (GST-Mid-mWT1, aa133-318) was amplified with a set primers, 5'-TTTTGAATTCTACCTGCCCAGCTGCCTG -3' and 5'-TATACTCGAGACTGGTTTCAGATGCTGACC -3', and cloned into the EcoRI/XhoI sites of pGEX-6P-1. The c-terminus of WT1 (GST-C-mWT1, aa265-449) was amplified with a set primers, 5'-ATATGAATTCAGCAACCACGGCACAGG -3' and 5'- TATTCTCGAGTCAAAGCGCCAGCTGGAG -3', and cloned into the EcoRI/XhoI sites of pGEX-6P-1. To isolate GST-WT1 protein, cell lysate was prepared from transformed Rosetta 2(DE3)pLysS (Novagen) cell cultures. Cells from a 1 L culture were harvested and centrifuged for 20 min at 4000*g* after a 3 h IPTG (1 mM) induction in Terrific broth at 30°C. Cultures producing GST-C-mWT1 were supplemented with 0.5 mM $ZnCl_2$. Pellets were resuspended in a total of 25 ml of lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 µM $ZnCl_2$, 0.3% NP-40 alternative, 0.1% Tween-20, 1 mM PMSF and Complete Mini (-EDTA) Protein Inhibitors (Roche)). Lysate was frozen in liquid nitrogen and thawed in a 37ºC water three times. Lysozyme (1 mg/ml, Sigma) was added, then the lysate was vortexed, and incubated on ice for 30 min. The lysate was sonicated three times and then centrifuged at 12,000*g* at 4°C for 10

min. Glutathione Sepharose 4B resin (GE Healthcare), 1 ml of a 50% slurry, was washed with 5 ml of TBS- NMGZn wash buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM $MgCl_2$, 10 µM $ZnCl_2$, 0.1% NP-40 alt., 10% Glycerol, and 1 mM PMSF). Supernatant was collected and incubated with pre-washed resin and rotated 60 min at 4°C. The resin was centrifuged (500*g*) and washed three times with 5 ml of TBS-NMGZn wash buffer before being eluted three times with 2.5 ml of TBS-NMGZn elution buffer (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2.5 mM $MgCl_2$, 10 µM $ZnCl_2$, 0.1% NP-40 alt., 10% Glycerol, 20 mM Glutathione, reduced), rotated for 10 min at room temperature. Elutions were combined, concentrated and buffer exchanged into PBS before being used for immunizations. Proteins were confirmed by western blot with anti-GST-HRP (GE Healthcare), and anti-WT1 (F-6, C-19, Santa Cruz Biotechnology) antibodies. Concentration was determined by Bio-Rad Protein Assay, and equal amounts of GST-N-mWT1, GST-Mid-mWT1, and GST-C-mWT1 were combined for GST-WT1 protein immunizations. C57BL/6 mice were injected intraperitoneally (IP) with 0.5 mg whole ovalbumin (Sigma-Aldrich) or 1 mg GST-WT1 (~0.5 mg WT1) with or without TLR agonists ((30 µg LPS or 50 µg poly(I:C), Sigma) and/or 50 µg of anti-CD40 mAb (FGK45 mAb was a kind gift of S. Schoenberger, LIAI, La Jolla, CA). Mice were immunized with a single injection IP and sacrificed 6 days later.

Bone marrow-derived dendritic cells (BMDC)

C57BL/6 mice were treated IP with 4 mg/mouse cyclophosphamide (Baxter Healthcare) and bone marrow was harvested 3 days after treatment. Femurs and tibiae of mice were removed and separated from the surrounding muscle tissue by rubbing with tissue. Thereafter intact bones were left in 70% ethanol for 1 min for disinfection and

washed with RPMI 1640. Then both ends were cut with scissors and the marrow flushed with RPMI 1640 using a syringe with a 25G needle. Clusters within the marrow suspension were disintegrated by vigorous pipetting. The bone marrow cell suspension was depleted of red blood cells by lysis with ACK lysing buffer (Quality Biologicals). After resuspension the cells were filtered through a 40 μm nylon filter and counted. At day 0, bone marrow cells were plated at $0.75x10^6$ per 100 mm dish (bacteriological) in 10 ml serum-free BMDC media (RPMI 1640 medium supplemented with 0.1% MITO+ Serum Extender (BD), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, non-essential amino acids, 10 mM HEPES buffer, and 100 μM 2-mercaptoethanol, 1 mM sodium pyruvate, and 800 U/ml of mGM-CSF). At day 4, another 10 ml of media (containing mGM-CSF) were added to the plates. At day 6, half of the culture supernatant was collected, centrifuged; the cell pellet was resuspended in 10 ml fresh media (containing mGM-CSF), and given back into the original plate. On day 7, non-adherent and loosely adherent immature BMDCs were harvested, washed twice and the phenotype of the BMDCs ($CD11c^+CD11b^+$) were confirmed by flow cytometry and the cells were transfected.

Transfection of immature BMDC was performed using the Nucleofector™ system (Mouse Dendritic Cell Kit, Program Y-001, (Lonza)) according to the manufacturer's instructions, with a few modifications. Briefly, each transfection consisted of 10 μg mRNA/$1x10^7$ cells in 100 μL of Nucleofector™ Mouse DC solution. BMDCs were harvested, and washed twice with room temperature PBS, counted, aliquoted, and pelleted. The supernatant was decanted and the remaining PBS aspirated. The pellet was broken with a tap of the tube, mRNA was added to the cells, and 100 μL of

Nucleofector™ Mouse DC solution was added to resuspend cells and mRNA, which was then transferred to a cuvette. The cells were shocked using program Y-001 on a Nucleofector™ II device, rescued with pre-warmed media, and transferred to a 100 mm dish containing 10 ml of pre-warmed serum-free BMDC media. On day 8, the media was supplemented with 25 μg/ml poly(I:C) to further mature the BMDCs. On day 9, mature BMDCs were harvested, washed twice and the phenotype of the BMDCs (CD11c, CD11b, $H2K^b$, $H2D^b$, IA^b, CD40, CD80, CD86 (BD Biosciences)) were confirmed by flow cytometry. After three washes in PBS, $1x10^6$ cells were resuspended in a total of 200 μl of PBS and administered by subcutaneous injection of 100 μl into each hind limb.

Listeria monocytogenes

Stocks of vaccine-ready attenuated *Listeria monocytogenes* were graciously provided by Thomas Dubensky and Pete Lauer (Aduro BioTech). Three strains were provided: Lm(-), the negative control; Lm Ova, a strain that express ovalbumin protein; and Lm WT1-SL8, a strain that expresses full length murine WT1 protein to which SIINFEKL is fused at the end. C57BL/6 mice were vaccinated, intravenously with a dose is $5x10^6$ CFU *L. monocytogenes* in 200 μL PBS.

Splenocyte isolation

Spleens and lymph nodes were isolated from mice, pressed through a 100 μm strainer to create a single-cell suspension, and red blood cells were lysed in ACK lysing buffer. Splenocytes were resuspended in complete media and filtered through a 40 μm strainer, before cell counting. In some experiments, T cells were used enriched by using Pan T Cell Isolation Kits (Miltenyi Biotech) following the manufacturer's protocol. Cells

were cultured in complete RPMI or serum free CTL media (RPMI 1640 medium supplemented with 0.1% MITO+ Serum Extender (BD), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, non-essential amino acids, 10 mM HEPES buffer, and 100 µM 2-mercaptoethanol, 1 mM sodium pyruvate).

IFNγ ELISA

Mice were sacrificed 1 week after the final vaccination, and splenocytes were isolated as mentioned previously. Splenocytes or T cells, from naïve and vaccinated mice were restimulated *in vitro* in 96-well flat bottom plates. Splenocytes were counted and added to wells at $4x10^5$ cells per well. Peptides (2 µg/ml SIINFEKL or 5 µg/ml RMFPNAPYL) or $1x10^5$ B cells (either untransfected, Ova or WT1 transfected B6BL#1153 B cells) were added to the wells. Wells reserved for positive and negative control received PMA/Ionomycin (eBioscience) or culture medium, respectively. Plates were subsequently placed in a 5% CO_2 atmosphere incubator for 48 to 72 h, after which supernatant was collected, frozen, and later analyzed using mouse IFNγ ELISA (R&D Systems or eBioscience). Ovalbumin class I peptide SIINFEKL, and WT1 class I peptide RMFPNAPYL were purchased from GenScript.

Flow cytometry and intracellular cytokine staining

Murine CD11c, CD11b, $H2K^b$, $H2D^b$, IA^b, CD40, CD80, CD86, CD8, CD4, B220, and IFNγ antibodies were purchased from BD Biosciences and used for flow cytometry. PE-labeled SAINFEKL/H-$2K^b$ tetramer (Ova), SIINFEKL/H-$2K^b$ monomer (Ova) and RMFPNAPYL/H-$2D^b$ monomer (WT1) were provided by the NIH Tetramer Core Facility and used for identification of Ova or WT1-specific $CD8^+$ T cells. Ova

monomers and WT1 monomers were tetramerized with streptavidin-PE or streptavidin-APC (BD Biosciences) as described[67]. Cells were stained with tetramer for 30 min at room temperature, in the dark, before staining with surface antibodies. Tetramer and surface antibodies were diluted in lx HBSS containing 1% FBS, 10 mM HEPES buffer and 0.1% sodium azide. For intracellular cytokine staining, cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (with BD GolgiPlug™ protein transport inhibitor containing brefeldin A), according to the manufacturer instructions. All flow data were collected using the FACSCaliber or LSR II (BD bioscience) and analyzed using FlowJo (TreeStar, Ashland, OR).

Cells to be analyzed by intracellular cytokine staining for IFNγ were cultured as follows. Splenocytes or T cells, from naïve and vaccinated mice were restimulated *in vitro* in 48-well flat bottom plates. Splenocytes were counted and added in wells at $2x10^6$ cells per well. Peptides (2 μg/ml SIINFEKL or 5 μg/ml RMFPNAPYL) or $1x10^6$ B cells (either untransfected, Ova or WT1 transfected B6BL#1153 B cells) were added to the wells. Wells reserved for positive and negative control received PMA/Ionomycin (eBioscience) or culture medium, respectively. Cell were stimulated in media containing GolgiPlug for 12 h, prior to intracellular cytokine staining.

IFNγ ELISpot assays with individual peptides or B cells

High-protein IP 96-well Multiscreen™ plates (Millipore, MAIPS4510) were coated with monoclonal mouse IFNγ Capture Antibody (R&D Systems, SEL485) and incubated overnight at 4°C. After three washes with 1x PBS, the plates were blocked with 1% BSA and 5% sucrose in 1x PBS for 2 h at ambient temperature. Mice were sacrificed 1 week after prime or boost vaccinations, and splenocytes were isolated as

mentioned previously. Splenocytes were counted and added in triplicate wells at $2.4x10^5$ cells per well. Peptides (2 µg/ml SIINFEKL or 5 µg/ml RMFPNAPYL) or $0.6x10^5$ B cells (either untransfected, Ova or WT1 transfected B6BL#1153 B cells) were added to the wells. Wells reserved for positive and negative control received PMA/Ionomycin (eBioscience) and culture medium in lieu of peptides, respectively. Plates were subsequently placed in a 5% CO_2 atmosphere incubator. After incubation for 18–24 h at 37°C, the wells were washed with 1x PBS. Biotinylated anti-mouse IFNγ Detection Antibody (R&D Systems, SEL485) was added to each well and then incubated overnight at 4°C. The plates were subsequently washed and processed per a color development protocol provided by R&D Systems using Streptavidin-AP and BCIP/NBT Plus (R&D Systems, SEL002). The wells were air-dried overnight and spots inside wells were scanned and counted using a Zeiss KS ELISpot Imaging system. Reported spot forming cell counts were converted to represent spot-forming units per $1x10^6$ splenocytes.

IFNγ ELISpot assays with pooled peptides

Mice in both treatment and control groups were sacrificed 1 week after the third immunization. Spleens were harvested from each mouse and transferred to R10 media (RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics). Using a stomacher (Seward Laboratory Systems), the spleens were pulverized and subsequently transferred through a 40 µm cell strainer. Erythrocytes were removed by adding ACK lysing buffer (Lonza). The splenocytes were isolated and resuspended in R10 media. High-protein IP 96-well Multiscreen™ plates (Millipore, S2EM004M99) were coated with monoclonal murine IFNγ Capture Antibody (R&D Systems, SEL485) and incubated overnight at 4°C. After three washes with 1x PBS, the plates were blocked

with 1% BSA and 5% sucrose in 1x PBS for 2 h at ambient temperature. Isolated splenocytes in R10 medium were counted and added in triplicate wells at $2x10^5$ cells per well. Peptides were added to the wells. A set of peptides spanning the wild type murine WT1 sequence (excluding the zinc finger region) were synthesized by GenScript. The peptides contained 15 amino acid sequences, of which 11 residues overlapped with each sequential peptide. The peptides were each divided into three pools at concentrations of 2 μg/mL/peptide. Wells reserved for positive and negative control received Concanavalin A (Sigma-Aldrich, C0412) and R10 culture medium in lieu of peptides, respectively. Plates were subsequently placed in a 5% CO_2 atmosphere incubator. After incubation for 18–24 h at 37°C, the wells were washed with 1x PBS. Biotinylated anti-mouse IFNγ Detection Antibody (R&D Systems, SEL485) was added to each well and then incubated overnight at 4°C. The plates were subsequently washed and processed per a color development protocol provided by R&D Systems using Streptavidin-AP and BCIP/NBT Plus (R&D Systems, SEL002). The wells were air-dried overnight and spots inside wells were scanned and counted by an ELISpot plate reader system with ImmunoSpot®3 and ImmunoSpot®4 software (Cellular Technology Ltd.). Reported spot forming cell counts were converted to represent spot-forming units per $1x10^6$ splenocytes.

Results

Characterization of B6BL#1153 B lymphoma (B cell) line

In order to study the efficacy of vaccines ex vivo, we measured IFNγ production in splenocytes from vaccinated mice upon restimulation, using ELISA, ICS and ELISpot. Splenocytes were restimulated by peptide for the dominant epitope for WT1 (Db126) or by peptide libraries for the in vitro assays. Responses to naturally processed and presented peptides were assessed using B6BL#1153, a C57BL/6 B lymphoma cell line. qRT-PCR ascertained that B6BL#1153 did not express WT1, while the assay reiterated that WT1 expression in the murine spleen was greater than that of unfractionated bone marrow, confirming the report of Fraizer et al[36] (Figure 1A). Flow cytometric analysis showed that B6BL#1153 was an ideal antigen presenting cell line as it had an activated phenotype: high levels of MHC class I and class II, as well as costimulatory markers such as CD40, CD80 and CD86, without any need for supplementary activation (Figure 1B). Since B6BL#1153 did not naturally express WT1, the ability to easily transfect the cells for each experiment was important. Electroporating the cells with mRNA transfected greater than 90% of the viable cells in each transfection and enabled quicker protein expression than transfecting with plasmid DNA would have achieved. Figure 1C showed typical results of transfecting with GFP mRNA, in that a majority of the cells were transfected and highly expressed GFP protein. Electroporation of B6BL#1153 with pWT1 plasmid, selection in antibiotic, and dilution cloning produced WT1 stably transfected B cell lines that expressed WT1 comparable to that of TRAMP-C2, a murine prostate adenocarcinoma cell line known to overexpress WT1[58] (Figure 1D,E).

Immunization utilizing wild type WT1 cDNA produces a muted immune response as compared to other antigens.

To activate the few WT1-specific T cells in the naïve repertoire, a DNA vaccine, using as much of the antigen as possible to provide a variety of epitopes for both CD8 and CD4 T cells, would be advantageous. Such a vaccine would bolster the both the cytotoxic and helper T cell response, but more importantly be available to a wider range of patients. Naked DNA vaccines are safe, easy to design and produce in volume making them an attractive method of immunotherapy. Plasmid DNA, intramuscularly injected, transfects muscle cells and tissue patrolling APCs, perturbs the tissue transiently causing inflammation. The plasmid itself triggers TLR9, invoking danger signals and activating the innate immune system, recruiting and maturing APCs[68–72]. T cells from mice vaccinated with pOva, a plasmid containing ovalbumin (Ova) sequence, responded to restimulation by Ova mRNA transfected B6BL#1153 cell (Ova B cells). In contrast, T cells from mice vaccinated with wild type murine WT1 construct, pWT1, did not produce IFNγ upon restimulation with WT1 mRNA transfected B6Bl#1153 cells (WT1 B cells) indicating that the vaccine was ineffective at activating and expanding WT1-specific T cells (Figure 2A).

One limitation of DNA vaccines, is that the DNA has to be efficiently taken up by the muscle cells after injection and antigen presenting cells have to be recruited to the injection site. Electroporation can enhance the quality and magnitude of response to DNA vaccination. Intramuscular DNA injection followed by electroporation, or electrovaccination, increases the amount of DNA entering the cells. This enhanced uptake is followed by increased expression, processed naturally for peptide/MHC

complexes, and includes an element of "danger" to stimulate the immune response[73–75]. Electroporation immediately following intramuscular injection causes more inflammation than a simple intramuscular injection[76–80]. Two WT1 DNA vaccines were compared, using *in vivo* electroporation and pooled peptide *ex vivo* restimulation. One group of mice received pWT1, while the second group received a construct in which an IgE leader sequence was placed in front of the murine WT1 sequence, pIgEL-WT1, to enhance expression of the antigen[75,81]. The addition of electroporation did not enhance the original vaccine, as splenocytes from naïve mice and mice vaccinated with pWT1 had the same response to murine WT1 peptides. However, splenocytes from mice vaccinated with pIgEL-WT1 produced a muted but slightly improved response as compared to splenocytes from naïve or pWT1 vaccinated mice (Figure 2B).

Immunization with GST-WT1, anti-CD40 and adjuvant modestly enhances the immune response.

Adjuvants enhance vaccines by improving the formulation or by activating the innate and, by extension, the adaptive immune system. TLR agonists trigger release of proinflammatory cytokines in monocytes, NK cells and DCs, while CD40 antibody activates B cells and matures DCs. Ahonen et al. and others reported that a triple combination of antigen, CD40 antibody, and TLR agonist, synergizes to expand antigen-specific CTLs and differentiate them into IFNγ producing effector cells[82,83]. In the case of Ova, brief restimulation by Ova peptide (SIINFEKL) showed that mice vaccinated with the combination of Ova protein, CD40 antibody, and LPS (a TLR4 agonist) had the most IFNγ producing T cells (Figure 3A, B). This was reiterated in the 72hr IFNγ ELISA, at which point responses in WT1 vaccinated mice were also demonstrated. In the ELISA, T

cells were restimulated with either peptide or B cells transfected with Ova or WT1 mRNA. T cells from Ova protein/αCD40/LPS vaccinated mice produced three times more IFNγ when stimulated with Ova B cells than with Ova peptide. Likewise, T cells from WT1 vaccinated mice responded better to WT1 B cells, rather than the peptide for the dominant epitope. However, unlike the Ova protein vaccination, the best response, though nearly 20-fold less, was from T cells from mice vaccinated with WT1 protein and poly(I:C) (a TLR3 agonist) (Figure 3D, E). The experiment was repeated with similar results.

Heterologous prime/boost vaccination utilizing GM-CSF expressing cells increases immune responses.

Vaccines for infectious diseases initially utilized heterologous prime/boost vaccination methods to present the same antigen but in different vectors to circumvent the effect of neutralizing antibodies to the vector backbone; however the same principle of heterologous prime/boost can be used for cancer vaccines. Although most cancer vaccines use a DNA prime followed by a viral boost vaccine, several reports demonstrate that a DNA prime followed by an antigen overexpressing cell line vaccine may be just as effective[84,85]. Rittich et al. showed that vaccination with E7 DNA and boosting with a cell line expressing E7 and GM-CSF (or IL-12), enhanced *in vitro* immune responses after vaccination, and delayed or inhibited tumor growth[85]. In our experiments, it was clear that repeated vaccinations with wild type murine WT1 DNA in a homologous prime boost barely produced an immune response. After boosting with TRAMP-C2/Gvax vaccine, in which TRAMP-C2 cells are admixed with a GM-CSF producing cell line before vaccination, splenocytes from heterologous DNA primed and cell line boosted

mice produced 250-fold more IFNγ, upon restimulation with WT1 B cells, than splenocytes from homologous DNA prime and boosted mice (Figure 4A); however, these splenocytes from TRAMP-C2/Gvax vaccinated mice also produced IFNγ when restimulated with B cells that were not transfected to express WT1. Since these cell lines were grown in serum-containing media, one possible explanation for this result was that fetal bovine serum (FBS) "contaminated" the vaccine[86]; that is along with WT1, mice developed an immune response to FBS. Upon restimulation, in media containing FBS, some splenocytes from TRAMP-C2/Gvax vaccinated mice secreted IFNγ in the absence of WT1 (background). To test this possibility, TRAMP-C2-GM, a cell line retrovirally transduced to express GM-CSF, was conditioned to grow in serum-free TRAMP media. However, heterologous prime boost vaccinations with a serum-free cell line showed elevated levels of non-WT1-specific IFNγ production (Figure 4B), suggesting that antigens, other than FBS, common to both TRAMP-C2 and the B6BL#1153 cells, were also in the immunization.

Bone marrow-derived dendritic cells (BMDCs) grown in serum-free media do immunize against Ova but not WT1.

Matured and activated dendritic cells are the ultimate antigen presenting cell[6], and previous experiments showed that cell based vaccines enhanced immune responses, albeit with associated high levels of background. To avoid priming T cells to FBS, bone marrow cells were grown in highly supplemented, but serum-free media[86–89]. The protocol was adapted from Lutz et al[90], with minor modifications from Salem et al[91], and grown in an excess of mGM-CSF. (Please see the Material and Methods for details.) Phenotypic analysis and comparison of cell surface markers, on immature BMDCs grown

with or without FBS, revealed the serum-free BMDCs had slightly higher percentages of $CD11b^{+}CDl1C^{+}$ and marginally higher expression of costimulatory markers (Figure 5A,B). Fortunately that did not hinder transfection, as greater than 50% of cells from each culture condition expressed GFP after electroporation with mRNA, which in itself has maturing effects. Incubation with TLR agonist poly(I:C) fully matured and activated BMDCs after 24hrs (Figure 5C, D). Before vaccination, more than 80% of the transfected BMDCs had very high levels of MHC II and CD80, CD86, and CD40 expression.

To assess the ability of serum-free BMDCs to activate and expand T cells *in vivo*, mice were vaccinated with either untransfected but mature BMDC (DC), Ova-transfected and matured BMDC (Ova DC), or WT1-transfected and matured BMDC (WT1 DC). Tetramer analysis of splenocytes from naïve and vaccinated mice once again illustrated how difficult it was to immunize against a self-antigen, as compared to a model antigen such as ovalbumin (Figure 5E). Further *in vitro* assays bore that out, as only splenocytes from mice vaccinated with Ova DC, and restimulated by Ova generated increased amounts of IFNγ; however as noted before when using cell based vaccines, background IFNγ was present when splenocytes were restimulated by untransfected B cells (Figure 5F).

Listeria monocytogenes (Lm) vaccines do not induce immune responses to WT1

As mammalian cell based vaccines tend to activate more T cells than those specific to the antigen of interest, we explored other types of vaccines. Active immunotherapies such as *Listeria monocytogenes,* a bacterial vaccine vector, are especially attractive as it is designed to express the antigen of interest, it activates the innate immune system, its

target cells are APCs and its mode of action enables cross presentation of antigens[92–95]. *Listeria* vaccines have been reported to break tolerance in HPV16 and Her2/neu tumor models[96,97]. We hypothesized that a WT1-expressing *Listeria* vaccine would elicit robust WT1-specific immune responses. Tetramer staining showed that vaccination by Lm Ova activated and expanded Ova-specific T cells, in both primary and boost responses (Figure 6A, B respectively). The boost response to Lm Ova generated nearly a 4-fold increase in $CD8^+$ Ova-specific T cells; in one mouse approximately 30% of the $CD8^+$ T cells were specific for Ova. Similarly, mice vaccinated with *Listeria* expressing WT1-SIINFEKL (Lm WT1-SL8, wherein the fusion protein is WT1 with an Ova class I peptide tag) also had Ova specific T cells that expanded with boost vaccination to over 10% of the $CD8^+$ T cells. ELISpot assays that restimulated the splenocytes with peptide or Ova B cells reiterated the tetramer analysis (Figure 6C, D). In contrast, no WT1-specific response was observed in the primary or boost responses. Mice that were subsequently challenged with $1x10^6$ or $3x10^6$ TRAMP-C2 cells did develop WT1-specifc T cells; however, prior immunization with LmWT1-SL8 had no significant effect on this response. The frequency of WT1-specific T cells in both naïve mice as well as *Listeria* vaccinated mice was the same (Figure 7). To test the hypothesis that a heterologous prime-boost regimen would be capable of breaking WT1 tolerance, mice were vaccinated with WT1-expressing BMDCs, then boosted with LmWT1-SL8[98]. Tetramer analysis identified an Ova-specific T cell response but not a WT1-specific T cell response (Figure 8A). Upon challenge with $0.25x10^6$ TRAMP-C2 cells, there was a slight increase in WT1-specific T cells in groups that were primed with WT1-expressing BMDCs (Figure 8B).

Discussion

It is evident that attempting to produce an immune response to full length wild type WT1 is very difficult. Each of the vaccine strategies tested elicited responses to ovalbumin, which is a foreign antigen, while none utilizing murine WT1, a self-antigen, produced a robust response. Vaccine strategies included cDNA vaccination, protein plus adjuvant, BMDCs, *Listeria* and WT1 overexpressing whole cell vaccination.

The first strategy, DNA vaccination, one the major immunotherapeutic tools, could not activate an immune response with wild type WT1 cDNA in spite of supplementing the vaccination with electroporation which should have increased cellular uptake of plasmid and produced localized transient inflammation. However, there are many improvements that can be made. Newer electroporation devices can electroporate not only muscle, but also subcutaneous and epidermal layers of the skin so that Langerhans DCs can be targeted. The plasmid DNA can be altered to include genetic adjuvants. In the next chapter, we test the hypothesis that altering the sequence of WT1 cDNA will increase immunogenicity.

A second vaccination strategy involved wild type murine WT1 protein, CD40 antibody and adjuvant. WT1 protein and poly(I:C) created a minor immune response, which was reproducible, but surprising as it was expected that the best vaccine formulation would have included CD40 antibody as was seen when Ova was the antigen. What was not surprising was that poly(I:C), a TLR3 agonist that mimics dsRNA, typical of viruses and intracellular pathogens, was the better adjuvant for a WT1 vaccine as WT1 is a self-antigen that normally shuttles between cytoplasm and nucleus and is not

secreted. As Ova is typically secreted, LPS, a TLR4 agonist found in bacteria and extracellular pathogens, delivered better results for the Ova vaccine.

The third vaccination strategy used whole cell vaccines, such as TRAMP-C2/Gvax. It provided the greatest immune response to WT1. The GM-CSF matured and activated monocytes and DCs. It also prepared them to produce proinflammatory cytokines, which would attract more immune cells to the site of vaccination. Given that WT1 expression in TRAMP-C2 is nearly two logs greater than WT1 expression in the spleen, it may be a simple matter of quantity of antigen presented; however, unless treated with IFNγ, which was not done here, TRAMP-C2 does not express great quantities of either MHC class I or class II. It may be pertinent to ascertain that, while likely, the WT1 overexpressed in this cell line is the wild type version.

The fourth vaccination strategy, immunization with mature WT1-expressing BMDC failed to elicit a response to WT1. As mature, activated *professional* antigen presenting cells, expressing high levels of MHC I and II, as well as costimulatory markers, and efficiently transfected with antigen, serum-free BMDCs activated naïve Ova-specific T cells, but not WT1-specific T cells. Even though the BMDCs were cultured, transfected, and matured with poly(I:C), in the exact same fashion, the tolerance to WT1 hindered any responses. Dannull et al. demonstrated that selective (and transient) *in vivo* depletion of regulatory T cells (T_{reg}), which suppress immune responses, help to break tolerance when used in conjunction with DC vaccines[99]. Reports also indicate that providing a third signal, proinflammatory cytokines such as IL-12, is necessary to break tolerance and prevent deletion or anergy[9]. It would be wise to check cytokine production of the serum-free BMDCs before utilizing them for any future vaccines. Such

modifications to the vaccination may help abrogate the profound tolerance to WT1. Additionally, whole cell vaccines, such as TRAMP-C2 or BMDCs, primed responses to antigens other than WT1. In clinical trials, that may be advantageous, if a patient's tumor is part of a GVAX® vaccine, but non-tumor-specific immune responses can also arise[100]. Non-Ova or non-WT1-specific IFNγ production detracted from the significance of the response in ELISpot, ELISA or other IFNγ *in vitro* assays.

The fifth strategy, *Listeria monocytogenes* vaccines did not elicit background IFNγ production, but splenocytes from LmWT1-SL8 vaccinated mice also had no detectable WT1 response, using either by tetramer staining or ELISpot assays. The same vaccine was able to prime and boost a response to the Ova tag at the end of the WT1 fusion protein. Goldberg et al have reported that profound tolerance to self-antigen can be mitigated if the PD-1/PD-L1 interaction is blocked by antibodies during immunization with self-antigen expressing *Listeria* monocytogenes[101].

Overall, these data show that future experiments should address not only presentation of antigen but mechanisms of peripheral tolerance, such PD-1/PD-L1, anergy and T_{reg} suppression, as well.

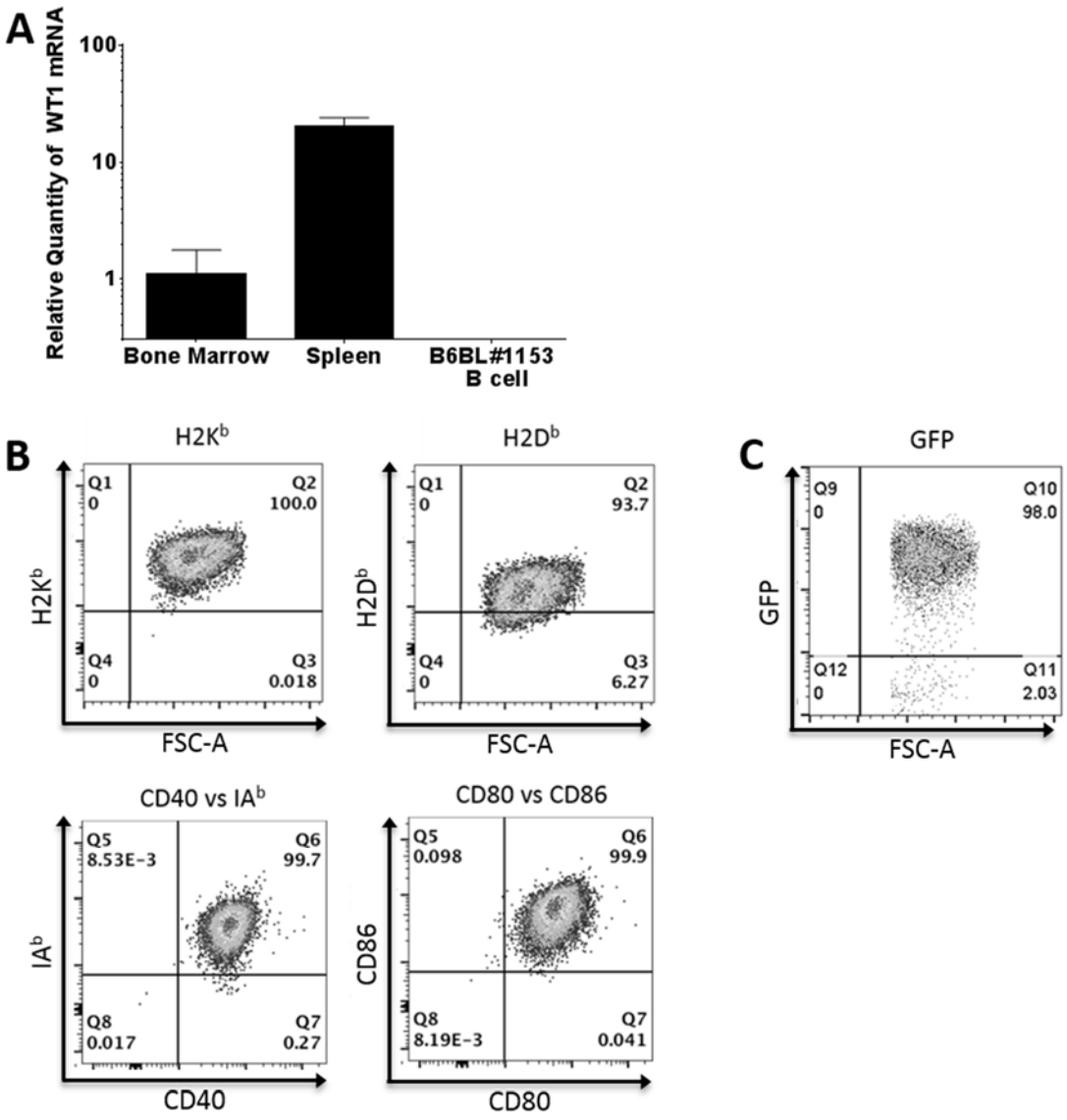


Figure 1. Characterization of B6BL#1153 B lymphoma (B cell) line.

(A) Relative expression of WT1 in C57BL/6 mouse bone marrow, spleen and B6 BL#1153 B cell line using mRNA isolated from tissues and cell line in a qRT-PCR assay. Bone marrow is normalized to 1. Each column represents the average of triplicates. Error bars show standard deviation of the mean. (B) B6BL#1153 B cell expression of MHC I, II, and costimulatory markers. (C) GFP expression 24 h after transfection (electroporation) of B6BL#1153 B cells with GFP mRNA. Data are representative of at least five experiments.

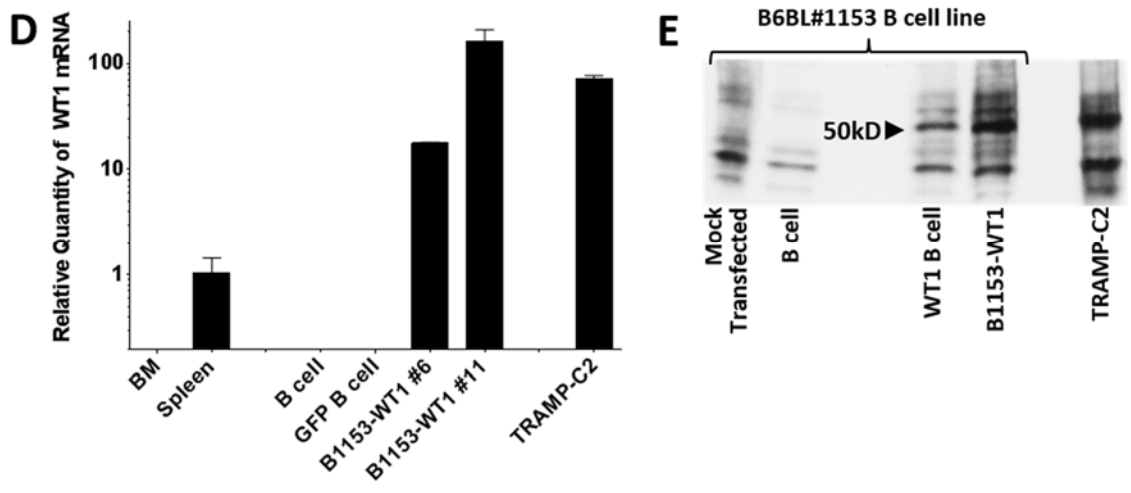


Figure 1. (continued)

(D) Relative expression of WT1 in murine bone marrow (BM) and spleen, B cells (untransfected B6BL#1153 B cells), GFP B cells (B6BL#1153 B cells transfected with GFP mRNA) and B1153-WT1 #6, #11 (two WT1 stably transfected B6BL#1153 B cell lines), and TRAMP-C2, a WT1-overexpressing prostate cancer cell line, using mRNA isolated from tissues and cell lines in a qRT-PCR assay. Spleen was normalized to 1. Each column represents the average of triplicates (+/-SD). Data are representative of at least three assays. (E) Western blot of mock transfected B6BL#1153 B cells (where cells are shocked without mRNA), B cells (untransfected B6BL#1153 B cells), WT1 B cells (B6BL#1153 B cells transfected WT1 mRNA) and B1153-WT1 (WT1 stably transfected B6BL#1153 B cell line), which are all compared to TRAMP-C2. WT1 is approximately 50kD.

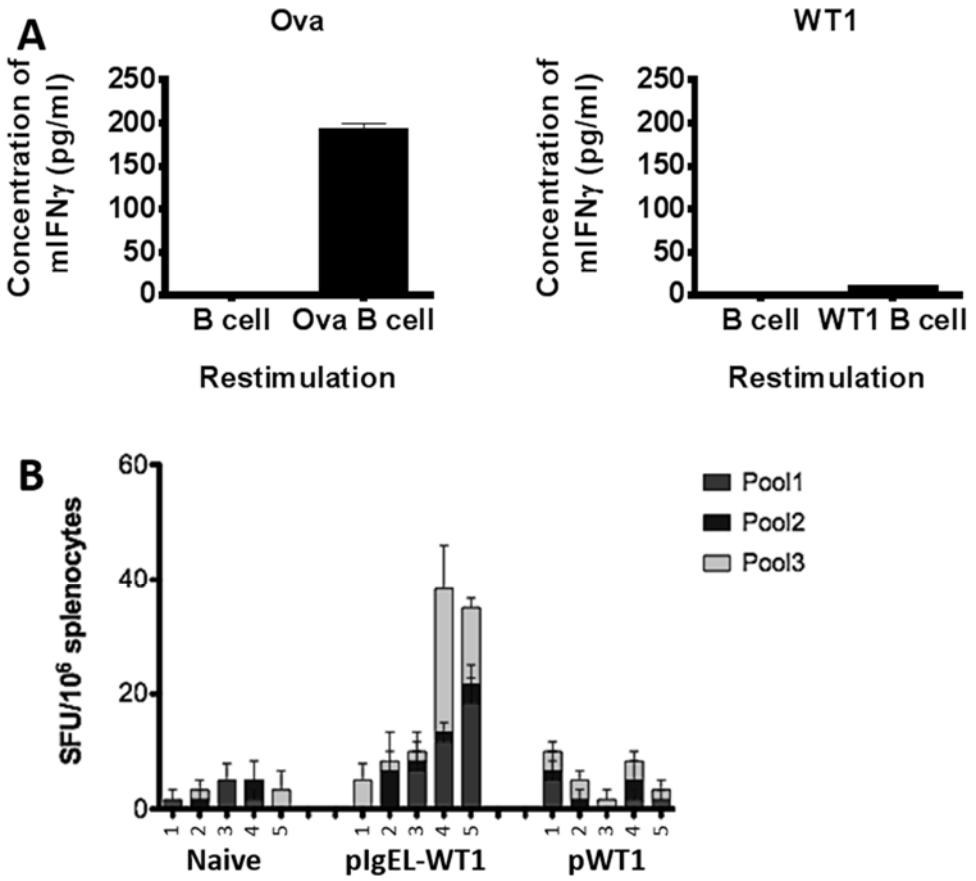


Figure 2. Immunization utilizing wild type WT1 cDNA produces a muted immune response as compared to other antigens.

(A) C57BL/6 mice were immunized by IM injection with 100 µg pOva or pWT1 plasmid per mouse on Days 0, 3, 6, 14, and seven days (Day 21) after the last vaccination mice were sacrificed, and T cells were restimulated by B cells (untransfected B6BL#1153 B cells), or Ova mRNA or WT1 mRNA transfected B6BL#1153 B cells, (Ova B cells or WT1 B cells) for 48 h, after which supernatant was collected and used in a mouse IFNγ ELISA. Data are representative of two independent experiments. Average (+/-SD) of three mice per group. (B) Two WT1 DNA vaccines are compared: 1) a construct in which an IgE leader is added to the murine WT1 sequence, pIgEL-WT1 and 2) an unaltered murine WT1 sequence, pWT1. One week after the fourth biweekly vaccination (30 µg of DNA by intramuscular injection followed by electroporation at injection site), mice were sacrificed, and splenocytes from BALB/c mice are restimulated with pools from a murine WT1 peptide library for 24 hours in 96 well ELISpot plates coated with antibody to IFNγ. Individual mice are shown for each group. Each column represents the average of triplicates (+/-SD). Data are from a pilot experiment and have not been repeated.

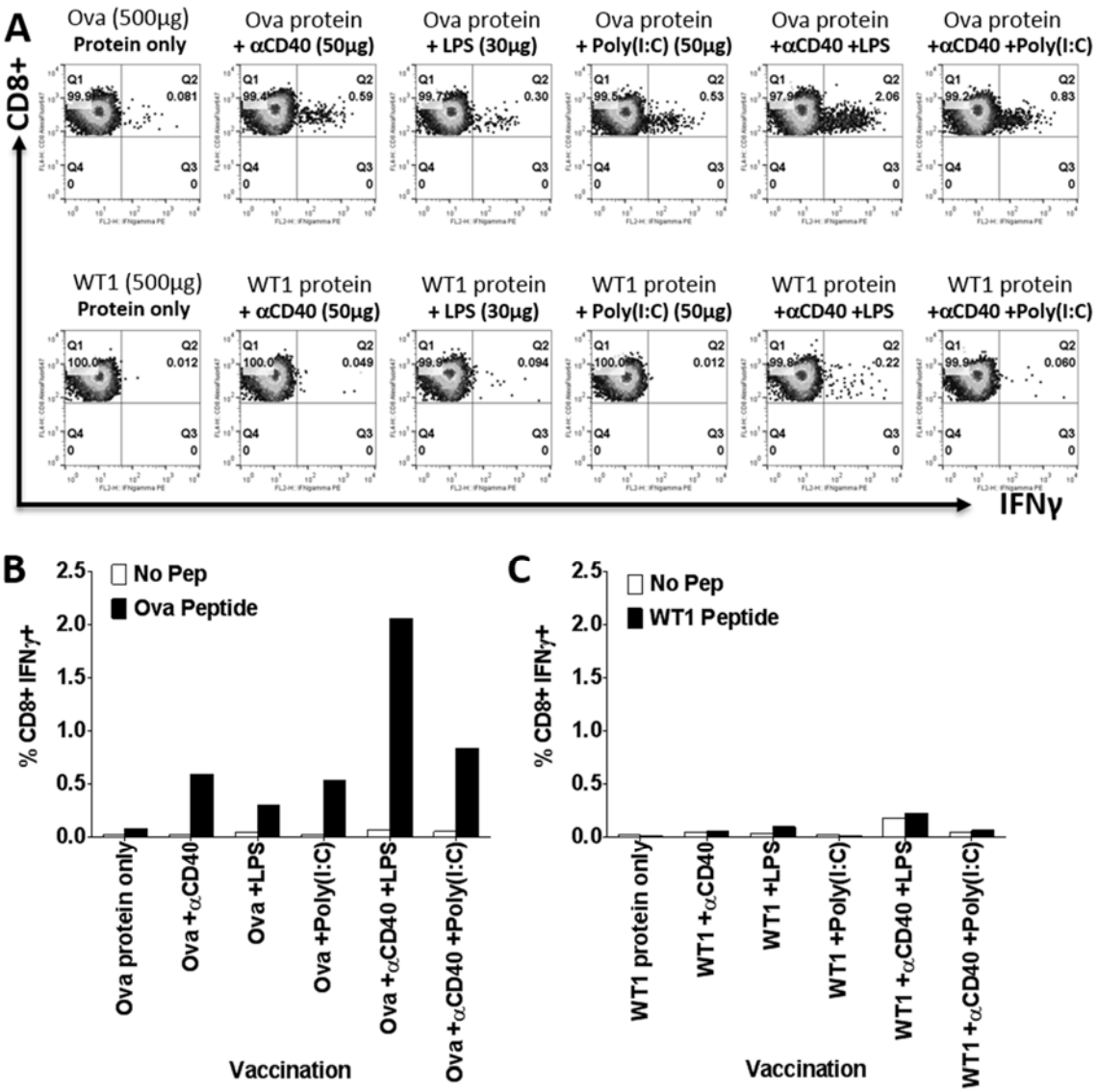


Figure 3. Immunization with GST-WT1, anti-CD40 and adjuvant modestly enhances the immune response.

C57BL/6 mice were immunized IP with 500 µg of whole ovalbumin or WT1 protein, with or without 50 µg of the anti-CD40 antibody (FGK45), and/or 30 µg of LPS, and/or 50 µg of poly(I:C) in the combinations indicated above. Mice were sacrificed 6 days after immunization, spleens were harvested and T cells isolated. T cells were restimulated with either peptide, untransfected B cells, or Ova or WT1 mRNA transfected B cells. (A-C) Frequency of $CD8^+$ $IFN\gamma^+$ T cells from representative mice, after restimulation by peptide for 12 h, and intracellular staining for IFNγ.

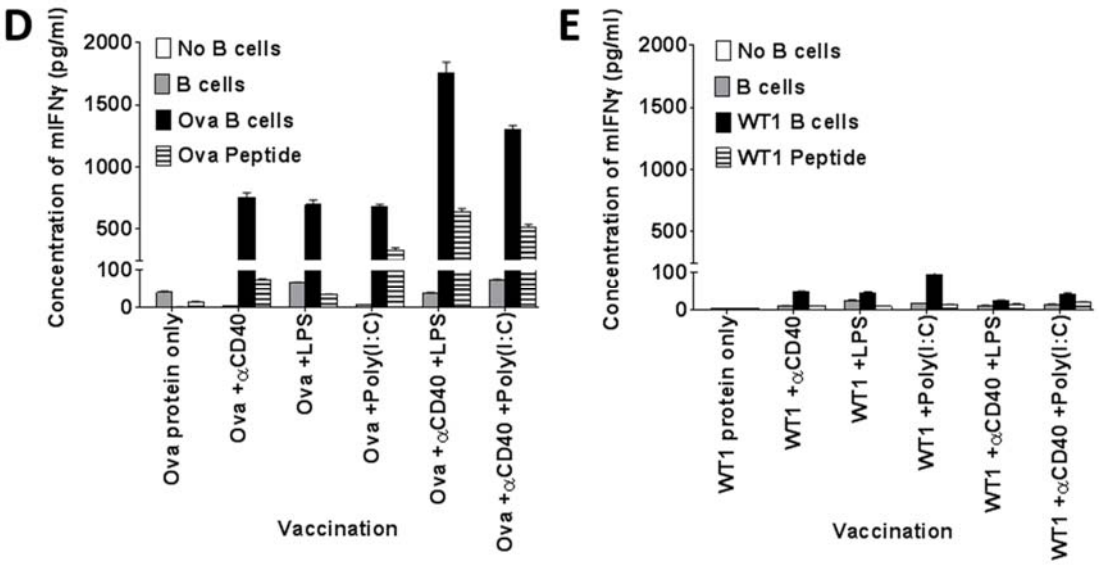


Figure 3. (continued)

(D, E) After 72 h of restimulation with peptide or B cells, supernatant was collected and assayed using mouse IFNγ ELISA. Each column represents the average of triplicates (+/-SD). Data are representative of two independent experiments.

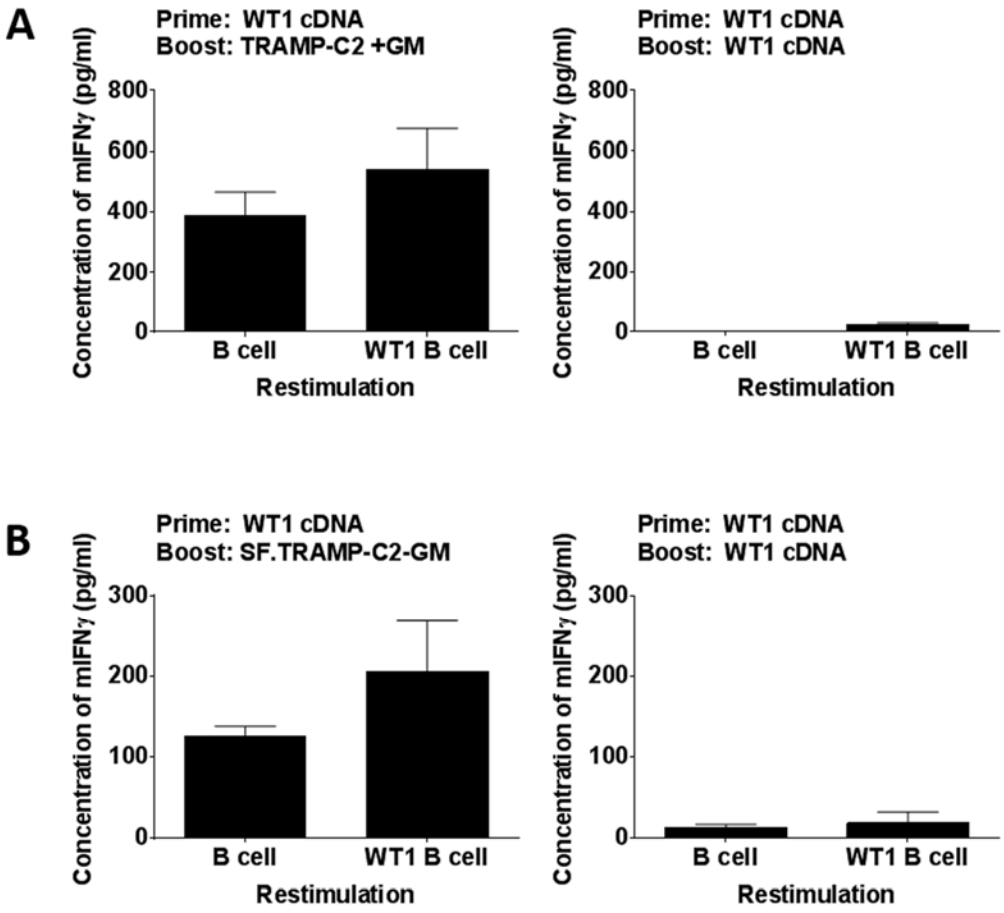


Figure 4. Heterologous prime/boost vaccination utilizing GM-CSF expressing cells increases immune responses.

C57BL/6 were immunized by IM injection with 100 μg pWT1 plasmid per mouse on Days 0, 3, 6, 14, and on Day 21 mice were immunized, subq, with either (A) TRAMP-C2/Gvax or (B) serum-free TRAMP-C2-GM. Seven days after the last vaccination mice were sacrificed, and T cells were restimulated by untransfected B6BL#1153 B cell (B cell) or transfected with WT1 mRNA (WT1 B cells) for 48 h, after which supernatant was collected and used in a mouse IFNγ ELISA. Data are representative of two independent experiments. Average (+/-SD) of three mice per group.

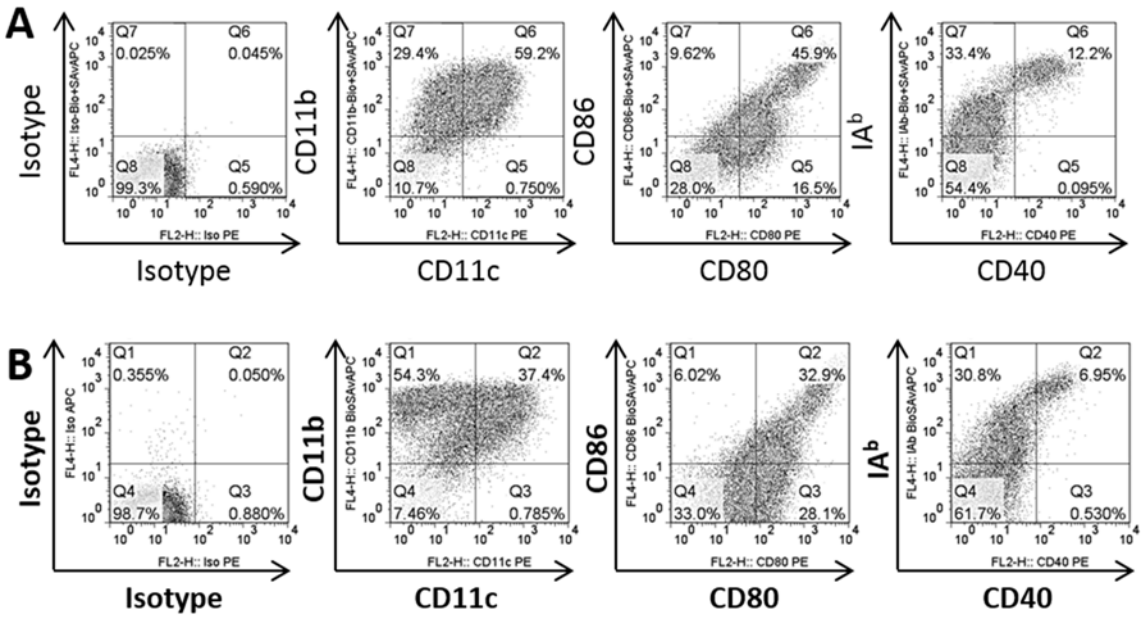
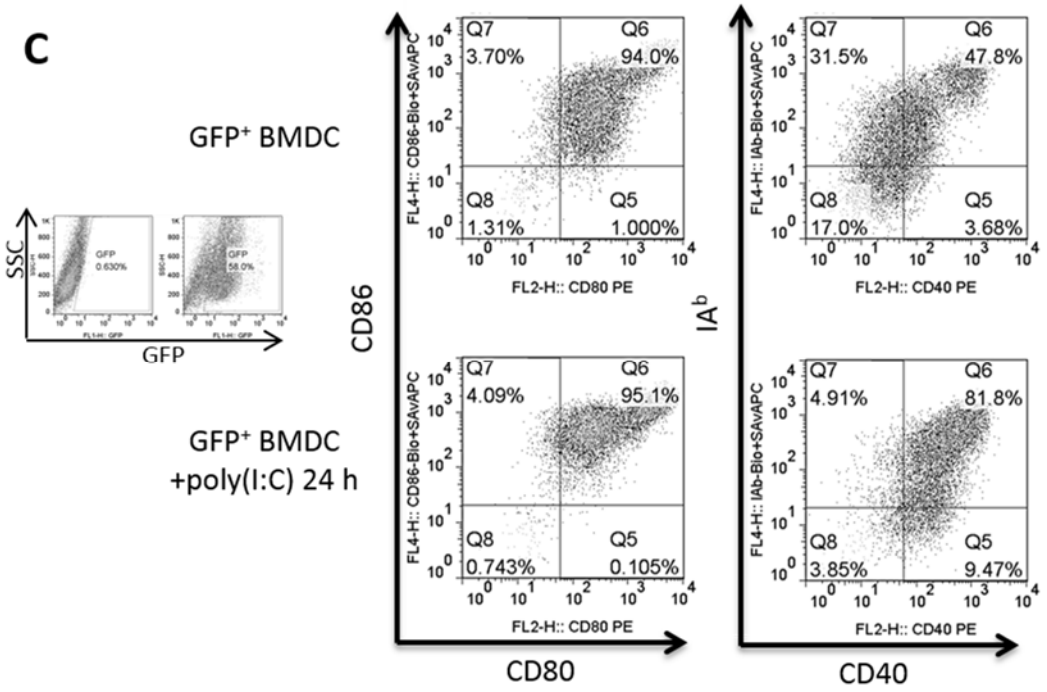
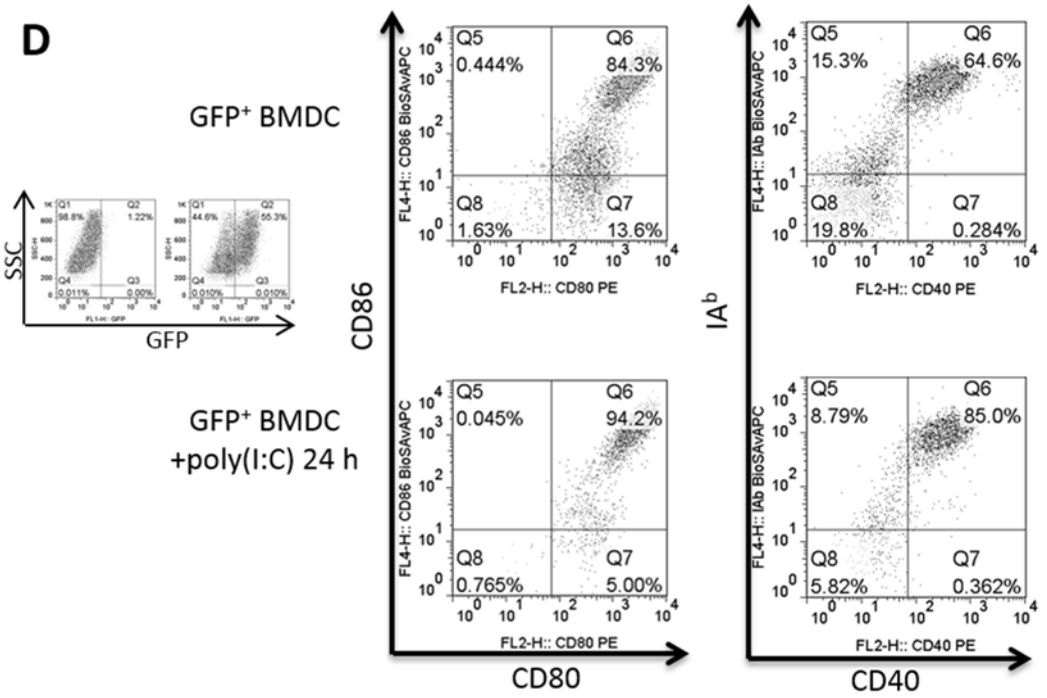


Figure 5. Bone marrow-derived dendritic cells (BMDCs) grown in serum-free media do immunize against Ova but not WT1.

Cell surface markers of immature BMDCs grown in (A) serum-free media or in (B) FBS-containing media before transfection with mRNA. Comparison of transfection efficiency and maturation using GFP mRNA to transfect immature BMDCs and poly(I:C) to mature BMDCs cultured without (C) or with FBS (D). Mice were immunized, subq, with serum-free, matured BMDCs, that were either untransfected (DC), or Ova or WT1 mRNA transfected (Ova DC and WT1 DC respectively). Mice were boosted 7 days later with the same type of BMDC, then sacrificed 7 days after the boost vaccination and spleen cells were isolated. (E) Tetramer analysis 7 days after boost vaccination. Splenocytes were stained with PE-labeled SAINFEKL/H-2K^b tetramer (OVA Tetramer) and APC-labeled RMFPNAPYL /H-2D^b tetramer (WT1 Tetramer) and were gated on B220$^-$ CD8$^+$ cells. (F) Frequency of CD8$^+$ IFNγ$^+$ T cells, after restimulation by peptide or B cells. Splenocytes were restimulated with either peptide, untransfected B6BL#1153 B cells (B cell), or Ova or WT1 mRNA transfected B6BL#1153 B cells (Ova B cell and WT1 B cell respectively), for 12 h in serum-free media and followed by intracellular staining for IFNγ. (G) Splenocytes were restimulated by indicated B cells for 24 hours in serum-free media in 96 well ELISpot plates coated with antibody to IFNγ. Data are representative of four independent experiments. Average (+/-SD) of 3-5 mice per group.





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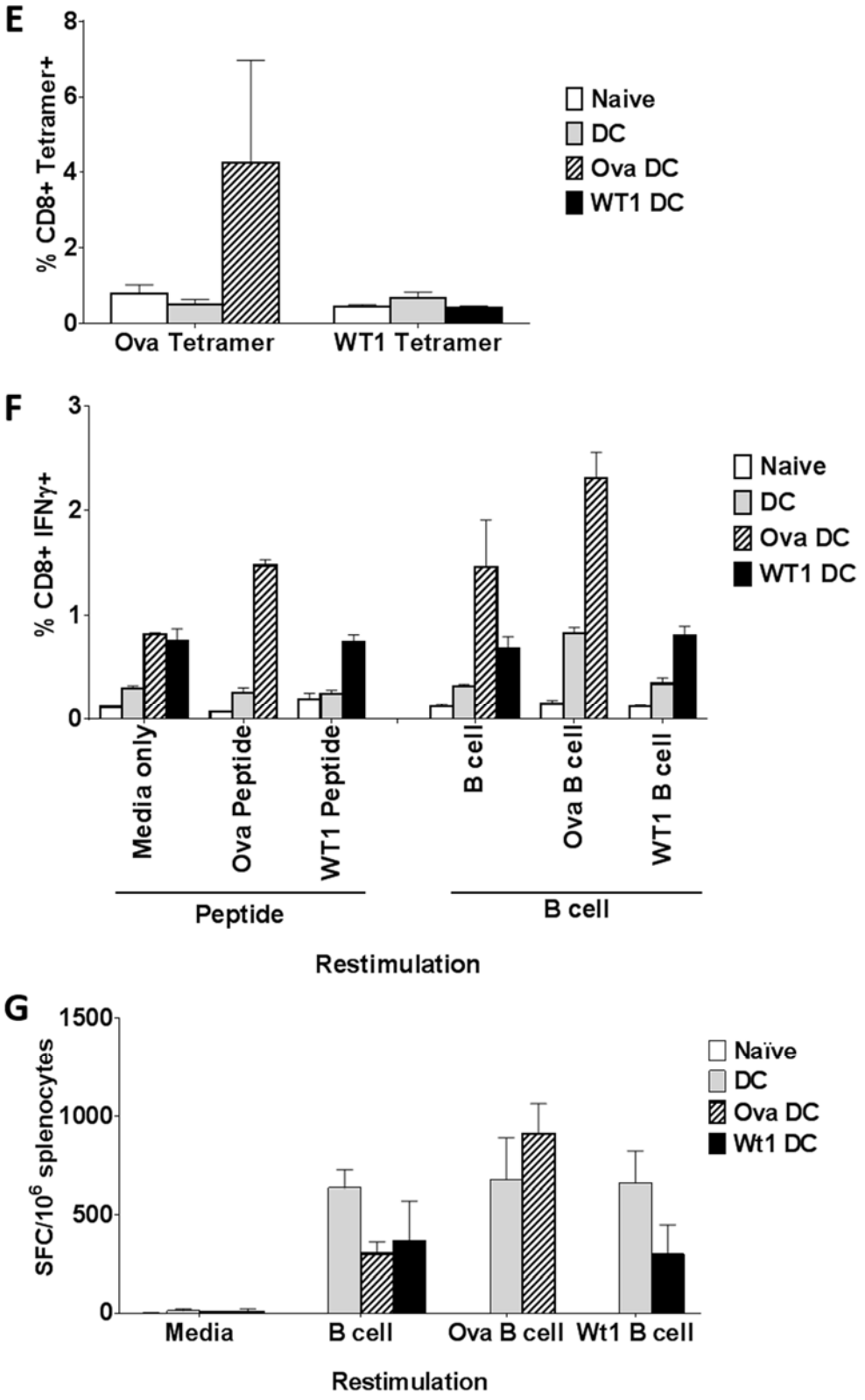


Figure 5. (continued)

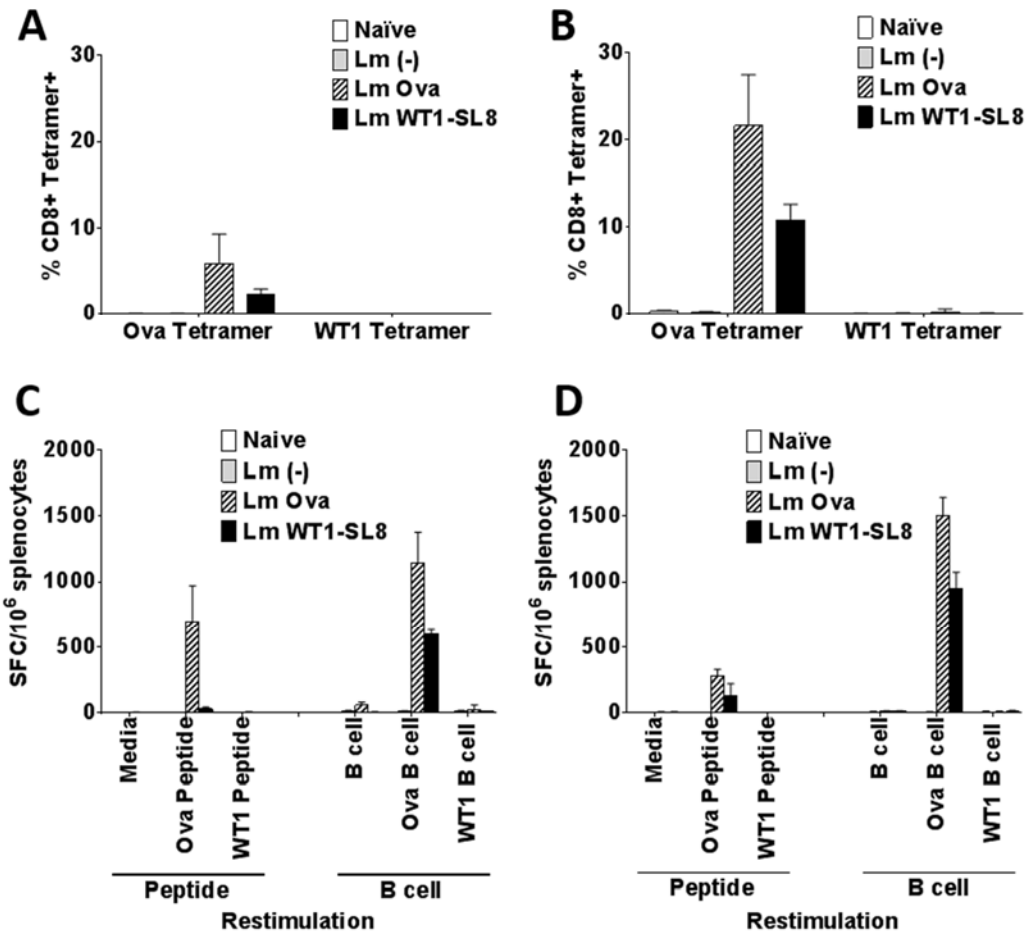


Figure 6. Comparison of immune response to foreign and self-antigens using *L. monocytogenes* vaccines.

Mice were primed with control *Listeria* (Lm (-)), ova-expressing *Listeria* (Lm Ova), WT1-SIINFEKL-expressing *Listeria* (Lm WT1-SL8) or unvaccinated (Naïve). Day 7 after priming, 3 mice from each group were sacrificed and splenocytes were assayed by tetramer and IFNγ ELISpot. The remainder of the mice were boosted 21 days after priming with the same *Listeria* vaccine with which they were primed. 6 days after boosting another 3 mice from each group were sacrificed and splenocytes were assayed. (A) Tetramer analysis of the primary response (day 7). Splenocytes were stained with PE-labeled SIINFEKL/H-2K^b tetramer (OVA Tetramer) and APC-labeled RMFPNAPYL/H-2D^b tetramer (WT1 Tetramer) and were gated on B220$^-$ CD8$^+$ cells. (B) Tetramer analysis of the boost response 6 days after boosting. (C) IFNγ ELISpot assay of the primary response (day 7). Splenocytes were restimulated with media, peptide (either Ova peptide (SIINKEKL) or WT1 peptide (RMFPNAPYL)), or with B6BL#1153 B cells (either untransfected (B cell), or transfected with either Ova mRNA (Ova B cell) or WT1 mRNA (WT1 B cell)). (D) IFNγ ELISpot assay of the boost response, 6 days after boosting. Data are from a pilot experiment and have not been repeated. Average (+/-SD) of three mice per group.

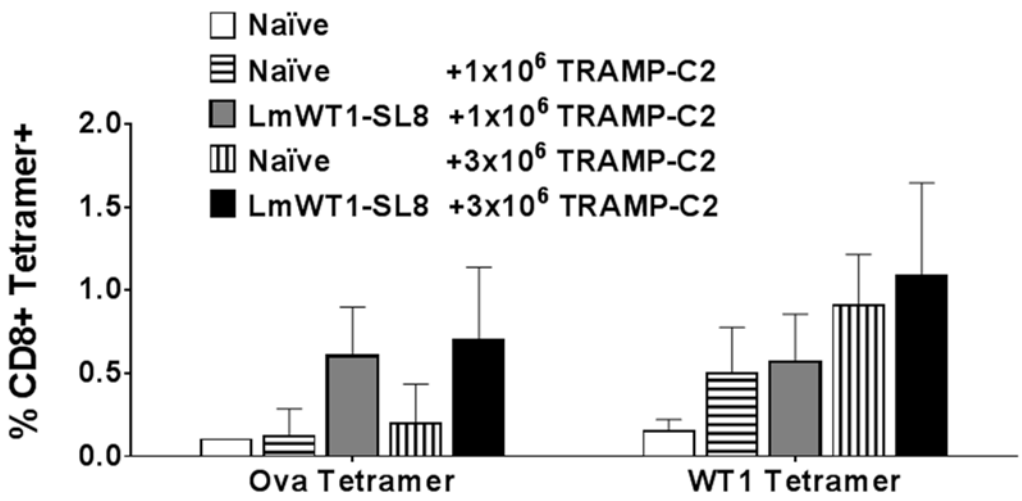


Figure 7. TRAMP-C2 tumor challenge, after *L. monocytogenes* vaccination, induced WT1-specific T cells.

Naïve mice or mice primed and boosted with LmWT1-SL8 vaccine were inoculated 7 days after the boost vaccination with either $1x10^6$ or $3x10^6$ TRAMP-C2 tumor cells, subq. Tetramer analysis of splenocytes from mice sacrificed after completion of the tumor challenge, 36 days after tumor inoculation. Splenocytes were stained with PE-labeled SIINFEKL/H-$2K^b$ tetramer (OVA Tetramer) and APC-labeled RMFPNAPYL/H-$2D^b$ tetramer (WT1 Tetramer) and were gated on $B220^-$ $CD8^+$ cells. Data are from a pilot experiment and have not been repeated. Average (+/-SD) of 5-6 mice per group.

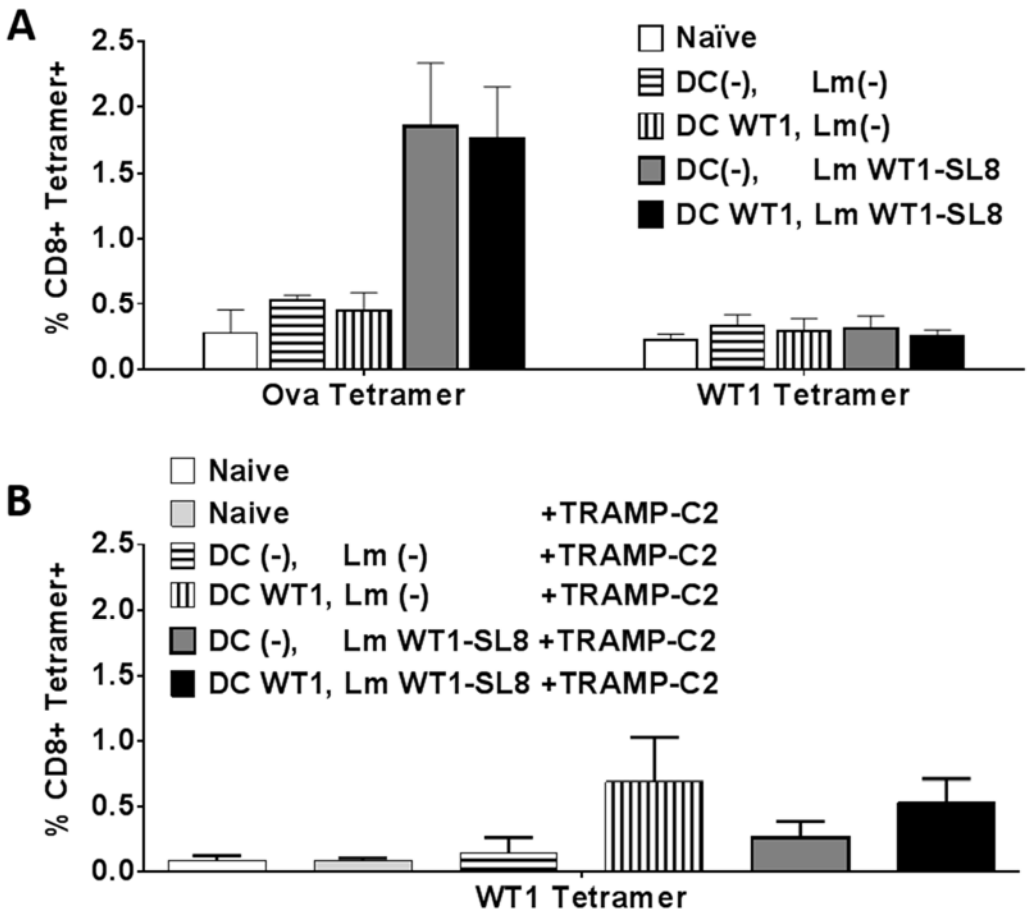


Figure 8. Heterologous Prime/Boost did not induce WT1-specific T cells while TRAMP-C2 tumor challenge activated WT1 primed T cells.

Mice were primed with $2.5x10^5$ matured and activated BMDC that were either untransfected (DC (-)) or transfected with WT1 mRNA (DC WT1) or remained unvaccinated (Naïve). Mice were boosted 6 days after priming with Lm(-), LmWT1-SL8 or remained unvaccinated. 5 days after boosting, 3 mice from each group were sacrificed and splenocytes were assayed with by tetramer. 24 days after the boost vaccination, the remainder of the mice (n=5 per group) were challenged with $0.25x10^6$ TRAMP-C2 tumor cells, subq. Tetramer analysis of the (A) boost response and (B) tumor challenge response. Splenocytes were stained with PE-labeled SIINFEKL/H-$2K^b$ tetramer (OVA Tetramer) and APC-labeled RMFPNAPYL/H-$2D^b$ tetramer (WT1 Tetramer) and were gated on $B220^-$ $CD8^+$ cells. Data are representative of two independent experiments. Average (+/-SD) of 3-5 mice per group.

Chapter 3: Vaccination with consensus WT1 cDNA intramuscular injection followed by electroporation induces an immune response to wild type WT1.

Introduction

As seen from the experiments detailed in chapter 2, mice are profoundly tolerant to wild type murine WT1. Matured and activated BMDC expressing wild type WT1 failed to induce WT1-specific T cells. Since even a fully functional professional antigen presenting cell was unable to elicit an immune response to the wild type antigen, we next hypothesized that altering the antigen would elicit an immune response that could recognize wild type WT1. Adding an IgE leader sequence to WT1 in a DNA vaccine construct, to facilitate expression, minutely improved the response (Figure 2B); more radical changes were necessary. Xenogeneic DNA vaccines are one approach to eliciting functional CTL that recognize self-antigens. Xenogeneic DNA are immunogenic because the antigen is similar enough that cross-reactive epitopes are utilized but different enough to circumvent barriers, such central and peripheral tolerance. Ideally, heteroclitic epitopes may be better able to bind peptide-MHC or the T cell receptor (TCR) and activate low avidity T cells. Subtle amino acids changes may affect the way the antigen is processed, possibly causing increased protein degradation, and presentation. Because the murine and human WT1 proteins are 97% identical, using human WT1 to vaccinate mice would be ineffective. To solve this problem, in collaboration with David Weiner and Jewell Walters (University of Pennsylvania, Philadelphia, PA) a consensus sequence DNA vaccine was created. The consensus is based on the alignment of homologous WT1 genes

from ten different species. The consensus WT1 sequence maintained conserved WT1 domains but was different enough at various amino acid positions to circumvent tolerance, as the consensus sequence is 91% identical to the wild type murine WT1 sequence. Lymphocytes from mice vaccinated with the consensus WT1 DNA vaccine responded to a peptide library based on the wild type murine WT1 sequence. Our findings suggest that using a consensus sequence DNA vaccine may be an effective method to produce an immune response to a self-antigen.

Materials and Methods

Construction of WT1 consensus sequence.

D. rerio, G. gallus, M. musculus, R. norvegicus, C. lupus, B. taurus, H. sapiens, P. troglodytes, and *A. mississippiensis* gene sequences were collected from GeneBank, and the consensus WT1 nucleotide sequence was obtained after performing multiple alignment. After obtaining the WT1 consensus sequence, an IgE leader sequence was added, and codon optimization and RNA optimization was performed by using GeneOptimizer® (GeneArt®, Life Technologies).

WT1 DNA immunogen.

The WT1 consensus sequence was synthesized and sequence verified by GENEART, and cloned into the expression vector pVAX (Invitrogen) and was named as WT1-pVAX-S but will be referred to as pConWT1 in this manuscript. Alternate constructs of the WT1 DNA vaccine have been previously mentioned. Briefly, wild type murine WT1 was cloned into pcDNA3.1, named pWT1, and a codon optimized murine WT1 (with an IgE leader sequence) was cloned into pVAX and named WT1-pVAX , but will be referred to as pIgEL-WT1 in this manuscript.

Mice

Female C57BL/6 mice between 6 to 8 weeks old were used in these experiments. Mice were obtained from the Jackson Laboratory. The mice were housed and maintained by the University Laboratory Animal Resources at the University of Pennsylvania in observance with the policies of the National Institutes of Health and the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). The mice used in

these experiments were separated into groups of five for immunization. Mice were immunized with pConWT1, pIgEL-WT1 or pWT1. Naïve mice served as negative control.

DNA vaccination and electroporation

Each mouse received four doses of DNA plasmid at 14-d intervals. Mice received 25μg to 75μg of DNA per vaccination. The DNA constructs were administered via intramuscular injection of the right quadriceps muscle, followed by square-wave pulses generated by the CELLECTRA® constant current electroporation device (Inovio Pharmaceuticals). The device was configured to deliver two 0.1 Amp pulses of 52 ms pulse width spaced apart by a 1 sec delay. Electroporation procedure was performed as described previously[64].

IFNγ ELISpot assays

Mice in both treatment and control groups were sacrificed 1 week after the third immunization. Spleens were harvested from each mouse and transferred to R10 media (RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics). Using a stomacher (Seward Laboratory Systems), the spleens were pulverized and subsequently transferred through a 40 μm cell strainer. Erythrocytes were removed by adding ACK lysing buffer (Lonza). The splenocytes were isolated and resuspended in R10 media. High-protein IP 96-well Multiscreen™ plates (Millipore, S2EM004M99) were coated with monoclonal murine IFNγ Capture Antibody (R&D Systems, SEL485) and incubated overnight at 4°C. After three washes with 1x PBS, the plates were blocked with 1% BSA and 5% sucrose in 1x PBS for 2 h at ambient temperature. Isolated

splenocytes in R10 medium were counted and added in triplicate wells at $2x10^5$ cells per well. Peptides or $0.5x10^5$ B cells (either untransfected, or WT1 mRNA transfected B6BL#1153 B cells, or B1153-WT1 stably transfected cell line) were added to the wells. Two sets of peptides spanning the consensus WT1 sequence and the wild type murine WT1 sequence (excluding the zinc finger region) were synthesized by GenScript. The peptides contained 15 amino acid sequences, of which 11 residues overlapped with each sequential peptide. The peptides for each WT1 set were each divided into three pools at concentrations of 2 μg/mL/peptide. Wells reserved for positive and negative control received Concanavalin A (Sigma-Aldrich, C0412) and R10 culture medium in lieu of peptides, respectively. Plates were subsequently placed in a 5% CO_2 atmosphere incubator. After incubation for 18–24 h at 37°C, the wells were washed with 1x PBS. Biotinylated anti-mouse IFNγ Detection Antibody (R&D Systems, SEL485) was added to each well and then incubated overnight at 4°C. The plates were subsequently washed and processed per a color development protocol provided by R&D Systems using Streptavidin-AP and BCIP/NBT Plus (R&D Systems, SEL002). The wells were air-dried overnight and spots inside wells were scanned and counted by an ELISpot plate reader system with ImmunoSpot®3 and ImmunoSpot®4 software (Cellular Technology Ltd.). Reported spot forming cell counts were converted to represent spot-forming units per $1x10^6$ splenocytes.

Epitope mapping

Epitope mapping studies were performed to determine the dominant epitopes within both consensus and mouse-matched WT1 peptide libraries. Combinatorial peptide libraries specific for wild type WT1 were established by setting up 18 peptide pools, each

pool consisting of eight to nine peptides combined in such a way that each individual library peptide was shared once by two particular pools. Combinatorial peptide libraries specific for consensus WT1 were established by setting up 17 peptide pools, each pool consisting of eight to nine peptides combined in such a way that each individual library peptide was shared once by two particular pools. The ELISpot assays were performed as previously described.

Results

Consensus Sequence Construct.

Initial experiments using wild type murine WT1 cDNA (either in its original pcDNA3.1 vector, or in a pVAX vector fused after an IgE leader sequence) to vaccinate mice elicited minimal immune responses, even when electroporation was included in the vaccination protocol. Therefore, a WT1 consensus sequence DNA vaccine was created by aligning homologues of WT1 from various species including human, mouse, rat, dog, and chimpanzee. The sequences were aligned to define the critically conserved domains, and identify positions of amino acid variability. For example, in *M. musculus* WT1 protein there is a 17 aa domain (exon 5) that is not conserved across the species and the exon is a commonly seen splice variant; therefore, this 17 aa domain was not included in the consensus sequence. Doubrovina et al. also reported finding epitopes across the breadth of human WT1 excepted for this 17 aa domain, even though their peptides were based on the full length sequence[47]. Another example, at amino acid position 136 where there is a serine in the *M. musculus* sequence that has been changed to the asparagine seen in the *D. rerio* sequence. The sequence was further modified by tRNA codon and RNA optimization, and a Kozak sequence and a highly efficient leader sequence was fused in frame upstream of the start codon to facilitate expression. Not included in the construct was a proline rich region, the variably spliced exon 5, and the zinc finger domain, which contains the nuclear localization signals[102] (Figure 9A). Removal of the zinc finger region that would normally sequester the protein in the nucleus, allows for more secretion of the protein from the muscle cells that have been transfected. This protein can be taken up by DCs resident in the tissue. Alignment of the final consensus

WT1 sequence shows a 91% similarity to the original wild type murine WT1 sequence and illustrates the amino acid differences between the two (Figure 9B). The dominant epitope reported in the literature $WT1_{126\text{-}134}$ (RMFPNAPYL) is unchanged. The final construct was cloned into pVAX vector, and is here referred to as pConWT1. Highly purified and concentrated DNA was used to vaccinate mice by intramuscular injection, immediately followed by electroporation.

Consensus WT1 DNA vaccine induces a stronger immune response than wild type murine WT1 DNA vaccine.

To compare the immunogenicity of the consensus WT1 construct to previous wild type WT1 constructs, mice received 25 μg of either pConWT1, pIgEL-WT1 or pWT1 vaccines (Figure 10). Upon restimulation, *in vitro* with pooled peptide from the consensus library sequence in an IFNγ ELISpot, splenocytes, from mice vaccinated with pConWT1 demonstrated clear unequivocal immune responses. Splenocytes from pIgEL-WT1 vaccinated or pWT1-vaccinated mice did not respond to consensus peptides. Although it is possible that these WT1-specific splenocytes could not recognize consensus peptide, but it is far more likely that those splenocytes lack WT1-specific T cells as seen previously in chapter two. To elicit a significant immune response to a self-antigen, actually changing portions of the antigen was necessary, whether it be truncation or individual amino acid substitutions.

Increasing the amount of consensus WT1 DNA increases the immune response to wild type WT1 peptides.

To further validate the use of pConWT1, splenocytes, from mice immunized with increasing doses of pConWT1, proved that they had increasing numbers of WT1-specific cells. Pools of wild type mouse-matched WT1 peptides restimulated these splenocytes, indicating that consensus WT1 vaccination activated and expanded WT1-specific T cells that recognized murine WT1. The dose-response curve shows increasing the amount of the consensus WT1 DNA electroporated into the muscle of the mouse does increase the number of IFNγ positive cells upon restimulation *in vitro* with a wild type murine WT1 peptide library (Figure 11). The immunogen is essentially xenogeneic but the response contains activated or memory T cells that can identify and respond to the murine WT1 antigen.

Splenocytes from pConWT1-vaccinated mice do not respond to endogenously expressed WT1.

Since splenocytes from pConWT1-vaccinated mice responded to wild type murine WT1 peptides, we restimulated these splenocytes with B cells expressing wild type murine WT1, either a stably transfected cell line or one that was transfected with mRNA the evening before mice were sacrificed. In either case, very few splenocytes from pConWT1-vaccinated mice produced any IFNγ, at least not above the level of background (Figure 12). This is not an unprecedented result as Ramirez et al. also observed a similar phenomenon, where peptide loaded BMDC vaccination induced WT1-specific CTLs, that could be restimulated *in vitro* by peptide, but could not recognize antigen on WT1-expressing cells[103].

Epitope mapping using the wild type mouse-matched WT1 peptide library identifies epitopes other than WT1 Db126

In order to define the epitopes within WT1 to which these T cells are responding, epitope mapping was done with combinatorial pools of peptide from either the consensus WT1 library (Figure 13) or the wild type murine WT1 library (Figure 14). The libraries were composed of 15mer peptides, overlapping by 11 amino acids. A matrix system was used in which peptides were pooled by row or columns, allowing each peptide to be represented twice, and tested in duplicate. Screening with the consensus WT1 library isolated peptide #28 ARMFPNAPYLPNCLE, which contains the well-characterized epitope RMFPNAPYL (aa 126-134). This is the dominant epitope for C57BL/6 and BALB/c mice and HLA-A0201 humans. Alignment of consensus peptide #28 that was identified in the consensus WT1 library, to consensus and wild type sequences revealed that the only difference between this 15mer peptide and the wild type murine WT1 sequence is the second asparagine is a serine in the wild type sequence (aa 136), outside of the known epitope (Figure 15A). Screening with the wild type murine library showed immune responses, above background, in three pools, #1, 9 and 12 which identified peptides #19 (EEQCLSAFTLHFSGQ) and 27 (PSQASSGQARMFPNA), which were then aligned to both sequences and compared (Figure 15B). Each of the wild type peptides differ from the consensus sequence at one amino acid. Wild type peptide #19 may contain a novel murine WT1 epitope as it has not been mentioned in the literature while peptide #27 contains QASSGQARM which was studied by Naylor et al[104] and Dai et al[105] with conflicting results. These epitope mapping assays need to be repeated to

confirm the results, but with splenocytes from mice vaccinated with 50 μg of pConWT1 so that CTL responses are significantly higher than the background.

Discussion

Given the overexpression of WT1 in a multitude of cancers, an effective immunotherapy targeting this tumor-associated antigen would be beneficial; however, because WT1 is a self-antigen, generating a substantial WT1-specific immune response is difficult due to tolerance barriers such as deletion of high avidity T cells in the thymus, and possibly anergy and suppression in the periphery as well. In a highly tolerogenic mouse model, in which WT1 is expressed not only in a few restricted cells of certain tissues, but in the stroma of the adult murine spleen as well, our consensus WT1 DNA vaccine may circumvent tolerance and generate a significant T cell immune response.

In the thymus, central tolerance is established whereby self-reactive T cells that have TCRs with a high affinity for self-peptide-MHC are deleted, anergized or differentiated into regulatory T cells to prevent autoimmunity[106]. The T cells that do escape the thymus have TCRs that recognize foreign antigens or have a low affinity for self-peptide-MHC. While this is normal, it poses a problem for cancer immunotherapies which target tumor-associated antigens, some of which are aberrantly expressed or overexpressed self-antigens. In a report by Aleksic et al., ten viral antigen (VA)-specific TCRs and 14 tumor-associated peptide antigen (TAPA)-specific TCRs (including 3 WT1-specific TCRs) were compared based on their affinity to their corresponding peptide-human leukocyte antigen complex (in this case pHLA-A0201). It was clear that VA-specific TCR-pHLA interactions tended to have lower dissociation constants (K_D) and on average longer half-lives ($t_{1/2}$) than their TAPA-specific counterparts[107]. For example, a HIV-specific TCR had a K_D of

0.18 μM and a $t_{1/2}$ of 27 seconds, while one of the WT1-specific TCRs had a K_D of 45μM and a $t_{1/2}$ of <0.5 seconds[107]. The HIV-specific TCR has a higher affinity for its pHLA as it binds it more tightly, requires less to reach equilibrium, and remains bound longer, than theWT1-specific TCR. The lower affinity WT1-specific TCR requires 250x more pHLA to reach equilibrium and even then the interaction may last less than half a second. Pinilla-Ibarz et al. reported that they were able to design analog heteroclitic WT1 peptides with a longer half-life and were able to elicit CTL responses; however, these peptides were designed to bind better specifically to HLA-A0201[108]. A higher affinity, and a longer TCR-pMHC interaction produced a stronger immune response, but it was relevant only to those who have the matching HLA.

In an effort to create an ideal WT1-specific TCR, Schmitt et al. designed two enhanced-affinity complementarity determining region (CDR) 3α mutants. Both mutants had higher affinity for WT1-D^b (RMFPNAPYL) than the wild type TCR, but one had higher functional avidity, in vitro, as well[109]. TCR transduced T cells were injected into mice that were subsequently immunized with WT1 peptide-pulsed splenocytes, then boosted with *Listeria monocytogenes* expressing the WT1 RMFPNAPYL epitope. They showed that the T cells expressing the enhanced-affinity TCRs expanded *in vivo* 1.5 to 1.6x greater than the T cells expressing the wild type WT1 TCR, without causing autoimmunity[109]. While this method of enhancing the WT1-specific immune response is encouraging as it doesn't depend on the immunocompetence of the subject and "replaces" the high avidity WT1-specific T cells that are deleted due to central tolerance, it focuses on one epitope, requires mutation of the TCR and

transfection of syngeneic T cells. If it were translated to the clinic, it would need to be tailored for each patient, or else may limit the type of patient for which it would be effective, due to histocompatibility issues.

If one cannot "replace" high avidity WT1-specific T cells, another approach to the problem would take into account that the low avidity T cells require more antigen to engage their TCRs sufficiently, and immunize subjects with more antigen. For example, C57/BL6 mice can be immunized with TRAMP-C2, a prostate adenocarcinoma that overexpresses WT1 several logs higher than what is seen in the murine spleen, and produce a significant immune response; however, the response is not only to WT1, but also to a plethora of other antigens expressed by the cell line and found in the culture media. While immunizing with a WT1 overexpressing cell line addresses the issue of quantity of antigen, it doesn't address the typically shorter half-life of the TCR-pMHC interaction of low avidity T cells.

To address both concerns, quantity of antigen and possibly quality of TCR-pMHC interaction, we created a consensus based WT1 DNA vaccine that is injected intramuscularly, immediately followed by electroporation. The beauty of DNA vaccines are they are easily designed, economical, and safe. The plasmid DNA can be engineered to express multiple antigens, linked epitopes, cytokines and adjuvants. In this report, the plasmid contains a Kozak sequence, an IgE leader sequence, and the codon-optimized WT1 consensus sequence. It excludes the zinc finger region, which can sequester the protein in the nucleus. These modifications along with in vivo electroporation, which increases the number of number of transfected cells and the number of plasmids per cell, greatly enhance protein expression of the antigen[75,76]. Additionally, the WT1 consensus

sequence is based on the alignment of WT1 homologs, which is used to create a more xenogeneic antigen that is 91% identical to the syngeneic antigen. Xenogeneic antigens are able to circumvent tolerance, because they are sufficiently different from the self-antigen, but similar enough to produce compatible peptides[46].

These peptides may be heteroclitic in nature and/or affect proteasomal cleavage. Heteroclitic peptides have amino acid differences which can enhance TCR affinity to its pMHC and can increase the half-life of TCR-pMHC interaction. These changes may increase the binding of the peptide to MHC, thereby increasing the density of that peptide's presentation on the surface of APCs, facilitating increased TCR engagement, T cell activation, and bolstering the immune response[46,110]. However, Speiser et al. would argue that *functional avidity*, gained by using the natural, unmodified peptide, is superior to the increased T cell frequencies gained by using the altered or heteroclitic peptide, when the vaccine formulation includes IFA and CpG[111]. If the peptides, identified in this study are not heteroclitic, the amino acids substitutions may still affect proteasomal cleavage.

In the cytoplasm, the constitutive proteasome regularly degrades ubiquitinated proteins, preferring to cleave proteins after certain amino acids. After cells are induced by IFNγ exposure, the proteasome is reconfigured into the immunoproteasome, which has slightly different specificities for cleavage sites[112,113]. In either case, these peptides are translocated by the transporter associated with antigen processing (TAP), into the lumen of the endoplasmic reticulum (ER), where they are loaded onto MHC I for eventual presentation to $CD8^+$ T cells[114,115]. Consensus peptide #28 which contains RMFPNAPYLP**N**CL, wherein the underlined "N" was a "S" in the murine WT1

sequence, has a better immunoproteasomal processing score with the substitution, according to T cell epitope prediction tools provided by the Immune Epitope Database and Analysis Resource (www.iedb.org)[116]. However, this does not take into account TAP transport and MHC binding, which then reveals that RMFPNAPYL is still the best epitope. In another example, murine peptide #19 contains LSAFTLHF, but the consensus sequence is LSAFT**V**HF, which again has a better immunoproteasomal processing score. Is it possible that better processing of the xenogeneic protein, more LSAFT**V**HF peptides produced and presented, activated LSAFTLHF-specific T cells? While T cell epitope prediction tools are useful, the epitope mapping assay needs to be repeated, and any proposed epitopes experimentally validated. Our current experiment identified two 15mer peptides with single amino acid substitutions that, upon further study, may reveal heteroclitic epitopes, or instead show how amino acids changes influence the peptides created by the constitutive and immunoproteasomes.

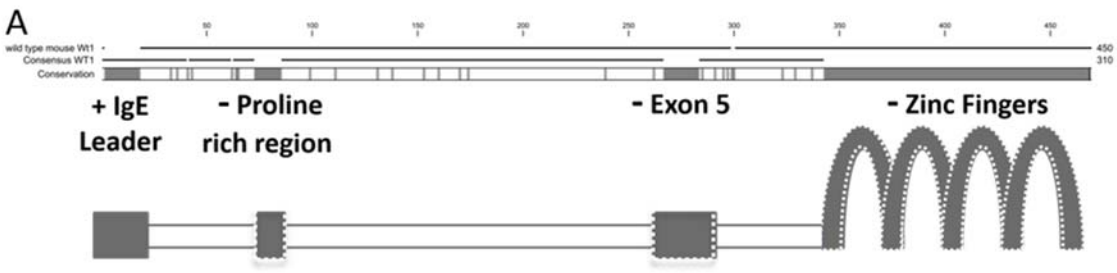


Figure 9. Schematic of the consensus WT1 construct, and comparison of the consensus and the wild-type murine WT1 sequences.

(A) Schematic of WT1 protein to show major differences between the murine WT1 and consensus WT1 sequences. The WT1 consensus sequence is based on the alignment of homologs of WT1 and further modified to include an IgE leader sequence and the removal of a proline rich region, the variably spliced exon 5, and zinc finger domains. (B) Amino acid alignment of the consensus sequence to the wild-type murine WT1 sequence. Dark grey regions along the conservation band indicate areas of conflict.

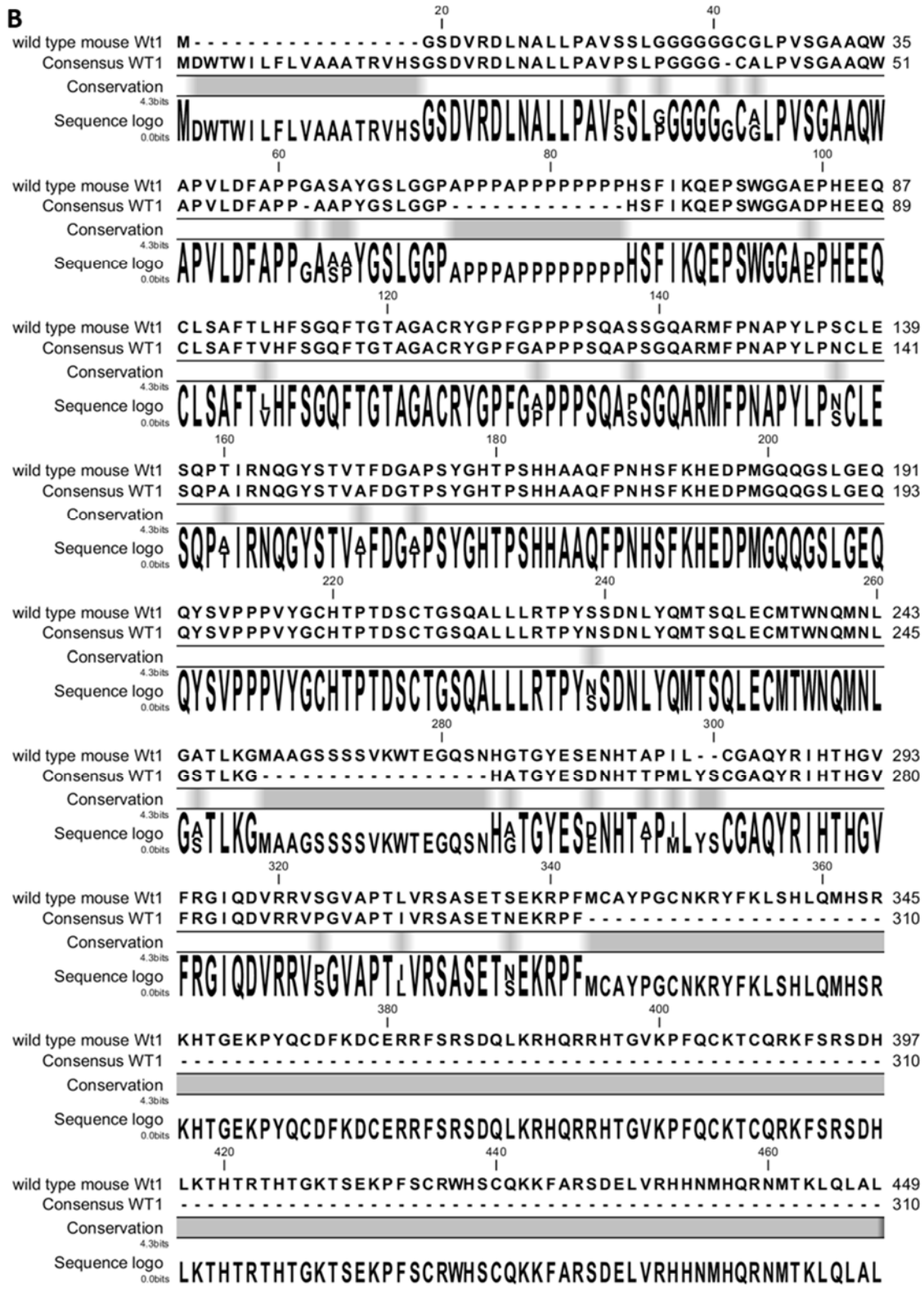


Figure 9. (continued)

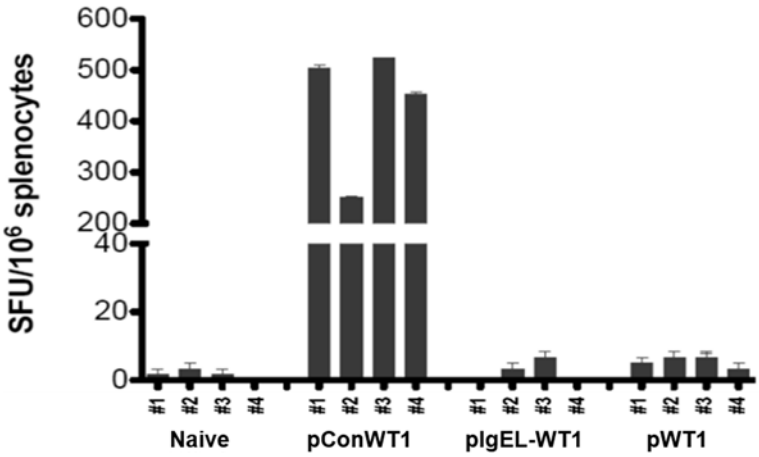


Figure 10. Consensus WT1 DNA vaccine induces a stronger immune response than wild type murine WT1 DNA vaccine.

Three WT1 DNA vaccines are compared: 1) the consensus WT1 construct pConWT1, 2) a construct in which an IgE leader is added to the murine WT1 sequence, pIgEL-WT1, and 3) the original wild type murine WT1 construct, pWT1. One week after the fourth vaccination (25 μg of DNA by IM injection followed by electroporation at injection site) splenocytes from C57BL/6 mice were stimulated *in vitro* with pooled consensus WT1 peptide for 24 hours in 96 well ELISpot plates coated with antibody to IFNγ. Individual mice are shown for each group. Each column represents the average of triplicates (+/-SD). Data are from a pilot experiment and have not been repeated.

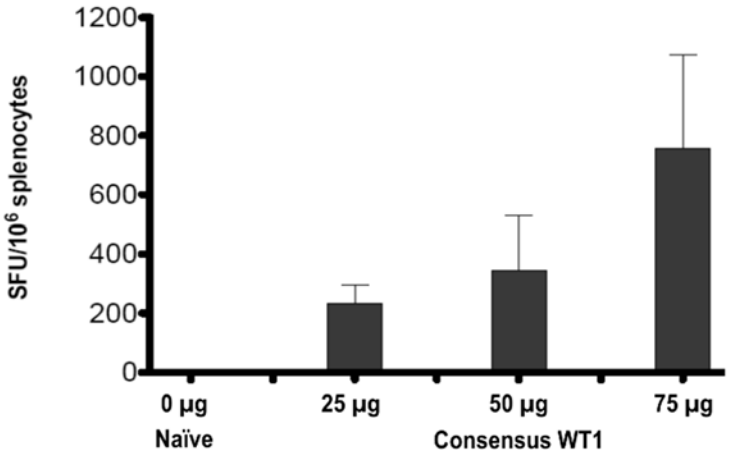


Figure 11. Increasing the amount of consensus WT1 DNA increases the immune response to wild type WT1 peptides.

Increasing amounts of consensus WT1 DNA, pConWT1, were used to vaccinate mice (25 µg, 50 µg, and 75 µg). One week after the fourth electrovaccination, splenocytes from C57BL/6 mice are stimulated *in vitro* with pooled wild type murine WT1 peptide for 24 hours in 96 well ELISpot plates coated with antibody to IFNγ, in triplicate. Data are representative of two independent experiments. Average (+/-SD) of five mice per group.

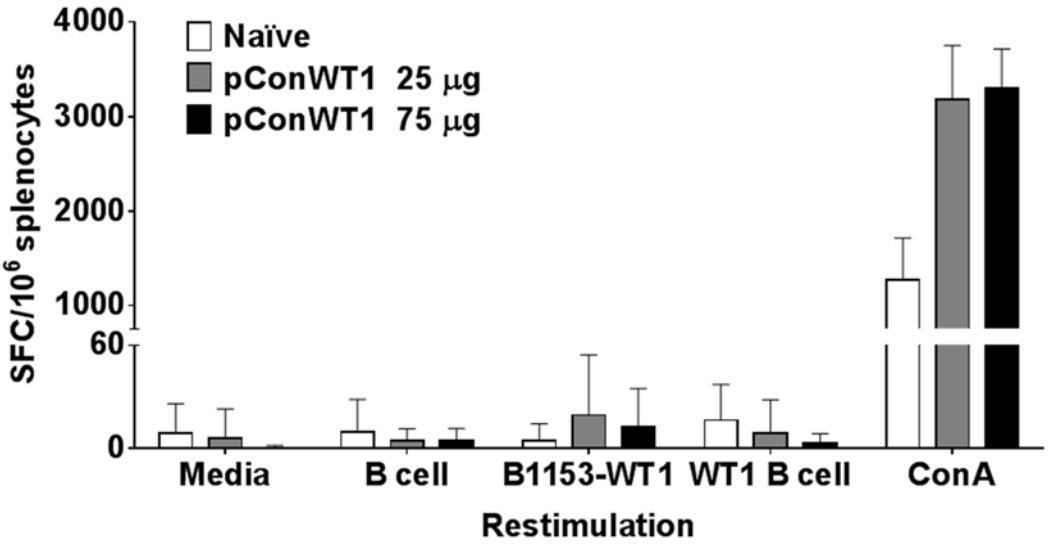
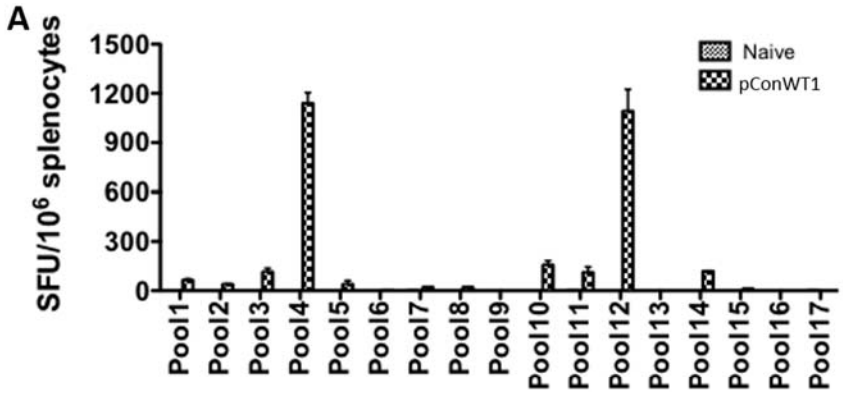


Figure 12. Splenocytes from pConWT1-vaccinated mice do not respond to endogenously expressed WT1.

Mice were vaccinated with 25 μg, or 75 μg of pConWT1. One week after the fourth vaccination, splenocytes from C57BL/6 mice were stimulated *in vitro* by B cells (untransfected B6BL#1153 B cells), a murine WT1 stably transfected B cell line (B1153-WT1) or WT1 mRNA transfected B6BL#1153 B cells (WT1 B cells) for 24 hours in 96 well ELISpot plates coated with antibody to IFNγ, in triplicate. Data are representative of two independent experiments. Average (+/-SD) of five mice per group.

A



B

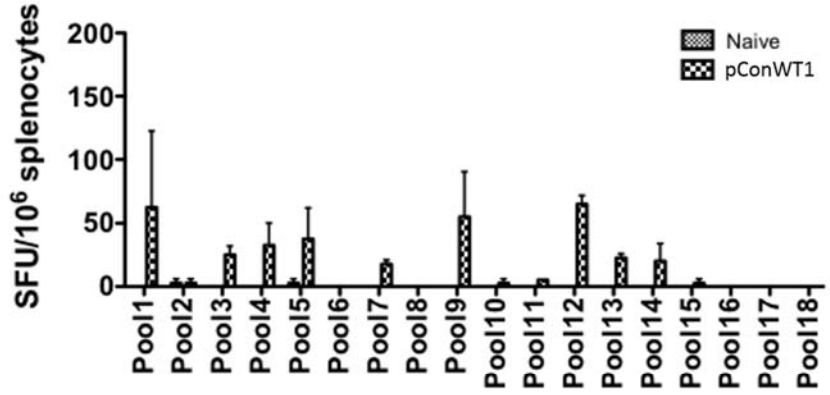
Consensus Peptides

	Pool 1	Pool 2	Pool 3	**Pool 4**	Pool 5	Pool 6	Pool 7	Pool 8
Pool 9	1	2	3	4	5	6	7	8
Pool 10	9	10	11	12	13	14	15	16
Pool 11	17	18	19	20	21	22	23	24
Pool 12	25	26	27	***28***	29	30	31	32
Pool 13	33	34	35	36	37	38	39	40
Pool 14	41	42	43	44	45	46	47	48
Pool 15	49	50	51	52	53	54	55	56
Pool 16	57	58	59	60	61	62	63	64
Pool 17	65	66	67	68	69	70		

Figure 13. Identification of a 15-mer peptide containing a dominant T cell epitope from the consensus WT1 library.

(A) Spleen cells from mice vaccinated with 25 µg pConWT1, and from naive mice were screened by an IFN-γ ELISpot epitope mapping assay for responses to consensus WT1 library peptides. Data are from a pilot experiment and have not been repeated. Average (+/-SD) of five mice per group. (B) Composition of the consensus WT1 library peptide pools 1 to 17 used for combinatorial screening of specific T cell responses. Individual library peptides identified by screening were darkly shaded. In italics was the number of the peptide containing epitope Db126.

A



B

Mouse Matched Peptides

	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Pool 6	Pool 7	Pool 8	Pool 9
Pool 10	1	2	3	4	5	6	7	8	9
Pool 11	10	11	12	13	14	15	16	17	18
Pool 12	**19**	20	21	22	23	24	25	26	**27**
Pool 13	*28*	29	30	31	32	33	34	35	36
Pool 14	37	38	39	40	41	42	43	44	45
Pool 15	46	47	48	49	50	51	52	53	54
Pool 16	55	56	57	58	59	60	61	62	63
Pool 17	64	65	66	67	68	69	70	71	72
Pool 18	73	74	75						

Figure 14. Identification of two 15-mer peptides containing T cell epitopes from the wild type murine WT1 library.

(A) Spleen cells from mice vaccinated with 25 µg pConWT1, and from naive mice were screened by an IFN-γ ELISpot epitope mapping assay for responses to wild type murine WT1 library peptides. Data are from a pilot experiment and have not been repeated. Average (+/-SD) of five mice per group. (B) Composition of the wild type WT1 library peptide pools 1 to 18 used for combinatorial screening of specific T cell responses. Individual library peptides determined by screening were darkly shaded. In italics was the number of the peptide containing epitope Db126.

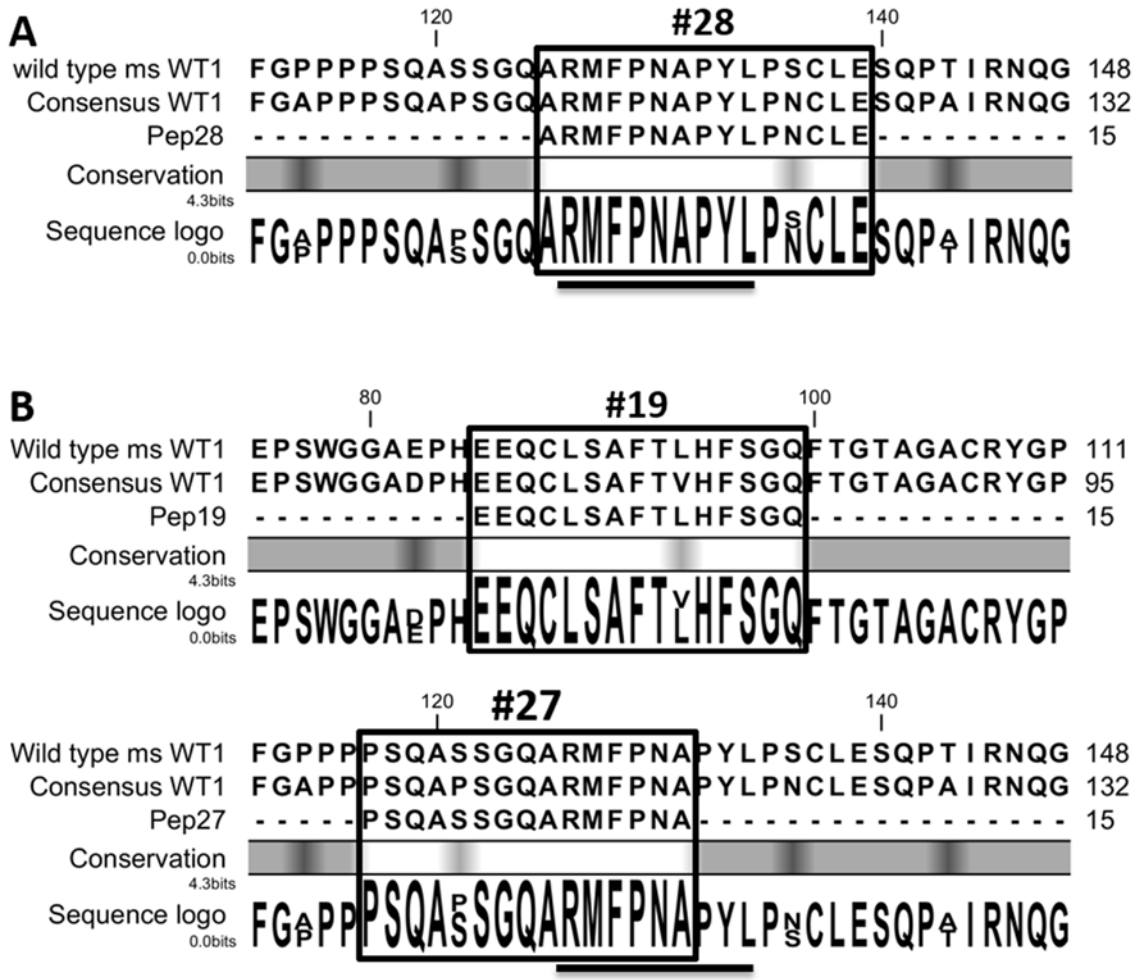


Figure 15. Alignments of the peptides identified from each of the peptide libraries.

Outlined in black and aligned to both the wild type murine WT1 and the consensus WT1 sequences was the identified 15-mers from the (A) consensus WT1 peptide library, and the (B) wild type murine WT1 peptide library. Grey and black sections along the conservation bar indicated regions of mismatch between the sequences; underlined in black was epitope Db126.

Chapter 4: Conclusions and Future directions

WT1, a transcription factor, essential for embryonic development, is highly homologous across species, and is unmutated and overexpressed in wide variety of cancers[30]. To create a vaccine that can potentially initiate tumor rejection to this self-antigen, a "mutated" or xenogeneic version of the antigen was designed. A reduction of 9% similarity to murine WT1 was able to induce a substantial CTL immune response in mice. In comparison to human WT1, the consensus WT1 sequence is 6% different, which could induce immune responses, but may be insufficient to circumvent tolerance. However, since this a DNA vaccine, further modifications, such as addition of a DC-LAMP sequence to enable presentation by class I and class II MHCs, can easily be engineered to make it more immunogenic, and potentially a potent tumor immunotherapy accessible to wide range of patients[117].

Future studies utilizing pConWT1 will feature tumor therapy and tumor challenge experiments, to determine whether pConWT1 can activate immune cells that recognize and kill tumor cells that endogenously express WT1. Previous reports, as well as our results showed that splenocytes from WT1-vaccinated mice responded to WT1 peptide but not to WT1-expressing cells. This may indicate that naturally processed WT1 peptide/MHC density, on the surface of the stimulator cell, is insufficient and is below the threshold necessary for cells predisposed towards tolerance[103,118]. Ramirez et al. experienced suboptimal results in that their peptide-loaded DCs were able to produce WT1-specific CTLs, but such CTLs were unable to recognize endogenously expressed WT1 from cell lines such as TRAMP-C2 or WT1 transfected RMA-S cells[103]. This may signify that for a potent and functional vaccine and rejection of tumor, pConWT1 may

have to be combined with genetic adjuvants such as IL-12, a proinflammatory cytokine, or be administered concurrently with PD-1 (or PD-L1) blockade to break peripheral tolerance.

Another interpretation of the negative result with WT1-expressing B cells is that the peptides antigens which primed the splenocytes from the pConWT1-vaccinated mice were likely from an immunoproteasome, while the peptides displayed on these WT1-expressing B cells may be from a constitutive proteasome[119,120]. Further characterization of the B6BL#1153 B cell line would be useful. Determining which proteasomal subunits are expressed may help explain our results. If it were true that splenocytes from pConWT1-vaccinated mice preferentially respond to peptides from the immunoproteasome, it would affect tumor challenge and therapy experiments, as manipulation of the WT1-expressing tumor microenvironment would need to occur. IFNγ is known to increase tumor immunogenicity[121,122]. Mejias et al have reported that, by using IFNγ adsorbed magnetic particles, and an external magnetic field, they were able to target and deliver the cytokine directly to the tumor and minimize systemic toxicity[123]. Among the multiple responses mediated by IFNγ, treatment with IFNγ enhanced tumor immunogenicity possibly by converting the constitutive proteasome to an immunoproteasome to create a different set of peptides.

A more direct method of identifying what peptides are displayed on the surface of the cell, as demonstrated by Riemer et al., uses a "predict/detect" technique[124]. This requires a set of peptides, defined by predictive programs, such as those available at Immune Epitope Database and Analysis Resource, and peptides eluted from cell lines of interest. By using an "MS3 Poisson detection mass spectrometry approach…[one can]

directly assess the physical presence of predicted CTL target epitopes on tumors" and quantify the number of copies of the epitope displayed on the cell[124]. Success with this method may hinge on the accuracy of the predicted peptides; however, to know precisely what peptide is being presented by an MHC in a particular cell is a powerful tool for vaccine development. Even though our consensus WT1 vaccine utilizes as much of the antigen as possible, not just predicted epitopes, such a tool could help elucidate the presentation of the consensus antigen on APCs, in comparison to the presentation of wild type antigen on tumor cells.

In this report, the consensus WT1 DNA vaccine substantially increased CTL immune responses as compared to the wild type murine WT1 DNA vaccine. Murine peptides, EEQCLSAFTLHFSGQ and PSQASSGQARMFPNA, have been identified that stimulate cells *ex vivo* from consensus WT1-vaccinated mice. Ideally, once low avidity T cells are activated by xenogeneic antigen, and differentiated into memory T cells, they may be restimulated by the same wild type antigen that could not activate their naïve counterparts, and therefore be better able to identify and to kill tumor expressing wild type WT1. While the RMFPNAPYL epitope was unchanged within the consensus sequence, a neighboring amino acid was changed from serine to asparagine, which has the potential to affect proteasomal processing of the protein. A potential experiment would involve making that one amino acid substitution in the WT1 mRNA used to transfect the B6BL#1153 B cell line, to determine whether that change could truly affect presentation of WT1 to splenocytes from consensus WT1-vaccinated mice. Further study will need to be done to confirm and define the minimal epitopes contained within these 15-mers, and to define CTL and T_H1 epitopes.

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CURRICULUM VITAE FOR Ph.D. CANDIDATES

The Johns Hopkins University School of Medicine

Ashley Saint-Fleur **March 2014**

Educational History

Ph.D. expected	2014	Program in Immunology, Johns Hopkins University Mentors: Hyam Levitsky, MD and Cornelia Trimble, MD
B.S.	2002	Molecular, Cellular, & Developmental Biology, Yale Univ.
A.A.	2000	Liberal Arts and Sciences, Rockland Community College

Professional Experience

Pre-Intramural (Cancer) Research Training Award Student	2003-2005	Lab of Ira Pastan, National Cancer Institute, NIH Sponsor: Tapan Bera
Research Assistant	2002-2003	Lab of Jon Morrow Yale University Mentors: Jon Morrow and Zenta Walther
Undergraduate Research Assistant	2001-2002	MAGUK and Tight Junction Lab Yale University Mentor: Zenta Walther
Undergraduate Research Assistant	2000-2001	Slack *C. elegans* Lab Yale University Mentor: Frank Slack
Summer Undergraduate Research Assistant	1999	Shepherd Tick Lab SUNY Binghamton Mentor: Julian Shepherd

Scholarships, Fellowships and External Funding

2005-2007	Fellowship (NIH/NIAID) Johns Hopkins Univ., Immunology Graduate Program

Awards and Recognition

2002	Graduated cum laude, Yale University
2002	Distinction in Molecular, Cellular & Developmental Biology, Yale
2000	Phi Sigma Omicron Honor Society, RCC
2000	Who's Who among Students in American Junior Colleges
1999	Best Overall Presenter—Natural Science, Beacon Conference

Peer Reviewed Publications

Bera TK, **Saint Fleur A**, Ha D, Yamada M, Lee Y, Lee B, Hahn Y, Kaufman DS, Pera M, Pastan I. (2008) Selective POTE paralogs on chromosome 2 are expressed in human embryonic stem cells. Stem Cells Dev. Apr;17(2):325-32.

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Lee Y, Ise T, Ha D, **Saint Fleur A**, Hahn Y, Liu XF, Nagata S, Lee B, Bera TK, Pastan I. (2006) Evolution and expression of chimeric POTE-actin genes in the human genome. Proc Natl Acad Sci U S A. Nov 21;103(47):17885-90.

Poster Presentations

Saint Fleur A, Walters J, Yan J, Weiner D, Trimble C, Levitsky HI. (2013) Induction of Cellular Immunity against Wilms Tumor 1 by Using a Consensus-based DNA Vaccine. Immunology Training Program Retreat. Baltimore, MD.

Saint Fleur A, Spence D, Levitsky HI (2011), Identifying Factors Influencing Immunogenicity by Screening Mutated Forms of Wilms Tumor 1. 19th Annual CRI Cancer Immunotherapy Symposium. New York, NY.