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Modulation Of Anti-Tumor Immune Response By Tgf-B-Inducible Early Gene 1 (tieg1)

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**MODULATION OF ANTI-TUMOR IMMUNE RESPONSE BY TGF- β -INDUCIBLE
EARLY GENE 1 (TIEG1)**

by

ANDI CANI

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

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for the degree of

MASTER OF SCIENCE

2012

MAJOR: BIOCHEMISTRY AND MOLECULAR
BIOLOGY

Approved by:

Advisor

Date

DEDICATION

To my family,

for their love and support in every step of my life.

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CHAPTER 1

INTRODUCTION

1.1 Cancer Immunotherapy

Cancer immunotherapy, or the use of the immune system to fight tumors, has emerged as a promising strategy in the war on cancer in the last decade. However, different forms of immunotherapy in clinical trials, aimed at boosting antitumor immunity, have been only partially successful and have generally failed to trigger an immune response sufficient to eradicate all cancer cells, especially in patients with advanced disease¹. This is usually attributed to the organism's inability to mount a robust effector antitumor immune response. A growing tumor does not usually go unnoticed by the immune system, although the response is relatively weak². Various immunotherapy techniques take advantage of this response by providing help in the form of vaccines, adjuvants or passive transfer of immune effector components. One major obstacle encountered however, is that the immune response induced is relatively weak because the immune system is somewhat tolerant to immunogenic molecules on the tumor surface named tumor associated antigens (TAA). This may be due to the fact that these antigens are simply normal self-proteins expressed in greater quantity or mutated self-proteins with only slight variations in structure. In addition, unlike a bacterial or viral infection, TAAs are usually found in a weakly immunogenic context, making them poor activators of the immune system. Vaccine and adjuvant strategies that are aimed at increasing their immunogenicity usually have only moderate effects in enhancing the immune response³. In a different mechanism, the tumor itself, with its high capability for

mutation, will adapt by “hiding” its TAAs and/or downregulating expression of the major histocompatibility complex (MHC), (a molecule that enables the immune system to “see” these antigens), via a phenomenon called immune evasion. This is the case in triple-negative breast cancer, which lacks expression the surface antigens: estrogen and progesterone receptors as well as the epidermal growth factor receptor Her-2⁴.

While poor response to self-proteins and immune evasion are important obstacles, perhaps the main barrier to cancer immunotherapy remains the suppression of the immune system, a mechanism that is directed in large part by the tumor itself and takes place in the surrounding environment⁵. The tumor microenvironment (TME) is an area of relative proximity to the tumor, characterized by an interaction of cancer cells with cells in the surrounding normal tissue, the immune system as well as various components of the extracellular matrix and soluble cell signaling factors. Tumor cells direct the components of the TME to work in its favor by suppressing the immune system, among other mechanisms⁶⁷. One way is by induction of enzymes such as indoleamine 2,3-dioxygenase or arginase that have the effect of starving the immune cells of important biochemical building blocks as well as triggering of direct immunosuppressive pathways⁸. Another important mechanism is the release of soluble factors (called cytokines) with suppressive function such as transforming growth factor beta (TGF- β) and interleukin-10 (IL-10)^{9 10}. These cytokines act primarily by recruiting or converting immune cells whose duty is to suppress the effector cells of the immune system. Prominent among these are the regulatory T cells (Treg)¹¹, which are characterized by expression of the master transcription factor Foxp3 and by their ability to dampen an overly stimulated immune response, maintain tolerance to self antigens

as well as enable tumor immune escape^{12 13}. Overcoming this suppression by Treg in order to generate a stronger immune response and better anti-tumor protection remains one of the main goals of cancer immunotherapy.

1.2 Her-2 positive cancers and DNA vaccination

Her-2, also known as ErbB-2 or E2, is an important proto-oncogene and tumor associated antigen (TAA). It is the second of the four-member family of ErbB receptors where another prominent member is Epidermal Growth Factor Receptor (EGFR or Her-1). The human ErbB-2 homologue Her-2, a 185 kDa transmembrane protein¹⁴ whose version in the rat is called neu, is found at moderate levels in normal cells and participates in tissue growth and organ formation. Excessive Her-2 signaling however contributes to malignant growth and this receptor was shown to be indeed overexpressed in various types of cancer, notably in 25-30% of breast malignancies as well as in ovarian tumors¹⁵. Gene amplification is responsible for Her-2 overexpression in some but not all tumor cases. Regardless of the mechanism however, excessive Her-2 expression has been associated with more aggressive cancers¹⁶. Upon dimerization with other ErbB family members on the cell surface, Her-2 will transduce signaling through its receptor tyrosine kinase activity to the PI3 and MAP kinase pathways to increase cell survival and proliferation and inhibit apoptosis (Fig. 1.1 A, B). Anti-Her-2 antibody has been detected in cancer patients and Her-2 has become an important immunogenic TAA. In fact, treatment with humanized monoclonal anti-Her-2 antibody Trastuzumab (Herceptin) that blocks receptor activity has shown some clinical efficacy

especially when combined with other chemotherapeutic agents¹⁷. Herceptin resistance has emerged in a portion of treated tumors however as cells evolve alterations in the PI3 kinase and PTEN pathways to overcome the blocked Her-2 signaling¹⁸.

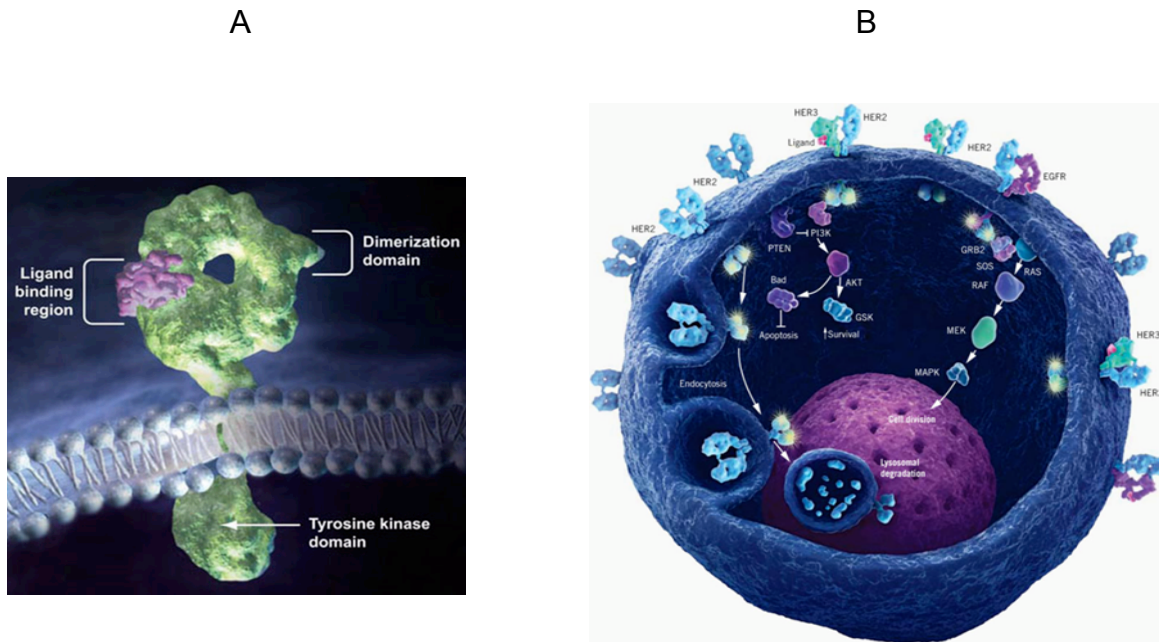


Figure 1.1 The Her-2 oncoprotein. **A** The Her-2 extracellular portion contains a dimerization domain that allows it to bind to other ErbB family members and signal through its intracellular tyrosine kinase domain. **B** Dimerization triggers signaling through the MAP kinase pathway to induce proliferation and through the PI3 kinase pathway (inhibited by PTEN) to increase survival and reduce apoptosis.

Due to the observed resistance to passively transferred antibody such as Trastuzumab, induction of a native anti-Her-2 immune response has become a sought-after alternative. One way to induce an immune response is via injection of a DNA plasmid that encodes the desired antigen. This has several advantages over native peptide immunization such as lower cost, the ability to induce a good T cell response in addition to antibody as well as the immunogenicity of DNA CpG-rich sequences themselves¹⁹. In an attempt to take advantage of these features our lab has developed

several variants of a Her-2 DNA vaccine. These include a version that encodes a mutated Her-2 at the intracellular domain tyrosine kinase active site (pE2A), a truncated protein that is missing the intracellular domain entirely (pE2TM) as well as a secreted version that has only part of the extracellular domain (psecE2)²⁰. In addition, a fusion DNA vaccine between Her-2 and its rat homologue neu was generated (pE2N), which induced the strongest anti-Her-2 immune response and protection from tumor in mice²¹. Vaccination of human Her-2 positive breast cancer patients with pE2A in a pilot clinical trial demonstrated vaccine safety and long-lasting anti-Her-2 immune response¹⁹.

Her-2 DNA vaccination is carried out in conjunction with pGM-CSF, a plasmid coding for Granulocyte-Monocyte Colony Stimulating Factor, a cytokine that helps induce a stronger immune response. In mice the plasmids are injected intramuscularly (i.m.) followed by electroporation, a technique by which an electric current runs through the injected muscle to increase DNA uptake (Fig. 1.2) Subsequent expression of the plasmids will attract cells of the immune system and induce an immune response to Her-2. If combined with other modulations of the immune system, DNA vaccination may prove to be a potent weapon in cancer therapy.

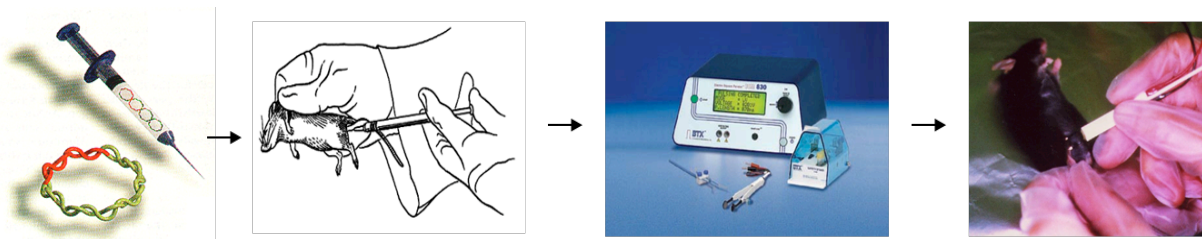


Figure 1.2 DNA vaccination. Plasmid encoding the desired antigen (Her-2) is injected into mouse hamstring muscles. An electroporator is used to deliver an electric current by electrodes placed on either side of the injected muscle. Subsequent uptake and expression of the plasmid by muscle cells will attract cells of the immune system and induce an immune response to the antigen (Her-2).

1.3 The Immune System: B cells

The most common role of the different components of the immune system is to clear infections by pathogenic microorganisms. It is these same cells and soluble products of the immune system that carry out an anti-cancer immune response as well. In either case the effector response can be categorized into humoral (soluble) or cellular²². The humoral immune response is based on the action of antibodies, small globular proteins produced by **B cells** that recognize and bind to their specific target. The cellular arm of the immune system on the other hand works by the direct action of **T cells** in killing specific target cells or by releasing mediators that direct other components of the immune system and coordinate the entire response. Both of these branches of an effector immune response are important for successful cancer immunotherapy²³.

The B cells are generated in the bone marrow from hematopoietic stem cells (HSC) and they undergo several maturation steps until they become capable of producing antibody in response to antigenic stimuli. Starting out as pro-B cells, they rearrange their antibody genes in a random fashion in order to generate antibodies specific to a particular portion of a foreign molecule (epitope), at which stage they are called pre-B cells. Once a productive antibody gene rearrangement is complete and can be expressed on the cell surface as a B cell receptor (BCR), the cell is defined as an immature B cell. After further differentiation and selection, the B cell leaves the bone marrow to go to the spleen and lymph nodes in the periphery as a functional, non-self-reactive, naïve (or mature) B cell where it will wait for eventual encounter with an antigen via its BCR. Upon proper stimulation the B cell will be induced to produce antibody which will bind with great affinity to the epitope on the target antigen. This can

cause elimination of the target cell simply by neutralizing it from carrying out its actions, calling in another system of plasma proteins called complement to lyse the cell or by marking the cell for phagocytosis by other cells of the immune system (opsonization) (Fig. 1.3 A).

An antibody is a protein composed of globular structures called immunoglobulin domains and contains two heavy and two light chains held together by disulfide bonds. Each has a variable N-terminus region which recognizes the antigen and a constant C-terminus region which, in the case of the heavy chain, determines the antibody type or class (Fig. 1.3 B, C). There are five classes of antibody, IgG, IgA, IgE, IgD and IgM corresponding to γ , α , ϵ , δ and μ heavy chains. The class determines the function of the antibody with respect to the stage of the immune response or the body tissue where the antibody is most active. In addition, each class can comprise more subclasses representing more minor variations in their heavy chain such as the IgG1, 2, 3 and 4 subclasses of IgG. These also differ in the type of response, their abundance as well as the stage of the immune response they partake in²².

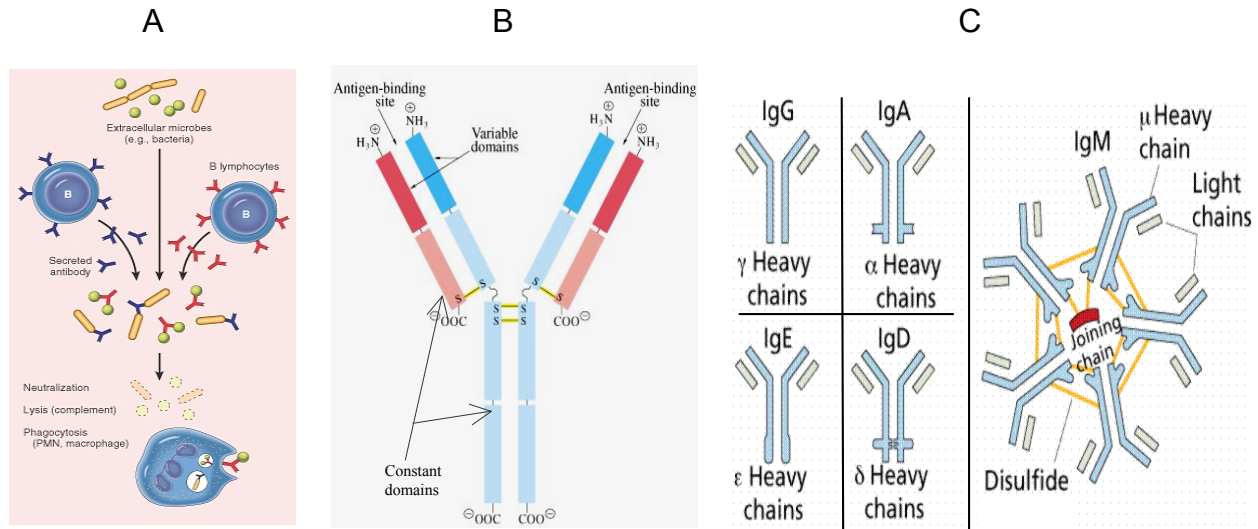


Figure 1.3 The humoral immune response. **A** Encounter with antigen and proper stimulation induces the B cell to produce antibodies with the same specificity that can bind and cause destruction of the target cell. **B** Structure of an antibody molecule with the variable and constant regions of heavy and light chains. **C** The five antibody classes are determined by the constant region of their heavy chain.

1.4 The Immune System: T cells

In contrast to the humoral immune system, the cellular arm of the immune response is represented by the T cells which are also generated from HSCs in the bone marrow but migrate to the thymus to undergo development and differentiation²². Their developmental stages are based on the presence of the surface marker CD3, (a component of the T cell receptor (TCR)), and the co-receptors CD4 and CD8. During these stages, there are gene rearrangements (similar to those in B cells) of the α and β chains of the TCR in order to make receptors specific for one foreign epitope. After several steps of differentiation and selection, functional, non-self-reactive naïve T cells will travel to the periphery expressing either CD4 or CD8 (single-positive cells). CD8+ cells have direct cytotoxic function to infected or cancer cells. CD4+ cells, also called T helper cells, act as coordinators of the entire immune response including that of B cells,

cytotoxic CD8 T cells and other cells by virtue of cytokines, the soluble products they release (Fig. 1.4 A).

While B cell receptors recognize antigen in its native form, T cell receptors can only recognize a portion of an antigen that has been derived from the native form after intracellular biochemical processing by and presentation on the cell surface of the so-called antigen-presenting cells (APC). In the case of a protein, this processed and presented portion can be as small as an 8 amino acid peptide. The surface molecules on APCs that present processed antigen are part of the major histocompatibility complex (MHC) and come in two kinds, MHC class I and II (Fig. 1.4 B). Antigen processing and presentation to the T cells via MHC I is carried out by virtually all cells of the body and requires binding of MHC I to the CD8 co-receptor on cytotoxic T cells. MHC II is only found on the 3 so-called professional APCs: 1) B cells, 2) the dendritic cells (DC) which specialize in antigen presentation and 3) macrophages, cells of the immune system with phagocytic function. MHC II has affinity for the CD4 co-receptor on T helper cells and activates these cells to produce cytokines that orchestrate the immune response. (Fig. 1.4 C)

T helper cells are divided into several groups depending on the cytokines they release and the type of immune response they orchestrate. T helper 1 (Th1) cells are mainly under the control of the transcription factor T-bet and are characterized by the elaboration of the cytokine interferon- γ (IFN- γ). This cytokine directs macrophages to kill their ingested intracellular pathogens among other functions. Th2 cells, via the transcription factor GATA-3, produce IL-4, IL-5 and IL-13 to activate B cells that have

already had contact with the antigen via their BCR. Another type of T helper cells, Th17, is involved in allergic or autoimmune responses via their cytokine IL-17²².

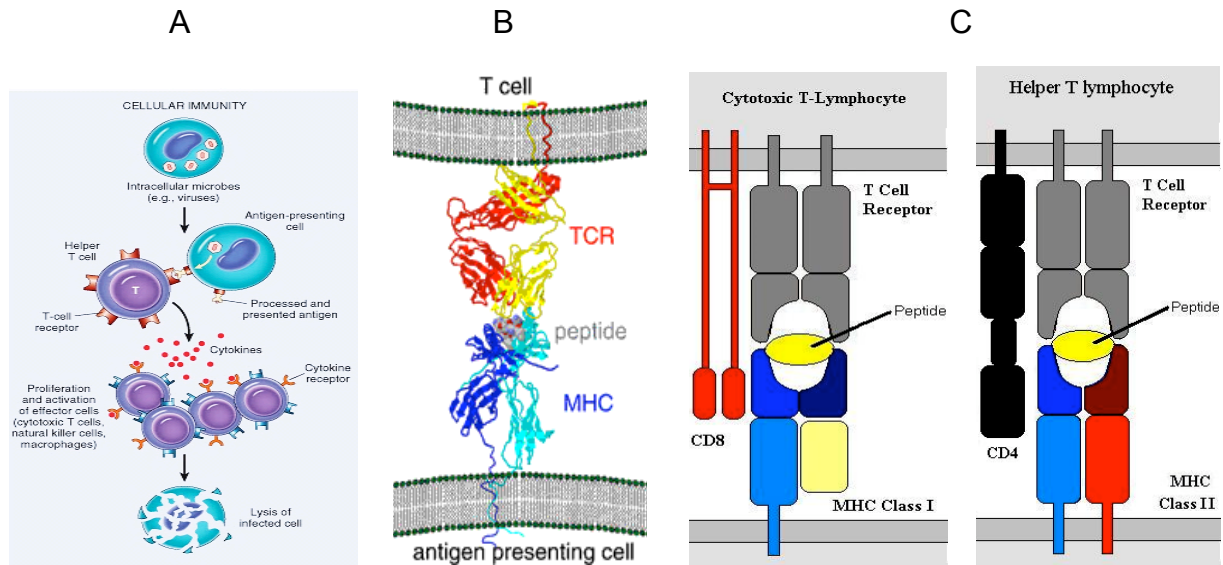


Figure 1.4 The cellular immune response. **A** Encounter with antigen presenting cells induces the T helper cell to produce cytokines that can activate B cells, cytotoxic T cells or macrophages. **B** Structure of the MHC – peptide – TCR complex. **C** CD4 and CD8 co-receptors on T cells restrict presentation to MHC II and MHC I respectively.

While engagement of the TCR with the antigen peptide-carrying MHC on antigen presenting cells is an essential signal for T cell activation, it is usually not sufficient. Additional signaling is required and that is accomplished by the interaction of co-stimulatory molecules on APCs with their counterparts on T cells. Some important co-stimulatory interactions are those of CD80 and CD86 (also called B7.1 and B7.2) on APCs with CD28 on T cells or binding of CD40 on APCs with CD40-L (ligand) on T cells. Other interactions may be inhibitory to T cells such as those between the same CD80/CD86 mentioned above with the co-inhibitor CTLA-4 on T cells.

1.5 The Immune System: Immune Suppression

While the cells described above have effector functions in an immune response, another type of T helper cell, regulatory T cells (Treg) carry out immune suppressive functions under the control of Foxp3, a member of the forkhead/winged-helix family of transcription factors^{24 25}. Treg express the CD25 surface marker in addition to CD4²⁶, although not exclusively. The evidence for Treg's suppressive function rests on 3 key pieces of evidence: 1) their ability to inhibit autoimmune pathological conditions *in vivo*, 2) the *in vitro* inhibition of proliferation of naïve T cells stimulated via the T cell receptor and 3) the observation that induced expression of Foxp3 in naïve T cells converts them into cells with suppressive Treg phenotype²⁷. The mechanisms by which Treg suppress are still somewhat debatable and evidence has been found for several of them. Treg can interact in a contact-dependent manner via surface molecules such as CTLA-4 to send negative signaling to antigen presenting cells or even have direct cytotoxic effects on cells of the immune system. Alternatively they can release immunosuppressive cytokines such as TGF- β , IL-10 and IL-35 in a contact-independent manner²⁷ (Fig. 1.5 A, B, C).

As T cells, Treg have T cell receptors with specific antigen recognition ability. Polyclonal populations of Treg with varying antigen specificities are perfectly capable of suppression, although there is evidence that monoclonal antigen-specific Treg may be even more efficient in inhibiting an immune response²⁸. Regardless of which of the above mechanisms are prevalent, there is little debate that Treg are essential in dampening an overly stimulated immune response, providing immune tolerance to self-antigens and inhibiting antitumor immunity^{29 30}. In fact depletion of Treg has been

accompanied by some autoimmune side effects. It has been shown however, that elimination of Treg greatly enhances anti-tumor immunity^{31 32 33}.

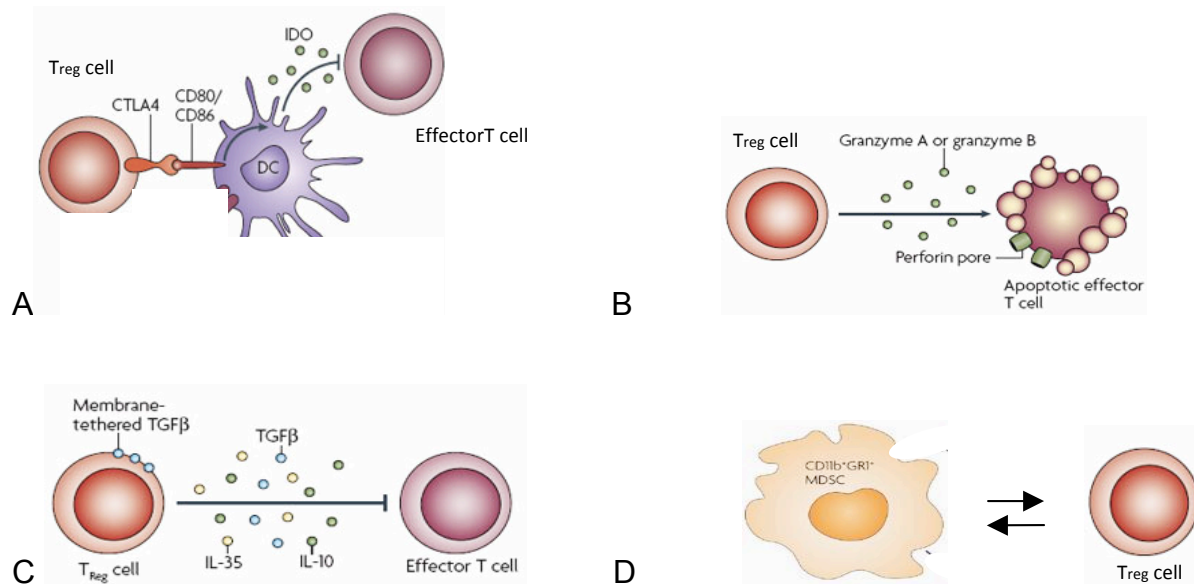


Figure 1.5 Immunosuppressive mechanisms. Treg are thought to exert their suppressive functions via contact mediated cell-to-cell signaling such as **A** through the co-inhibitory molecule CTLA-4, **B** cytotoxic activity by a Granzyme B mechanism or **C** via soluble cytokines such as TGF- β , IL-35 and IL-10, among other mechanisms. **D** Myeloid-Derived Suppressor Cells (MDSC) are immature cells of myeloid origin that are CD11b⁺ Gr-1(Ly6G)⁺ and suppress T cells. MDSC and Treg are thought to have mutually beneficial interactions.

Treg are divided into two groups, natural Treg (nTreg) generated in the thymus during T cell development and adaptive or induced Treg (iTreg) induced by TGF- β with IL-2 in the periphery from naïve CD4⁺CD25⁻ T cells³⁴. nTreg are maintained at rather constant levels in circulation and have stable expression of Foxp3. iTreg on the other hand are converted from naïve T cells usually during an immune response by inducing expression of Foxp3 via the TGF- β signaling pathway. Although iTreg are immunosuppressive like nTreg, their Foxp3 expression is more transient, allowing thus

the immune system to have a temporary or localized suppression of the immune response. The stable vs. transient expression of Foxp3 in Treg is controlled by epigenetic regulation at its promoter. CpG methylation at a certain enhancer element is important in preventing stable Foxp3 expression in all non-nTreg cells^{35 36}. Another enhancer region has been shown to undergo histone acetylation in order to induce expression of Foxp3. Signaling via the TGF- β receptor, which participates in iTreg conversion, has been implicated in this epigenetic-level control of Foxp3 expression³⁷. Interestingly, conditions that combine TGF- β with IL-6, an inflammatory cytokine, direct naïve CD4+CD25- T cells to convert to Th17 cells, thus diverting the immune response from a suppressive into a pro-inflammatory one^{38 39 40 41}.

The immunosuppressive functions described above are carried out by T cells, which are part of the *lymphoid* lineage of immune cells (as are B cells). The *myeloid* lineage is another important branch of bone marrow-derived cells and it includes cells mentioned earlier such as macrophages and dendritic cells as well as the granulocytes, comprising the neutrophils, basophils, eosinophils etc. A more recently discovered population of the myeloid lineage was shown to be a potent inhibitor of T cell effector functions and is recognized as an important branch of the suppressive immune system. This population of cells is named Myeloid-Derived Suppressor Cells (MDSC) and was initially shown to expand in cancer⁴². These cells are characterized by expression of the surface markers CD11b and Gr-1/Ly6G in mice and have since been implicated in immune suppression during inflammation as well as infection⁴³. Rather than being a distinct subset of myeloid cells, MDSC are a heterogeneous mixture of macrophages, dendritic cells and granulocytes maintained at an immature stage of their development. MDSC are

activated by cytokines such as IFN- γ , Granulocyte-Monocyte Colony-Stimulating Factor (GM-CSF) and TGF- β . MDSCs themselves may be a source of TGF- β and are thought to have a mutually beneficial interaction with Treg⁴⁴ (Fig. 1.5 D).

1.6 Inducible Regulatory T cells, TGF- β and Cancer

As mentioned earlier, Treg play an important role in helping tumors escape the immune system and are considered a major target in cancer immunotherapy. In fact, depletion of Treg with antibodies directed at CD25 has shown improvement in antitumor response⁴⁵. Although CD25 is generally a reliable marker for Treg, it is also transiently expressed on all activated T cells, including the effector anti-tumor T cells one hopes to generate, thus making depletion of all CD25-bearing cells problematic⁴⁶. In addition, pan-depletion of Treg carries with it the risk of autoimmunity especially in a susceptible organism^{31 32}. Evidence shows that the naturally occurring nTreg are not the only immunosuppressive cells associated with cancer. In fact iTreg play a very important role in this regard and their contribution is independent of that of nTreg^{47 48}. As previously described, iTreg are formerly naïve CD4+CD25- T cells that have been converted to suppressive, Foxp3-expressing CD4+CD25+ Treg by the action of TGF- β and IL-2 following TCR stimulation. It is conceivable that naïve T cells in the TME are induced to express Foxp3 in the right cytokine environment and that this would be beneficial to a growing tumor trying to evade immune control. The tumor would have an advantage in releasing or causing other cells of the surrounding stroma to release cytokines that create an environment that is conducive to iTreg conversion. In fact iTreg are present in

the TME and have been shown to contribute to this tumor-directed immunosuppression^{49 50 51}.

The action of TGF- β is key in order for the tumor to hi-jack the iTreg immunosuppressive mechanism and the presence of this cytokine has been documented in the tumor microenvironment^{52 53}. Different kinds of cells elaborate TGF- β in the tumor vicinity and these include the tumor cells themselves as well as the surrounding stroma, with tumor-infiltrating immune cells prominent among them⁴⁹. Targeting TGF- β in cancer immunotherapy however is not straightforward since it is a ubiquitous molecule with rather pleiotropic effects. The actions of TGF- β in cancer can be pro-tumorigenic as well as tumor-suppressive. In addition to aiding tumors to evade the immune response as described above, TGF- β also helps cancer spread by assisting in the tumor epithelial to mesenchymal transition (EMT), whereby epithelial cancer cells break the barrier of the lamina propria and invade the connective tissue underneath paving the way for metastasis⁵⁴. On the other hand, activation of the TGF- β pathway in cancer cells sends tumor suppressive signals that cause decrease in proliferation among other actions⁵³. This is demonstrated by the fact that inactivation of the TGF- β receptor or other upstream components of the pathway induces malignant transformation in cells that already have other oncogenic mutations⁵⁵. These pro and anti-tumorigenic effects depend on the context and make specific targeting of the iTreg-dependent immunosuppressive function of TGF- β difficult to tease out. Targeting downstream elements of the TGF- β pathway that lead to induction of Foxp3 in iTreg becomes a more feasible alternative.

1.7 TGF- β -inducible early gene 1 (TIEG1)

The TGF- β pathway evolved in vertebrates to participate in various processes such as development, wound-repair and the immune system⁵³. The ligand family includes TGF- β itself which is active in immune modulation among other functions, as well as factors such as bone morphogenetic proteins (BMP) that participate in bone formation, nodal and lefty that determine left-right asymmetry during organogenesis etc. The active TGF- β ligand is a 25 kDa dimeric protein that binds to and brings together two serine/threonine kinase receptors, the TGF- β receptors type I and II thus triggering their autophosphorylation. The receptors then phosphorylate and activate members 2 and 3 of the Smad family which form a complex and after partnering with Smad4 are translocated to the nucleus. Upon associating with or activating other general or tissue-specific transcription factors from various families (bHLH, homeobox, zinc-finger etc.), the Smad complex triggers a specific pattern of gene expression⁵⁶.

One of the earliest proteins to be activated by TGF- β signaling (within 30 minutes) is **TIEG1** (TGF- β -Inducible Early Gene 1) also known as KLF10 (Krüppel-Like Factor 10). TIEG1 (KLF10) is a transcription factor that was originally discovered in the Spelsberg lab at the Mayo clinic as a gene important in human bone mineralization by osteoblasts and differentiation of osteoclasts⁵⁷. This 72 kDa protein is a member of the KLF family, a subgroup of the zinc-finger transcription factors that is characterized by a C-terminal DNA binding domain composed of 3 C₂H₂ zinc-finger motifs with a highly conserved 7-residue sequence (TGEKP(Y/F)X) between them⁵⁸. This gene has been shown to mimic some actions of TGF- β by being pro-apoptotic and anti-proliferative^{59 60}. Deficiency of

TIEG1 has been linked to some signs of cardiac hypertrophy⁶¹ and skeletal weakness⁶²
⁶³.

In a T cell context, Venuprasad et al. have shown that upon signaling from the TGF- β -Smad pathway, cytoplasmic TIEG1 is mono-ubiquitinated by the E3 ubiquitin ligase Itch and is thus marked for nuclear localization where it can carry out its function as a transcription factor. A major function of TIEG1 in the nucleus is activation of the promoter of Foxp3, the master regulator of T cells with a suppressive phenotype (Fig. 1.6) They also demonstrated that when TGF- β is combined with IL-6, (a condition which directs the naïve T cell away from the Treg phenotype and toward Th17) this will in fact cause poly-ubiquitination of TIEG1 (as opposed to mono-ubiquitination) and prevent its nuclear translocation and Foxp3 induction⁶⁴. Knocking out TIEG1 or Itch in CD4+ T cells severely impairs their ability to induce expression of Foxp3 and to convert iTreg from naïve CD4+CD25- T cells. As a result, TGF- β -treated naïve T cells from TIEG1-/- mice had reduced suppressive activity in vitro and were unable to keep in check the immune response in a model of induced allergic inflammation of the airways⁶⁵. Furthermore Cao et al. showed that in addition to Foxp3, the TGF- β promoter itself is another activation target of TIEG1 in a positive feedback mechanism of iTreg conversion. In fact overexpression of TIEG1 induced expression of both TGF- β and Foxp3 and this caused a reduction of markers for other competing effector T cell lineages, such as Th1 and Th2, whereas TIEG1 ablation had the opposite effect. Transfer of CD4+CD25- naïve T cells from TIEG1-/- mice into a mouse model of inflammatory atherosclerosis increased atherosclerotic lesions, indicating inability of TIEG1-/- naïve T cells to be converted to the immunosuppressive iTreg^{66 58}. Further studies by the Venuprasad group showed

that growth of TRAMP-C2 tumor in unimmunized TIEG1^{-/-} mice was inhibited, indicating a stronger antitumor immune response, an effect attributed to the absence of immunosuppression by iTreg⁶⁴.

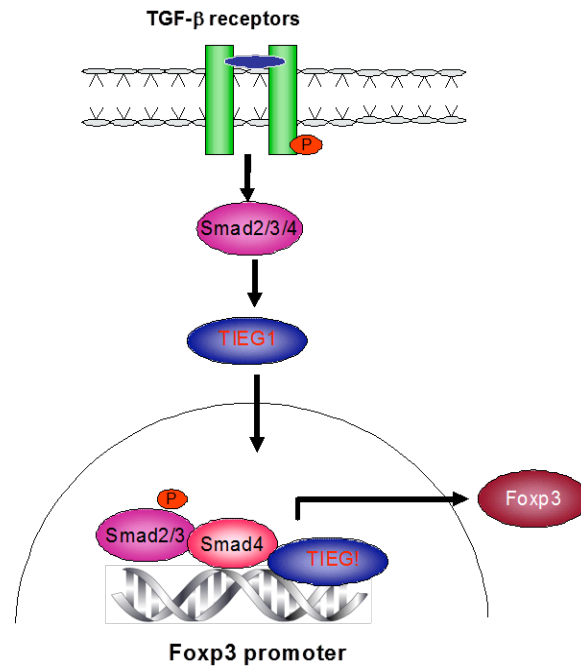


Figure 1.6 TGF- β induction of Foxp3 via TIEG1 in naïve CD4⁺ T cells. Upon ligand binding and autophosphorylation of the TGF- β receptor, a Smad2/3 complex, later helped by Smad4, will eventually activate TIEG1. This transcription factor will be marked for nuclear transport where it will bind to the Foxp3 promoter and will be essential in inducing its expression.

Thanks to Dr. K. Venuprasad, our lab has obtained the TIEG1 knock-out line of mice, (generated and generously provided by the laboratory of Dr. Thomas C. Spelsberg⁶³) a line which, as mentioned above, is unable to induce expression of Foxp3 via TGF- β signaling and thus cannot convert iTreg from naïve CD4⁺CD25⁻ T cells. This project aims to combine the absence of immunosuppression by iTreg in TIEG1^{-/-} mice with our Her-2 DNA vaccination platform in order to induce a potent antitumor immune response.

TIEG1^{-/-} and wild type mice will be vaccinated with Her-2 + GM-CSF DNA, and their B and T cell immune response as well as tumor growth will be assessed. In addition, *in vitro* testing of spleen cells from TIEG1^{-/-} and wild type mice will be performed.

CHAPTER 2

MATERIALS AND METHODS

Mice

All procedures involving mice were conducted in accordance with institutional guidelines and under the supervision and/or approval of the Wayne State University Division of Laboratory Animal Resources (WSU DLAR). Wild type C57Bl/6 and BALB/C mice were purchased from the National Institutes of Health (NIH) or generated in house from breeding. C57BL/6 TIEG1 knockout mice, generated in the laboratory of Dr. T. C. Spelsberg, were generously provided by Dr. K. Venuprasad. TIEG1^{-/-} were maintained by inbreeding with other TIEG1^{-/-} mice. A selected sub strain of TEIG1^{-/-} mice with severely impaired antibody response was generated from the original TIEG1^{-/-} line and is maintained by inbreeding with other TIEG1^{-/-} mice from this selected sub strain. C57BL/6 Her-2 transgenic mice were generated in our laboratory and are maintained as heterozygous mice by back-cross breeding with wild type C57Bl/6. C57BL/6 TIEG1^{-/-} Her-2 Tg mice were generated in our laboratory by two rounds of breeding from TIEG1^{-/-} males and Her-2 Tg females and are maintained by breeding of TIEG1^{-/-} Her-2 Tg males with TIEG1^{-/-} females.

Plasmids and Vaccinations

pE2TM, pE2N and empty vector pVax (control) were previously described^{20 21}. pGM-CSF encoding murine GM-CSF was provided by Dr. N. Nishisaki at Osaka University, Osaka, Japan. In the allogeneic immunization, mice received 5×10^6 splenocytes from BALB/C or C57Bl/6 (control) background via i.p. injection 3 times at 2 week intervals. In the tumor cell immunization mice received 3×10^6 irradiated TC-1/E2 cells (20×10^3 rad) i.p. twice with a 10 week interval between injections. In the DNA vaccination mice received 50 μ g of pE2TM/pE2N/pVax and pGM-CSF plasmid DNA each (in 50 μ l PBS) via i.m. injection in the hamstring muscles 3 times at 2 week intervals. Each vaccination was immediately followed by square wave electroporation over the injection site using a BTX830 (BTX Harvard Apparatus, Holliston, MA) electroporator apparatus. A tweezer electrode was used to deliver 8 pulses at 100 V at 25 ms per pulse, 4 in each direction.

Cell Lines and Reagents

All tissue culture reagents were purchased from Invitrogen and Gibco BRL. Cell lines were maintained *in vitro* in a starting medium composed of DMEM supplemented with 5% cosmic calf serum (HyClone, Logan, UT), 5% fetal bovine serum (Hyclone, Logan, UT), 2 mM l-glutamine, 0.1 mM non-essential amino acids, 100 units/ml penicillin, 100 μ g/ml streptomycin, 0.5 mM sodium pyruvate (SDME 5+5). Variations from this medium are noted below for each cell line. E0771/Her-2 cells were maintained in DMEM with 20% fetal bovine serum and 0.1mg/ml neomycin. TC-1 cells (generously provided by Dr. T. C. Wu, The Johns Hopkins University, Baltimore, MD) were maintained in SDME 5+5

with 0.4 mg/ml neomycin. TC-1/E2 cells were generated by transfecting TC-1 cells with Her-2 and maintained in TC-1 media (see above) + 7.5 µg/ml puromycin. 3T3 cells were maintained in SDME 5+5 media. 3T3/KB cells were maintained in SDME 5+5 with 0.6 mg/ml neomycin and 7.5 µg/ml puromycin. Other reagents were purchased from Sigma. Antibodies were purchased from BD biosciences and Invitrogen. PCR reagents were purchased from Qiagen.

CD25 Depletion

To deplete CD25+ regulatory T cells (Treg), mice were injected twice i.p. with 0.5 mg of PC61 mAb (in 0.3 ml volume) at a one week interval with the second injection given 3 days before the first vaccination.

Tumor Challenge and Measurement

Ten days after the third vaccination, mice were inoculated s.c. in the flank with 2×10^5 E0771/Her-2 cells. Tumor growth was monitored by palpation, and tumor diameter was measured in two perpendicular dimensions with a caliper at 3-day or weekly intervals. Tumor volume was calculated as $\text{width}^2 \times \text{length} \times \frac{1}{2}$. Mice were sacrificed when any one dimension of the tumor reached 20mm.

Measurement of Antibody Response by Flow Cytometry

In allogeneic immunization, sera from the immunized mice were used to stain BALB/C splenocytes and detected with fluorophore-conjugated Fc_γ secondary antibody by a BD Canto II flow cytometer. We constructed the standard curve by staining BALB/C splenocytes with anti-Kd (clone SF1-1.1) antibody and used regression analysis to calculate serum IgG antibody concentrations (see note in text). In the Her-2 tumor cell and DNA vaccine experiments sera from the immunized mice were used to stain TC-1/E2 cells and detected with fluorophore-conjugated Fc_γ secondary antibody. We constructed the standard curve by staining TC-1/E2 cells with anti-Her-2 (clone Ab-5) antibody and used regression analysis to calculate serum IgG antibody concentrations.

Measurement of T Cell Response by IFN-γ ELISPOT Assay

96-well HTS IP plates (Millipore, Bedford, MA), were pre-coated with 2.5 μg/ml rat anti-mouse IFN-γ. Spleen cells were added to the wells in the presence of 3T3 or 3T3/KB cells for allogeneic immunization and TC-1 or TC-1/E2 cells for Her-2 immunization at 1:10 APC : lymphocyte ratio in R10 media (RPMI1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 10mM HEPES). Other control wells had medium or Concanavalin A. Following 48 hr incubation, cells were removed and captured IFN-γ was detected with biotinylated rat anti-mouse IFN-γ, avidin-HRP and the substrate 3-amino-9-ethylcarbazole. The visualized cytokine spots were counted with the ImmunoSpot analyzer (CTL, Cleveland, OH), and the results were expressed as number of cytokine-producing cells per 10⁶ splenocytes.

Statistical Analysis

Data were analyzed using the student's t-test (or Kruskal-Wallis test where appropriate, see note in text). Select data were analyzed with the help of Dr. Judith Abrams, (Biostatistics Core, Karmanos Cancer Institute, Detroit, MI).

CHAPTER 3

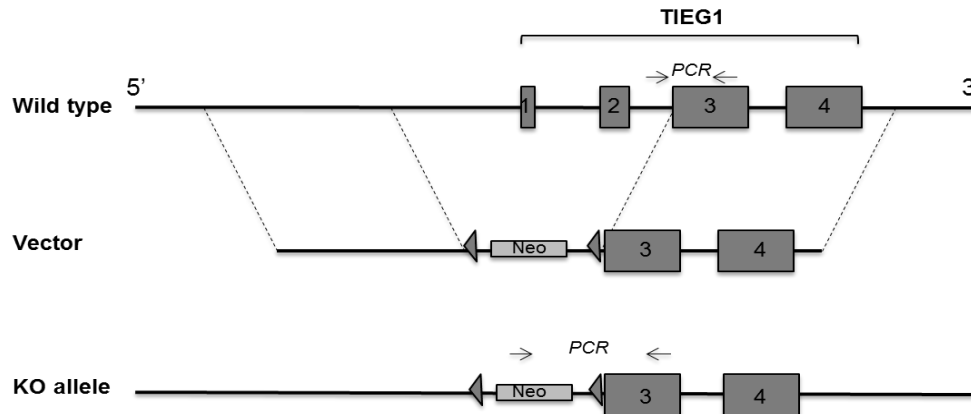
RESULTS

3.1 PCR genotyping of TIEG1^{-/-} line

In order to test our hypothesis that inhibition of iTreg will improve antitumor immunity, we used TIEG1^{-/-} mice, a complete and stable knockout line that was generously provided by Dr. T. C. Spelsberg and Dr. K. Venuprasad. This KO line was generated in the Spelsberg laboratory⁶³ by homologous recombination with a targeting vector comprising two homology regions flanking an insertion cassette. The two homology regions in the wild type allele are located upstream of the TIEG1 coding sequence on the 5' end and downstream of the second TIEG1 intron, a region that includes exons 3 and 4 as well as regions downstream of the gene on the 3' end (Fig. 3.1 A). The insertion cassette is composed of the Neomycin resistance gene flanked by loxP sequences and has been inserted into the wild type allele replacing regulatory sequences immediately upstream of TIEG1 as well as exons 1 and 2, thus inactivating the gene. We maintained the KO allele in a homozygous mouse line of C57Bl/6 genetic background and we genotyped the KO or wild type mice by PCR of genomic DNA extracted from tail tissue. The reaction was carried out using the three primers positioned as shown in Fig. 3.1 A. A common reverse primer is complementary to a region in exon 3 and two forward primers for the KO and wild type alleles bind to the Neo region and intron 2 respectively. This yielded a KO amplicon that was longer than

that of the wild type allele (Fig. 3.1 B). Heterozygous mice (TIEG1^{+/-}) yielded both PCR products.

A TIEG1^{-/-} allele generation (adapted from Subramaniam et al., 2005)



B PCR genotyping

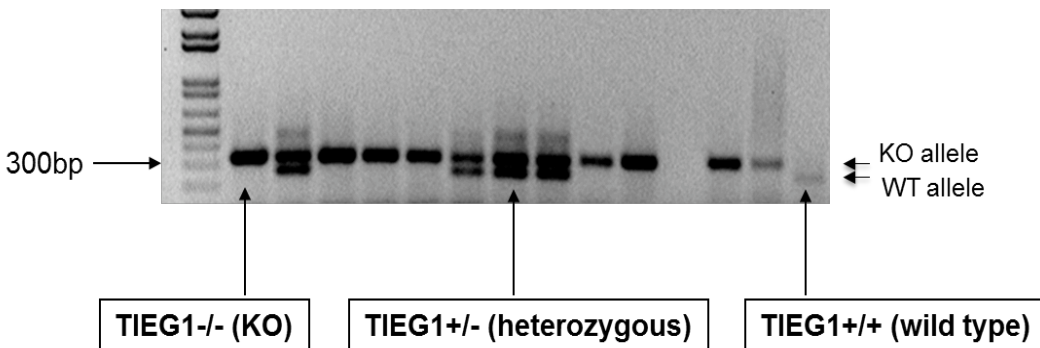


Figure 3.1 Generation and genotyping of TIEG1^{-/-} mouse line. **A** Schematic depiction for the generation of TIEG1 Knock Out allele by homologous recombination between the wild type allele and the targeting vector. (Generated in and generously provided by the T. C. Spelsberg laboratory at the Mayo Clinic, Rochester, MN; scheme adapted with simplifications from Subramaniam et al.⁶³) **B** Mouse tail tissue was genotyped for the TIEG1 null or wild type allele by PCR using two different forward and one common reverse primers (see scheme). The PCR was carried out at 94°C x 1 min denaturation, 55°C x 2 min annealing and 72°C x 3 min extension, repeated for 41 cycles. The KO allele amplicon was slightly longer than the product of the wild type allele based on the positions of the forward primers (see A). Heterozygous mice show both PCR products. Examples of each allele combination are marked.

3.2 Stronger T cell response but comparable antibody response to immunization with allogeneic splenocytes in TIEG1^{-/-} mice

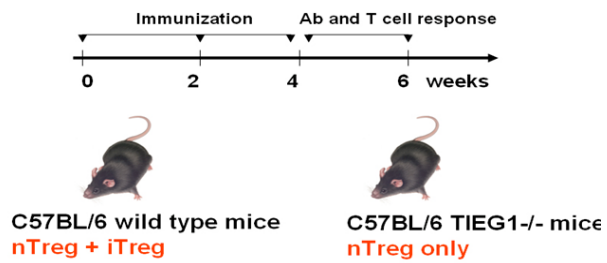
TIEG1^{-/-} mice were shown to be incapable of converting naïve CD4⁺ T cells into Foxp3-expressing iTreg with immunosuppressive phenotype. In order to test whether this impairment in immune suppression has an effect on our antitumor vaccination we first tested the immune response of TIEG1^{-/-} mice to a common antigen such as allogeneic (foreign) MHC. MHC is the cell surface protein used to present antigen to T cells and is encoded by a gene with great allelic variability between individuals making it a strong immunogen and the basis for immunological transplant rejection. TIEG1^{-/-} and wild type mice were immunized with spleen cells from mice of a different genetic background (BALB/C) where the MHC molecules serve as the main immunogenic molecule. 5×10^6 cells/mouse were injected i.p. 3 times, at 2-week intervals. T cell and antibody responses specific to the allogeneic antigens were measured 2 weeks after each immunization (Fig. 3.2 A).

T cell response was measured by ELISPOT, an assay that detects antigen-specific T cells, in our case to the K^d allele of BALB/C MHC class I. In this assay, T cells specific for the desired antigen will be activated to produce cytokine when the antigen is presented to them (Fig. 3.2 B). Splenocytes from the immunized mice are co-cultured with other cells that present the K^d protein or release it into the culture medium. K^d-specific T cells will be activated either directly by this presentation or by professional APC from the splenocyte population that phagocytose, process and present the released K^d antigen to them. Activated T cells produce cytokine (in our case IFN_γ) that is captured by the anti-IFN_γ antibody coating the membrane and later detected with a

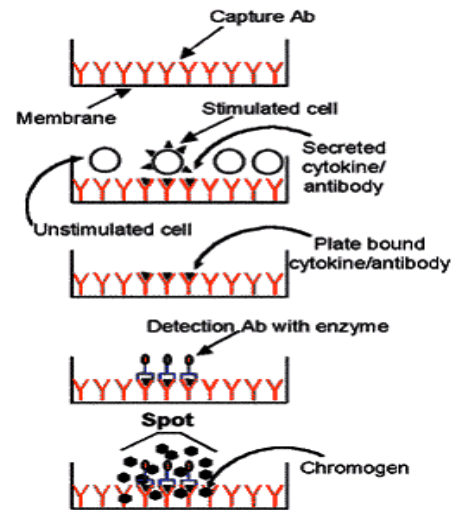
secondary antibody linked to an enzymatic colorimetric reaction that creates a spot on the membrane. The density of spots is proportional to the concentration of K^d-specific T cells generated during the immunization.

Antibody response was measured by flow cytometry, which we used to detect antibody specific to the antigens on BALB/C splenocytes. In this assay, the antibody specific for the desired antigen binds to antigen-expressing cells and is detected by fluorescence (Fig. 3.2 C). Sera from the immunized mice are used to stain BALB/C splenocytes that express the various BALB/C MHC molecules and other antigens that elicit the immune response in the mouse. The bound serum antibody is stained with a fluorophore-conjugated secondary antibody specific for the IgG constant region of the heavy chain of the serum antibody and the cells are fed into a flow cytometer in which they flow in single file and are excited by a laser. The strength of the emitted signal is proportional to the concentration of antigen-specific IgG antibody in the sera generated during the immunization. In order to convert the emitted fluorescence into serum antibody concentration we construct a standard curve by staining the antigen-expressing cells with varying concentrations of antigen-specific, purified, commercial antibody. The curve obtained is fitted to a linear or polynomial equation, which is then used to calculate serum antibody concentrations corresponding to a particular mean fluorescence value (Fig. 3.2 D). (Note: for our allogeneic immunization, we constructed the standard curve by staining BALB/C splenocytes with anti-K^d (clone SF1-1.1) antibody. This represents only one of several MHC and other molecules that serve as antigens so an absolute antibody concentration cannot be easily obtained and the values given are only relative to the anti-K^d antibody.)

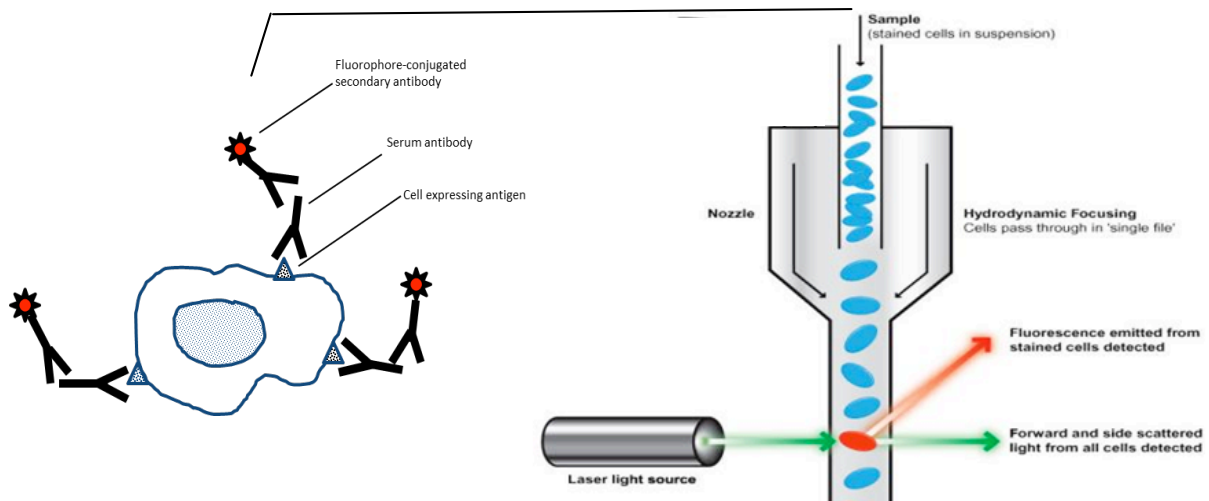
A Experimental time scheme

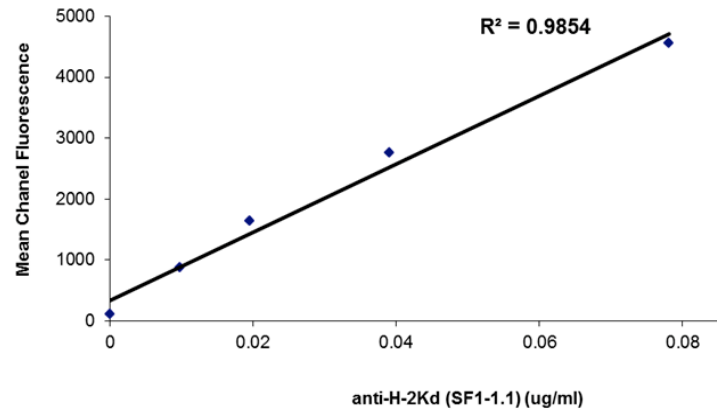
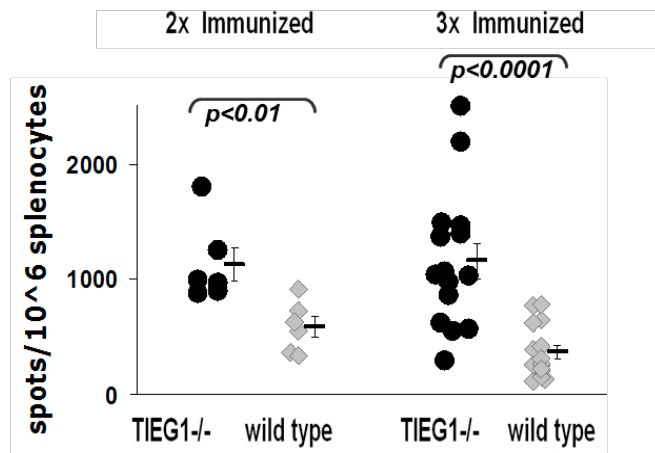
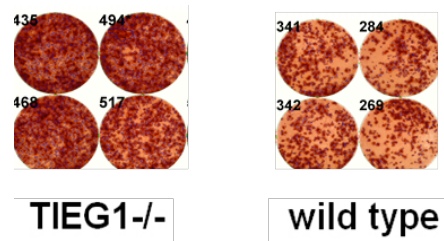


B ELISPOT assay scheme



C Flow cytometry scheme



D Flow cytometry standard curve**E H-2K^d-specific IFN- γ -producing T cells in spleen****F Representative ELISPOT wells**

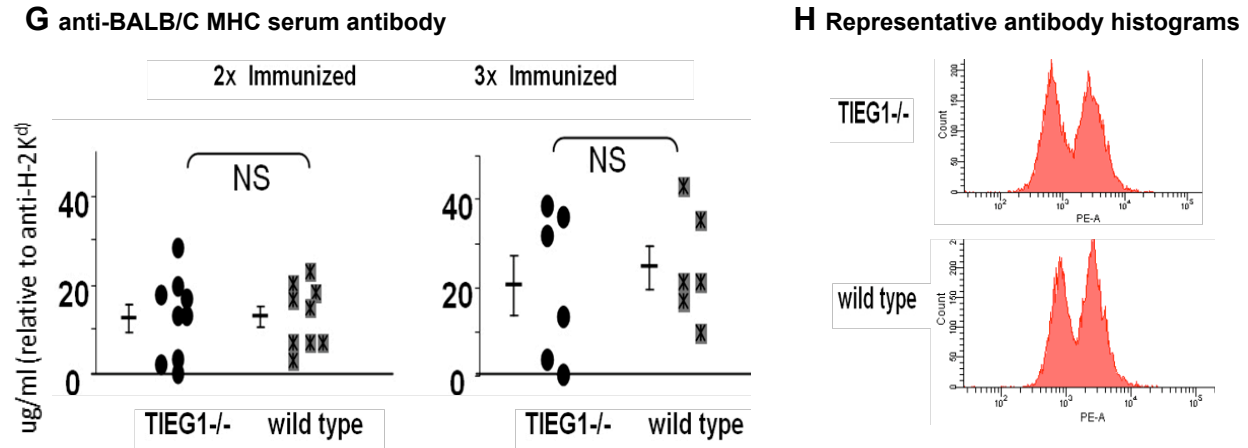


Figure 3.2 Superior IFN- γ T cell response but similar antibody levels in TIEG1^{-/-} mice immunized with allogeneic splenocytes. **A** TIEG1^{-/-} and wild type mice, both of C57Bl/6 genetic background, were immunized with 5×10^6 splenocytes from BALB/C mice via i.p. injection 3 times at 2 week intervals. Allogeneic MHC-specific antibody and T cell responses were measured at 2 weeks following each injection via flow cytometry and ELISPOT respectively. **B** ELISPOT assay to measure concentration of T cells that are specific for the desired antigen. Splenocytes from immunized animals are loaded onto a membrane coated with antibody to desired cytokine (IFN- γ) and are stimulated with the purified antigen or cells expressing and presenting the antigen. The cytokine produced by the stimulated antigen-specific T cells is captured on the membrane and detected with secondary antibody linked to an enzyme-substrate system that leaves a spot on the membrane. **C** Flow cytometry to measure concentration of serum antibody that is specific for the desired antigen. Cells expressing antigen transfected into them are stained with diluted sera from immunized animals then with a secondary antibody conjugated to a fluorophore. Laser excites fluorophores on cells flowing in single file and fluorescence is detected. **D** Standard curve for calculation of antibody concentration built by staining the same cells as above with known concentrations of antigen-specific antibody (anti-H-2K^d clone SF1-1.1) **(E)** Plot and **(F)** representative ELISPOT well images of IFN γ -producing T cells in spleens of immunized TIEG1^{-/-} and wild type mice. T cells are specific to BALB/C MHC molecule H-2K^d. **(G)** Plot and **(H)** representative flow cytometry histograms of anti-BALB/C MHC polyclonal antibody levels in sera of immunized TIEG1^{-/-} and wild type mice.

Immunization with allogeneic (BALB/C) splenocytes resulted in a considerably stronger IFN γ T cell response in TIEG1^{-/-} mice as measured by ELISPOT of K^d-specific T cells (Fig. 3.2 E, F). No significant difference in T cell response was observed after the first immunization (data not shown), but it became apparent after the second round

of immunizations and especially after the third. The concentration of K^d-specific T cells in spleens of some TIEG1^{-/-} mice was as high as 2500 cells/10⁶ splenocytes or approximately 0.85% of all T cells in the spleen whereas in the wild type mice the concentration did not exceed 800 cells/10⁶ splenocytes or 0.27% of T cells. (T cell response in TIEG1^{-/-} mice showed a greater variability than in wild type mice also.) Absence of iTreg however, did not have the same effect in the IgG antibody response of TIEG1^{-/-} mice as compared to their wild type counterparts (Fig. 3.2 G, H). No significant difference between the two strains of mice was observed. Each successive immunization increased the antibody response in both the knockout and wild type strains but any possible difference between the two groups was not noticeable with the flow cytometric method we used.

3.3 Stronger T cell response but comparable antibody response to immunization with Her-2-expressing tumor cells in TIEG1^{-/-} mice

In order to test whether absence of iTreg has an effect on immunity to the oncoprotein Her-2, we conducted a small, pilot experiment to test the immune response of TIEG1^{-/-} mice to injection with Her-2 in the context of tumor cells expressing this protein. TIEG1^{-/-} and wild type mice were vaccinated with TC-1/E2, a mouse epithelial lung cell line that has been transformed with Human Papilloma Virus-16 oncogenes E6 and E7 as well as ras to become immortalized (generously provided by Dr. T. C. Wu) and then transfected to express the Her-2 (E2) protein. Mice received 3x10⁶ TC-1/E2 cells (irradiated to prevent tumor growth), i.p., twice with a 10 week interval between

injections. Her-2-specific antibody and T cell responses were measured by flow cytometry and ELISPOT, two weeks post injection. T cell response was measured with ELISPOT by stimulating splenocytes with TC-1/E2 cells. Antibody response was measured with flow cytometry by staining TC-1/E2 cells with sera from the vaccinated mice. We constructed the standard curve by staining TC-1/E2 cells with purified commercial anti-Her-2 (clone Ab-5) antibody to obtain absolute serum Her-2 antibody concentrations.

As in the case of allogeneic splenocytes, immunization with irradiated TC-1/E2 cells resulted in a stronger IFN γ T cell response in TIEG1 $^{-/-}$ mice (after the second immunization) (Fig. 3.3 A). The concentration of Her-2-specific T cells in spleens of 3 TIEG1 $^{-/-}$ mice was nearly 2000 cells/ 10^6 splenocytes with only one mouse responding at around 800cells/ 10^6 splenocytes. On the other hand, T cell response in the two wild type mice tested was around 700 cells/ 10^6 splenocytes. As with allogeneic splenocyte immunization, absence of iTreg or TIEG1 did not make a difference in the antibody response of TIEG1 $^{-/-}$ mice as compared to their wild type counterparts (Fig. 3.3 B). Serum IgG concentrations in response to TC-1/E2 were comparable in the two groups after the first and second immunizations. In conclusion, a pilot experiment testing anti-Her-2 immunity via immunization with Her-2-expressing irradiated tumor cells yielded a more robust T cell response but comparable antibody levels.

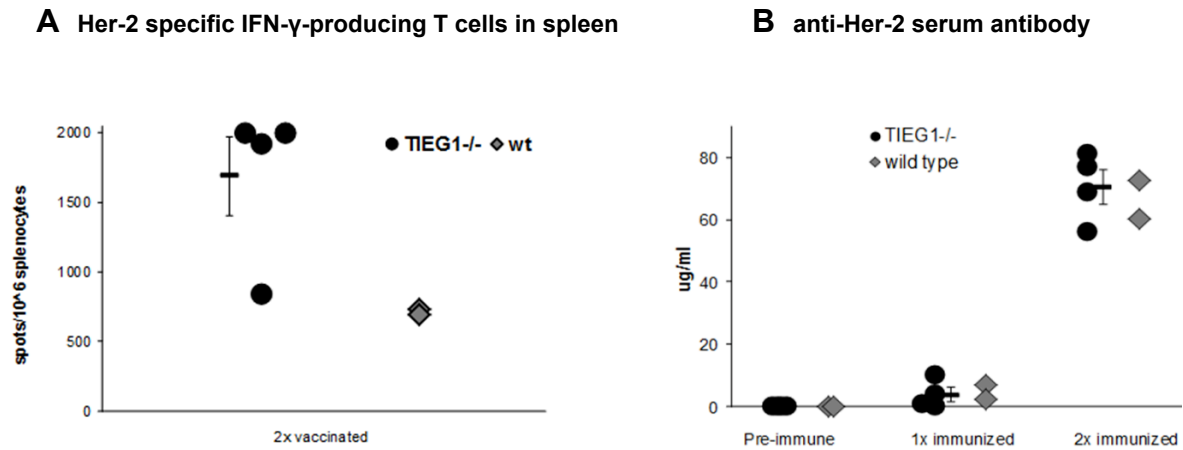


Figure 3.3 Stronger IFN- γ T cell response and equal antibody production in TIEG1^{-/-} mice vaccinated with TC-1/E2 cells. TIEG1^{-/-} and wild type mice were immunized with 3×10^6 irradiated TC-1/E2 cells i.p. twice, with a 10-week interval between injections. Her-2-specific antibody and T cell responses were measured by flow cytometry and ELISPOT respectively. **A** Her-2-specific IFN- γ -producing T cell concentration in ELISPOT of spleens from immunized TIEG1 KO and wild type mice, (no statistical analysis was performed due to the small number of mice). **B** Anti-Her-2 IgG antibody levels in flow cytometry-analyzed sera from immunized TIEG1^{-/-} and wild type mice.

3.4 Stronger T cell response but lower antibody response to Her-2 DNA vaccination in TIEG1^{-/-} mice

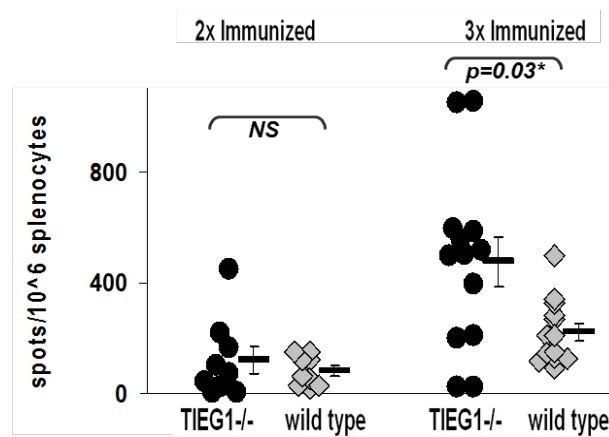
In order to test whether the absence of iTreg has an effect on DNA vaccination we tested the immune response of TIEG1^{-/-} mice to our Her-2 DNA vaccine. TIEG1^{-/-} and wild type mice were vaccinated with a cocktail of two plasmids pE2TM and pGM-CSF. pE2TM codes for a truncated portion of the Her-2 tumor antigen that comprises its extracellular and transmembrane domains. pGM-CSF codes for Granulocyte Monocyte Colony-Stimulating Factor, a growth factor for immune cells of myeloid lineage that will serve as antigen presenting cells. 50 μ g DNA from each plasmid was delivered via i.m.

injection in the hamstring region and electroporation for 3 times at 2 week intervals. Her-2-specific antibody and T cell responses were measured at 2 weeks following each injection via flow cytometry and ELISPOT as before. T cell response was measured with ELISPOT by stimulating splenocytes with the TC-1/E2 cell line which expresses Her-2. Antibody response was measured with flow cytometry, by staining TC-1/E2 cells with sera from the vaccinated mice. We constructed the standard curve by staining TC-1/E2 cells with anti-Her-2 (clone Ab-5) antibody to obtain absolute serum Her-2 antibody concentrations.

As in the case of allogeneic splenocyte immunization, vaccination with pE2TM + pGM-CSF resulted in a stronger IFN_γ T cell response in TIEG1^{-/-} mice (Fig. 3.4 A). No significant difference in T cell response was observed after the first (data not shown) or second vaccination, but it became apparent after the third immunization. The concentration of Her-2-specific T cells in spleens of TIEG1^{-/-} mice was as high as 1100 cells/10⁶ splenocytes with a mean of approximately 500 cells/10⁶ splenocytes. T cell response in the wild type mice on the other hand did not exceed 500 cells/10⁶ splenocytes with a mean of only 200 cells/10⁶ splenocytes. As with the allogeneic splenocyte immunization, T cell response to DNA vaccine in TIEG1^{-/-} mice showed a greater variability than in wild type mice with a few non-responders present. In fact the Kruskal-Wallis test showed good statistical significance of the difference in the third vaccination T cell response between the two groups (p-value shown in figure), whereas a student's t-test, a statistical test that is more sensitive to outlying data, did not, (not shown).

As in the case of allogeneic splenocyte immunization, absence of iTreg or TIEG1 did not have the same positive effect in the antibody response of TIEG1^{-/-} mice as compared to their wild type counterparts (Fig. 3.4 B). In fact, IgG response to Her-2 DNA vaccine was surprisingly lower in TIEG1^{-/-} mice starting as early as the first vaccination and continuing throughout the experiment with strong statistical significance. Several of the TIEG1^{-/-} mice in particular showed a strikingly impaired initial antibody response after one vaccination. The same mice tracked throughout the experiment were very slow to initiate production of anti Her-2 IgG, showing any detectable antibody levels only after 3 vaccinations. This indicated that, while absence of iTreg may allow a more robust T cell response, TIEG1 might be involved in other pathways important in the immune response, specifically that of B cells.

A Her-2 specific IFN- γ -producing T cells in spleen



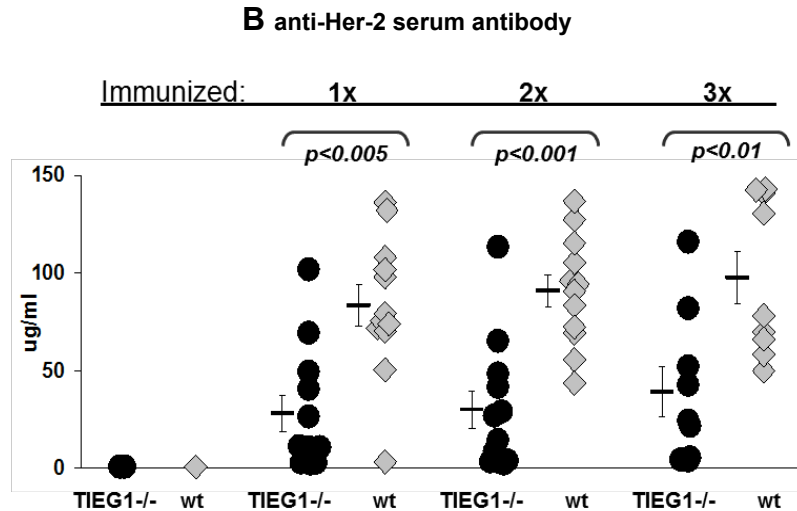


Figure 3.4 Stronger IFN- γ T cell response and lower antibody production in TIEG1^{-/-} mice vaccinated with Her-2 DNA. TIEG1^{-/-} and wild type mice were vaccinated with 50 μ g of pE2TM (Her-2 extracellular and transmembrane domains) and pGM-CSF (Granulocyte Monocyte Colony-Stimulating Factor) plasmid DNA each via i.m. injection and electroporation 3 times at 2 week intervals. Her-2-specific antibody and T cell responses were measured at 2 weeks following each injection via flow cytometry and ELISPOT respectively. **A** Plot of Her-2-specific IFN- γ -producing T cells in ELISPOT of spleens from immunized TIEG1 KO and wild type mice, (*p value shown for Kruskal-Wallis test). **B** Plot of anti-Her-2 IgG antibody levels in flow cytometry-analyzed sera from immunized TIEG1^{-/-} and wild type mice.

3.5 Deficient IgG1 subclass antibody production and impaired switch to subclass IgG2c in Her-2 DNA vaccination of TIEG1^{-/-} mice

Given that the IgG response to Her-2 DNA vaccination was impaired in TIEG1^{-/-} mice, we further investigated whether this disparity was due to different contributions among the IgG subclasses. We measured levels of IgG1, IgG2c and IgG3 in the sera of the vaccinated mice by flow cytometry using labeled secondary antibodies specific for each subclass. (Mice of the C57Bl/6 strain have only the c version of the IgG2 gene). Given that the purified commercial anti-Her-2 antibody (Ab-5) used to construct the standard curve is an IgG1, we could calculate the real IgG1 serum concentrations,

whereas the other IgG subclasses could only be measured as relative fluorescence intensities.

IgG1 was the subclass that appeared earliest in the anti-Her-2 IgG response in both the TIEG1^{-/-} and wild type strains (Fig. 3.5 A). In keeping with the trend observed in total IgG (Fig. 3.4 B), IgG1 levels were significantly lower in TIEG1^{-/-} mice starting with the first vaccination and continuing to stay impaired for the rest of the experiment. Levels of IgG2c were negligible after the first and second vaccinations for both the TIEG1^{-/-} and wild type groups, indicating that a switch to this subclass happens in later stages of Her-2 DNA vaccination (Fig. 3.5 B). Indeed IgG2c showed a strong response in wild type mice after the third vaccination. However, this increase was almost absent in the TIEG1^{-/-} group indicating an inability of TIEG1^{-/-} mice to switch to production of an antibody of a different subclass. IgG3 is a relatively minor component of the IgG response and its levels in our sera were negligible (data not shown). In conclusion, lower IgG levels in TIEG1^{-/-} mice are due to an impaired IgG1 response as well as a later inability to switch on production of IgG2c.

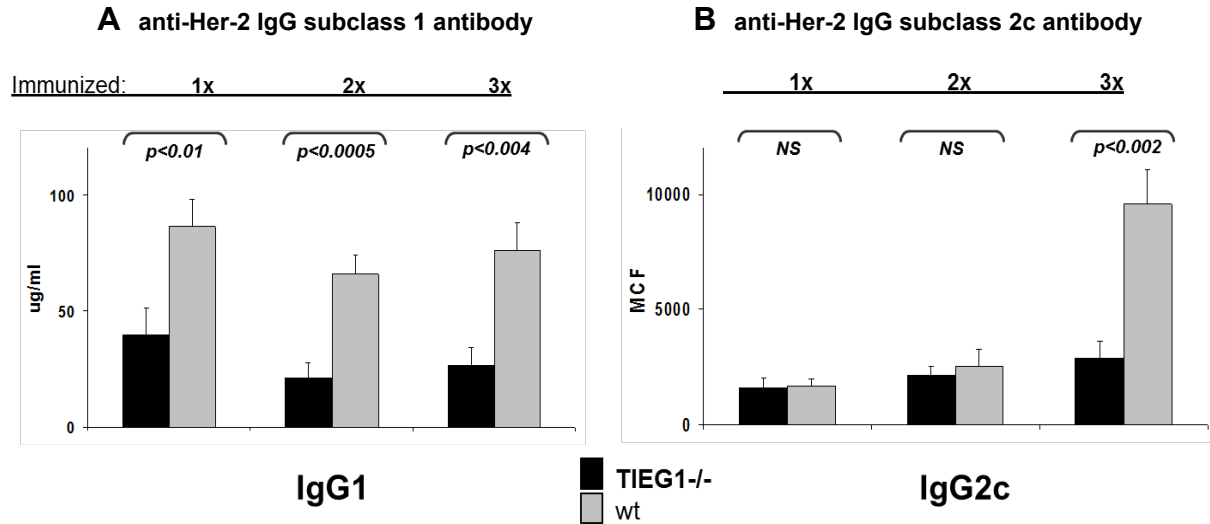


Figure 3.5 Impaired anti-Her-2 IgG response in TIEG1^{-/-} mice vaccinated with Her-2 DNA is due to lower initial IgG1 levels and a later inability to switch on IgG2c production. Sera from mice in Figure 3.4 were analyzed by flow cytometry for anti-Her-2 IgG subclass 1 and 2c antibody. Shown are plots of anti-Her-2 antibody levels in sera of immunized TIEG1^{-/-} and wild type mice for (A) IgG1 and (B) IgG2c subclasses.

3.6 Normal B cell numbers and *in vitro* B cell proliferation in TIEG1^{-/-} mice

Although iTreg-deficient TIEG1 knockout mice had a superior IFN γ T cell response to both allogeneic splenocyte and Her-2 DNA and tumor cell vaccination, this did not also translate to a stronger antibody response. In fact we observed severely impaired antibody production in the case of DNA vaccination. In order to elucidate the mechanism behind this observation we began to investigate whether TIEG1^{-/-} B cells have intrinsic defects that are unrelated to the action of iTreg. We first checked B cell levels in naïve mouse spleens and a collection of lymph nodes (inguinal and mesentery) as these organs are major resting stations for B cells in the body. Freshly collected and untreated TIEG1^{-/-} and wild type spleen and lymph node cells were stained with antibody to CD19, a pan-B cell surface marker and activating receptor. Flow cytometry

analysis revealed that B cell counts in TIEG1^{-/-} mice were not different than those in wild type mice as percentage of total cells (Fig. 3.6 A). Thus B cells in TIEG1^{-/-} mice appear to be present at adequate levels relative to other cell types in the spleen and lymph nodes. We next tested B cell stimulation in spleen and lymph nodes via PMA (Phorbol Myristate Acetate) + Ionomycin treatment *in vitro*. These molecules in combination act as inducers of cell activation and proliferation by stimulation of Protein Kinase C by PMA and increase of intracellular Ca²⁺ levels by Ionomycin. A 48-hour stimulation of whole spleen and lymph node cell populations from both TIEG1^{-/-} and wild type mice increased B cell levels as percentage of total cells in both groups, but no difference between the groups was observed (Fig. 3.6 B). In conclusion, B cells in TIEG1^{-/-} mice have adequate counts and they proliferate normally *in vitro* in response to a PMA + Ionomycin stimulation, thus these factors cannot explain the impaired antibody production we observed in our vaccination.

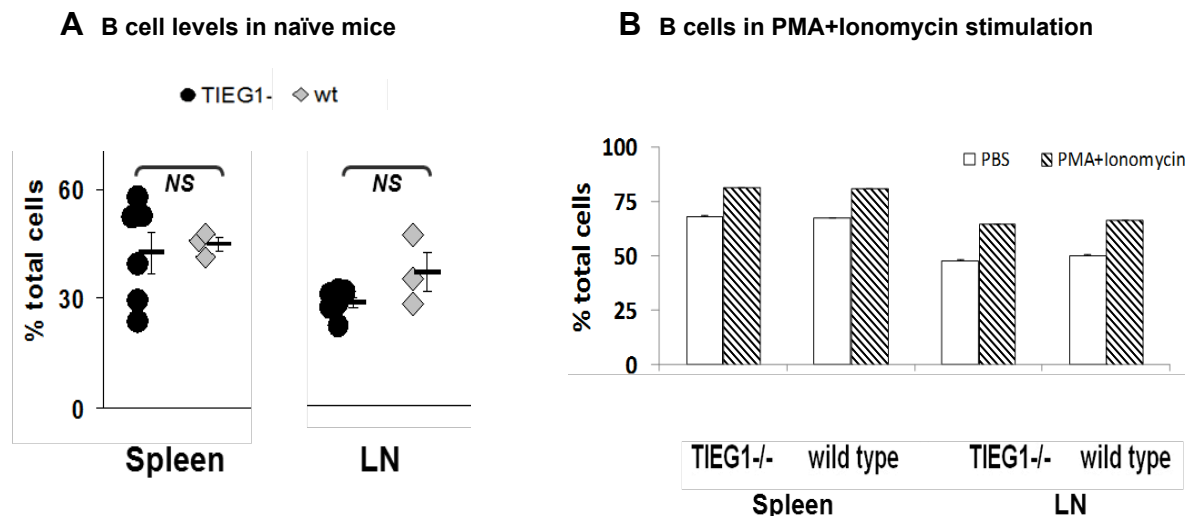
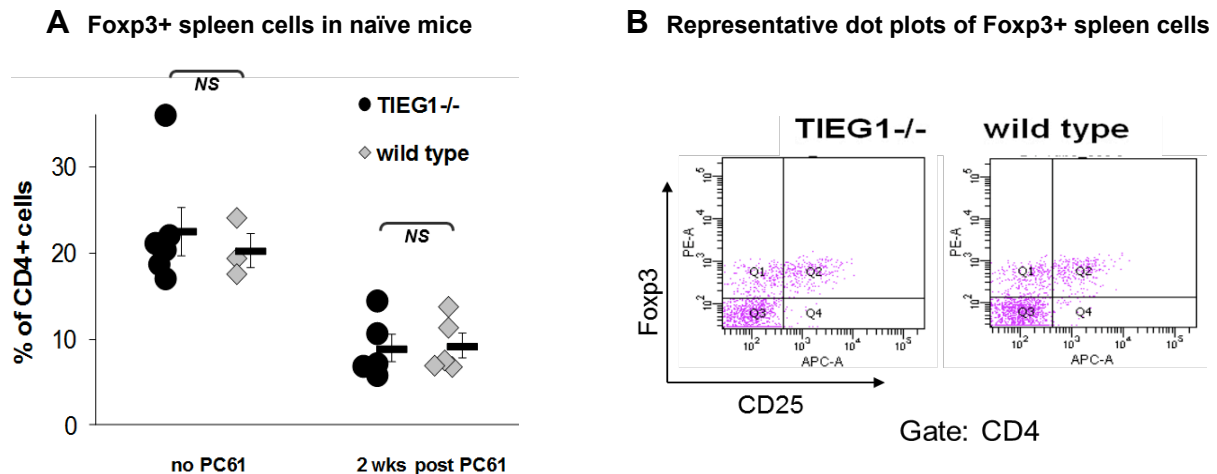


Figure 3.6 *In vivo* B cell count and *in vitro* B cell proliferation after PMA+Ionomycin stimulation. Antibody surface staining and flow cytometry measurement of (A) CD19⁺ cells (B cells) from spleens and lymph nodes of naïve TIEG1^{-/-} and wild type mice as % of total cells, (B) CD19⁺ cells (B cells) as % of total cells after stimulation of spleen and lymph node whole cell populations from naïve TIEG1^{-/-} and wild type mice with PMA (10 ng/ml) + Ionomycin (500 ng/ml) for 48 hrs.

3.7 TIEG1^{-/-} mice have normal levels of natural Treg but more resilient Myeloid-Derived Suppressor Cells

Given that TIEG1 is involved in immune suppression, we investigated whether other aspects of the suppressive arm of the immune system were affected in TIEG1 knockout mice. As natural Treg (nTreg) are by far the most abundant regulatory T cells in naïve animals, we measured nTreg levels in mouse spleens. Freshly collected and untreated TIEG1^{-/-} and wild type spleen cells were surface stained as before with antibodies to CD4 and CD25 and underwent overnight membrane permeabilization and intracellular staining for the Treg transcription factor Foxp3. Flow cytometry analysis revealed that Foxp3⁺ cell counts in naïve TIEG1^{-/-} mice were not different than those in wild type mice as percentage of CD4⁺ cells (Fig. 3.7 A, B) which had also been shown before by Venuprasad et al⁶⁵. Furthermore Foxp3⁺ cells were depleted equally well by treatment with the anti-CD25 antibody PC61 with counts in both TIEG1^{-/-} and wild type spleens being reduced by over 50%. Thus no difference in nTreg levels was present in TIEG1^{-/-} mouse spleens in naïve mice and after CD25 depletion.



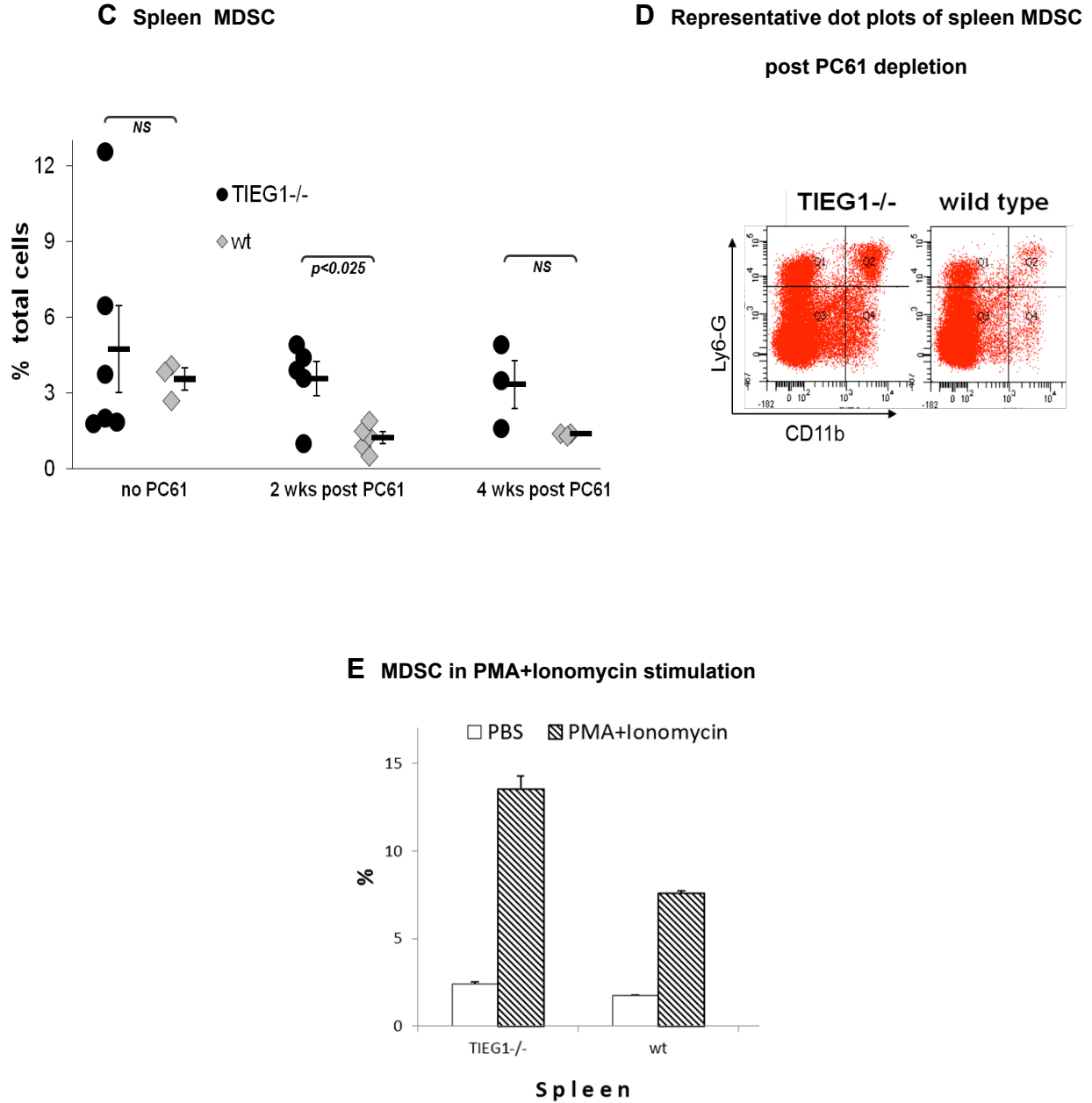


Figure 3.7 TIEG1^{-/-} have normal levels of Foxp3⁺ cells but more resilient MDSC. TIEG1^{-/-} and wild type mice were depleted with 0.5 mg anti-CD25 (clone PC61) i.p. twice, one week apart. Intracellular and surface markers were measured with flow cytometry (**A**) Plot and (**B**) representative dot plot of Foxp3⁺ cells as % of CD4⁺ T cells in spleens of TIEG1^{-/-} and wild type mice before and 2 weeks post PC61 treatment (**C**) Plot and (**D**) representative dot plot of CD11b⁺ Ly6G⁺ cells (MDSC) as % of total cells in spleens of TIEG1^{-/-} and wild type mice before, 2 weeks and 4 weeks post PC61 treatment. **E** CD11b⁺ Ly6G⁺ cells (MDSC) as % of total cells after stimulation of spleen whole cell populations from naïve TIEG1^{-/-} and wild type mice with 10 ng/ml PMA + 500 ng/ml Ionomycin for 48 hrs.

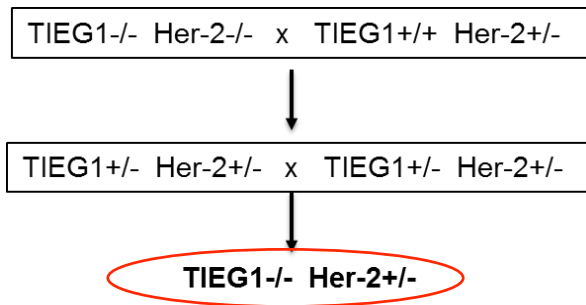
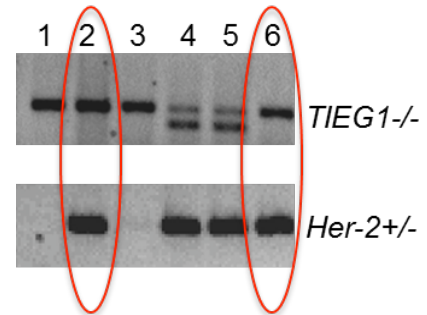
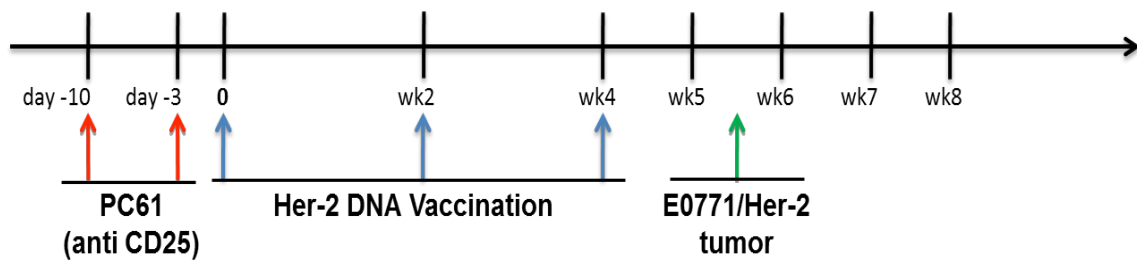
Next we tested whether Myeloid-Derived Suppressor Cells (MDSC), the other component of the suppressive immune system was normal in TIEG1^{-/-} mice. Freshly collected and untreated TIEG1^{-/-} and wild type spleen cells were stained for the MDSC surface markers CD11b and Ly6G (Gr-1). Flow cytometry analysis revealed that MDSC counts in naïve TIEG1^{-/-} mice were not statistically different than those in their wild type counterparts as percent of total cells, although the knockout group showed a large degree of variability. Treatment with the CD25-depleting antibody PC61 also showed decreased levels of MDSC at two and four weeks after depletion in wild type mice. This was possibly due to depletion of Treg as these cells are known to cooperate with MDSC in a mutually beneficial relationship. Interestingly however, MDSC in TIEG1^{-/-} mice maintained their count to pre-depletion levels without showing any reduction in association with decreasing nTreg (Fig. 3.7 C, D). Furthermore, a 48-hour PMA + Ionomycin stimulation of whole spleen cell populations from TIEG1^{-/-} and wild type mice boosted MDSC levels as percentage of total cells in both groups but the knock out group showed a greater increase (Fig. 3.7 E). Thus Myeloid-Derived Suppressor Cells in TIEG1^{-/-} mice appear to be more resilient and eager to proliferate upon PMA + Ionomycin stimulation.

3.8 Comparable overall antitumor immune response in TIEG1^{-/-} and wild type mice

In order to test whether absence of TIEG1/iTreg had an effect on antitumor immunity we tested tumor growth in vaccinated TIEG1^{-/-} and wild type mice. To mimic a system

where a tumor associated antigen such as the human oncoprotein Her-2 is tolerated by the immune system as self (similar to a human patient with Her-2+ cancer), we used a human Her-2 transgenic (Tg) line of mice generated in our lab. In these mice, expression of the Her-2 transgene is under the control of the whey acidic protein (WAP) promoter⁶⁷. We first generated mice that are both TIEG1 knock out and Her-2 Tg by two rounds of breeding of TIEG1^{-/-} mice with Her-2 Tg heterozygous mice (both of C57Bl/6 genetic background) as shown in Fig. 3.8 A. Mice were genotyped by TIEG1 and Her-2 PCR and knock out-transgenic offspring were selected to test the antitumor response with Her-2 Tg mice as control (Fig. 3.8 B).

In order to induce an adequate immune response to a self antigen such as Her-2 in transgenic mice it is necessary to temporarily deplete nTreg prior to DNA vaccination. TIEG1 KO Her-2 Tg mice and Her-2 Tg controls were injected i.p. with 0.5 mg anti-CD25 (PC61) antibody twice with a 7 day interval between treatments (Fig. 3.8 C). Three days after the second injection mice were started on a vaccination scheme with pE2N plasmid which codes for a hybrid fusion between human Her-2 and its rat homologue neu as well as pGM-CSF plasmid as in the earlier vaccination experiment. 50 µg DNA of each plasmid was delivered via i.m. injection in the hamstring region and electroporation for 3 times at 2 week intervals as described earlier. 10 days after the third vaccination mice were challenged with 2×10^5 E0771/Her-2 cells, a mouse C57Bl/6 tumor line that has been transfected to express Her-2 thus mimicking a Her-2 positive malignancy. By this time the effect of PC61 on CD25+ cells is over and CD25+ iTreg can be normally converted in wild type mice but are unable to do so in TIEG1 knock outs. Tumor growth was measured over the next several weeks at 3-7 day intervals.

A Genetic breeding scheme to generate TIEG1^{-/-} Her-2 Tg mice**B PCR genotyping****C Experimental time scheme**

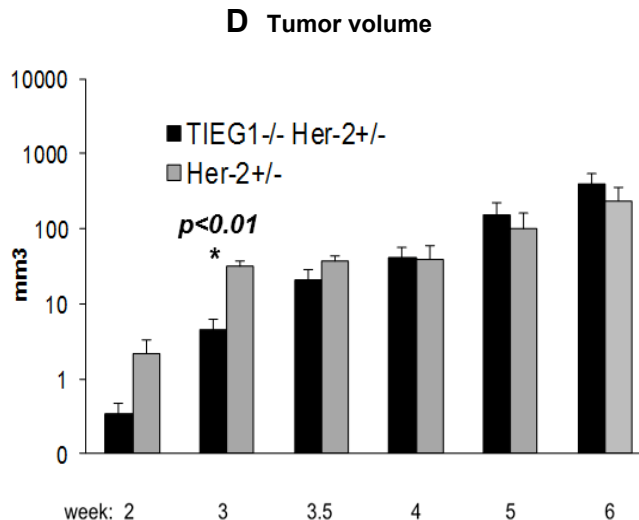


Figure 3.8 TIEG1^{-/-} mice have a comparable antitumor immune response to wild type mice. **A** TIEG1 KO Her-2 Tg mice were generated by two rounds of breeding starting with TIEG1^{-/-} and Her-2^{+/-} mice to make TIEG1^{-/-} Her2^{+/-} offspring. **B** Pups were screened separately by PCR for the TIEG1 KO allele as described in Figure 3.1 and for the Her-2 transgene (94°C x 30 sec denaturation, 65°C x 1 min annealing and 72°C x 1 min extension, repeated for 33 cycles). **C** TIEG1 KO Her-2 Tg mice (and Her-2 Tg controls) were depleted with 0.5 mg anti-CD25 (clone PC61) i.p. twice, one week apart. 3 days later they started Her-2 DNA vaccination with 50 µg of pE2N (human Her-2 - rat neu hybrid) and pGM-CSF (Granulocyte Monocyte Colony-Stimulating Factor) plasmid DNA each via i.m. injection and electroporation 3 times at 2-week intervals. 10 days later mice were challenged with 2x10⁵ E0771/Her2 tumor cells at the flank and tumor size was measured at 3-day or weekly intervals after that. **D** Tumor volume in mm³ (log scale) calculated as the product of tumor width² x length x 1/2, showed no major difference between TIEG1^{-/-} and wild type mice.

Initially tumors became palpable and grew more slowly in TIEG1^{-/-} mice compared to wild type controls up to the third week post challenge when the tumor burden in these mice was smaller than in the wild type group in a statistically significant manner (Fig. 3.8 D). After this point, tumor growth in TIEG1^{-/-} mice accelerated and caught up with that in control mice and the two groups remained not different statistically for the rest of the experiment. The slight advantage in initial antitumor immune response to Her-2 DNA vaccination that we observed in TIEG1^{-/-} mice, possibly due to absence of iTreg, may have been erased later by inadequate anti-Her-2 antibodies and perhaps greater

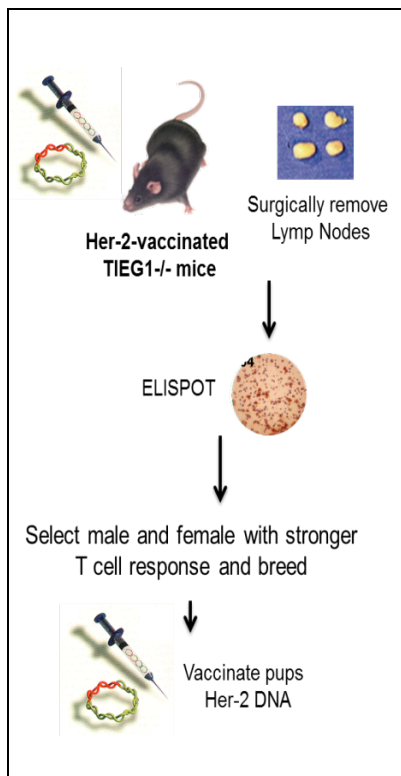
immune suppression by Myeloid-Derived Suppressor Cells in these mice as was shown earlier. In conclusion, TIEG1^{-/-} mice were not able to mount a stronger overall antitumor immune response.

3.9 A selection process yielded a sub strain of TIEG1^{-/-} mice with a more severely impaired antibody response

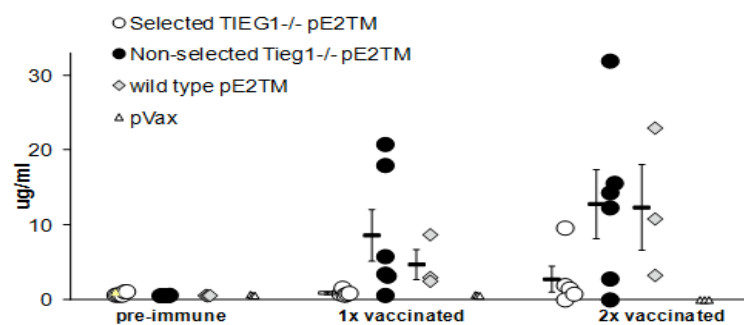
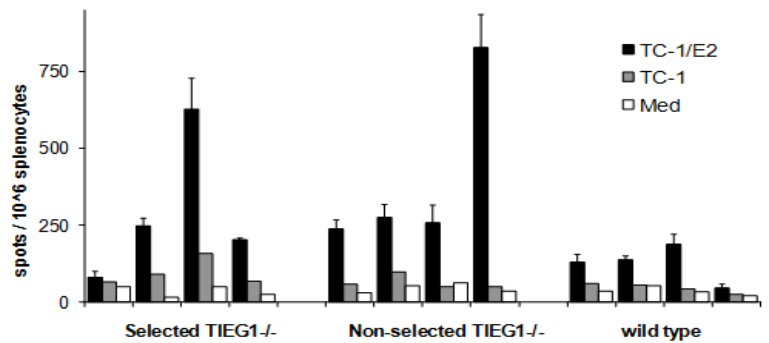
Although TIEG1 KO mice had a generally stronger T cell response, as noted earlier we observed a greater degree of variability in these mice compared to their wild type counterparts. An analysis of the earlier Her-2 DNA vaccination experiment showed that strong IFN_γ response in individual mice did not correlate with excessively impaired antibody production in the same animal (data not shown). A possible explanation for the variability in T cell response could be genetic factors unknown to us. For this reason we sought to select for TIEG1^{-/-} mice that have a particularly robust T cell response to DNA vaccination. Given that the splenectomy we perform in mice in order to obtain cells for our ELISPOT assay is a terminal procedure, we used inguinal lymph nodes obtained by survival surgery. Mice were vaccinated similarly to earlier experiments with 50 μg DNA each of pE2TM + pGM-CSF delivered via i.m. injection in the hamstring region followed by electroporation for 3 times at 2 week intervals. T cell response was measured at 2 weeks following the third vaccination via ELISPOT as before (Fig. 3.9 A). One male and two females with the strongest IFN_γ response (data not shown) were selected for breeding. Offspring of the selected mice as well as non-selected TIEG1^{-/-} and wild type

controls underwent Her-2 DNA vaccination according to the same scheme as above, 3 times at 2-week intervals.

A Selection scheme to generate strong TIEG1^{-/-} T cell responders



B Her-2 specific IFN- γ -producing T cells in spleen



C anti-Her-2 antibody

Figure 3.9 A selection process yields a sub strain of TIEG1^{-/-} mice with more severely impaired antibody response. **A** Selection scheme for strong TIEG1^{-/-} T cell responders. TIEG1^{-/-} mice were vaccinated with 50 μ g of pE2TM (Her-2 extracellular and transmembrane domains) and pGM-CSF (Granulocyte Monocyte Colony-Stimulating Factor) plasmid DNA each via i.m. injection and electroporation 3 times at 2-week intervals. Inguinal lymph nodes were collected via survival surgery and Her-2 ELISPOT was performed on lymph node cells. One male and 2 females with the strongest response were bred and the offspring (as well as regular non-selected TIEG1^{-/-} and wild type controls) were vaccinated with Her-2 DNA three times at 2-week intervals as above. Her-2-specific antibody and T cell responses were measured at 2 weeks following each vaccination via flow cytometry and ELISPOT respectively. **B** IFN γ response of splenocytes (after 3 vaccinations), stimulated with Her-2-expressing TC-1/E2 (dark bars) and control TC-1 cells (gray bars) as well as unstimulated media (Med) (open bars). Selected TIEG1^{-/-} mice do not show a superior T cell response compared to non-selected mice. **C** Selected TIEG1^{-/-} mice have a severely impaired antibody response after 1 and 2 vaccinations.

As observed earlier, TIEG1^{-/-} mice as a whole had a stronger T cell response than wild type controls (Fig. 3.9 B). Selected TIEG1^{-/-} mice however, did not show any superiority in their T cell response compared to the non-selected, knockout group. In addition, the variability in IFN- γ T cell response in the selected group seemed to be just as great among the non-selected mice. Interestingly however, selected mice had severely impaired levels of anti-Her-2 IgG that continued to stay almost at baseline for the duration of the experiment (Fig. 3.9 C). That the selection process yielded mice with almost inexistent antibody response to Her-2 DNA vaccination was unlikely to be due to the selection criteria for strong T cell response. As mentioned earlier, strong IFN γ response did not correlate with low IgG levels in individual mice. Furthermore, as mentioned above, the selected pups themselves did not show a superior ELISPOT response. Earlier in the project we identified a group of TIEG1^{-/-} mice with particularly low antibody response to DNA vaccination. We believe that the selection process inadvertently yielded TIEG1^{-/-} mice with more severely impaired antibody response.

CHAPTER 4

DISCUSSION

In this study we tested tumor growth and immune response to various antigens and immunization methods in mice lacking the TGF- β -Inducible Early Gene 1 (TIEG1). These mice have normal development and lifespan and showed no signs of disease. One important immune system defect in TIEG1^{-/-} mice however is their inability to convert naïve CD4⁺ T cells into inducible regulatory T cells (iTreg) with immune suppressive function under the action of the cytokine TGF- β . This conversion happens normally during an immune response and is an important one among the few immune suppressive mechanisms an organism employs in order to prevent immune-mediated tissue damage. Since many suppressive effects of Treg are geared toward T cells directly or indirectly (via antigen presenting cells), we tested T cell INF γ production in response to allogeneic splenocytes, Her-2 expressing tumor cells and Her-2 DNA vaccine. T cells in TIEG1^{-/-} mice responded more strongly to all three immunizations. This was in line with our hypothesis that iTreg are important in keeping these immune responses in check and their absence will allow greater activation of T cells. Upon encountering antigen such as various MHC molecules or Her-2 fragments, antigen presenting cells will travel to lymph nodes where they will interact with T cells that have receptors specific for these antigens normally present in the T cell repertoire. How many and how strongly T cells will be activated and will proliferate depends in part on the effector or suppressive character of the local microenvironment which is determined by

a variety of cell types and the cytokines they produce. We showed that after a few immunizations, mice lacking iTreg allowed a more robust specific effector T cell activation and proliferation without any observable adverse effects. It is likely that the IFN γ -producing T cells we detected in our ELISPOT assay are from a mixture of CD4 $^{+}$ (helper) and CD8 $^{+}$ (cytotoxic) cells since the antigen is probably both presented by our APC to CD8 $^{+}$ cells via MHC I as well as released in the medium where it can be picked up by native APC among the mouse splenocytes and presented to CD4 $^{+}$ cells via MHC II. Of note, although the T cell response was generally higher in TIEG1 $^{-/-}$ mice, there was a great variability in the response in all three types of immunization with a few mice responding very weakly. In fact, although the T cell difference showed strong statistical significance for the allogeneic immunization, the same difference was not straightforward to establish statistically in Her-2 DNA vaccination due to this variation. Out of two statistical tests employed, student's t-test and the Kruskal-Wallis test, only the latter yielded a p-value of less than 0.05 in the case of Her-2 DNA vaccination. We saw however that the great majority of TIEG1 $^{-/-}$ mice had levels of Her-2-specific IFN γ -producing T cells in their spleens that were much higher than those in the wild type group.

If absence of iTreg made a difference in T cell response, the effect on B cells was not as straightforward. The antibody levels we observed in response to the cell-based immunizations with allogeneic splenocytes and Her-2 expressing tumor cells were not higher in TIEG1 $^{-/-}$ mice compared to wild type controls. Specific IgG levels in the two groups were in fact equivalent. In the case of DNA vaccination we observed an antibody response that was actually inferior in TIEG1 $^{-/-}$ mice. Furthermore, we showed that this

lower IgG concentration in TIEG1^{-/-} sera was due to an initial impaired IgG1 subtype response as well as an inability to switch to IgG2c after a few rounds of vaccination. Deficiency in iTreg conversion is unlikely to be responsible for any of these observations regarding the B cell response in TIEG1^{-/-} mice. As mentioned previously, the first step for B cell activation and eventual antibody production is encounter with antigen. In respect to B cells, antigens can be T cell independent (T-I) or -dependent (T-D)²². A T-I antigen, usually a bacterial cell wall polysaccharide with many repeating units (called a Type 2 T-I antigen), can crosslink many B cell receptors on the cell surface and does not require further help. A T-D antigen, usually of protein origin, in addition to recognition by the BCR, also requires processing and presentation by the B cell to a CD4⁺ T helper cell with TCR specific for the same antigen. The three methods of immunization we performed will eventually produce protein antigens that will require CD4⁺ T cell help and are thus T-D antigens. As observed, the immunization-induced antigen-specific T cells in TIEG1^{-/-} mice were more abundant than in wild types. This would argue for a stronger B cell response and not an equal or weaker one, which is what we observed. In addition, the inability of the TIEG1^{-/-} humoral immune system to later switch to IgG2c, an IgG subclass associated with a Th1 response in mice, also argues against our observations. Th1-type responses are orchestrated by CD4⁺ T cells that elaborate cytokines such as IFN γ which was abundant in our T cell response to Her-2 DNA vaccination, especially later in the experiment. Thus an explanation for our defective antibody response based on ineffective T cell help and iTreg deficiency becomes implausible.

The observed deficiency in antibody production indicates rather that TIEG1 might be involved in other cellular pathways intrinsic to the B cell response. Since we observed no abnormalities in *in vivo* B cell numbers or *in vitro* pan-stimulation via PMA + Ionomycin, we looked for reported effects of TIEG1 on B cells in literature that were previously unknown to us. A report by Rian et al.⁶⁸ showed that TIEG1 is temporally expressed during B cell development with high levels in early B and Pro-B cells but undergoes a marked downregulation thereafter, namely in the pre-B and immature B cell stages. As mentioned earlier, TGF- β and TIEG1 have antiproliferative effects. In fact the above study showed that TGF- β /TIEG1 stimulation or ectopic overexpression in pre-B acute lymphoblastic leukemia (ALL) cells inhibited their proliferation and allowed them to escape chemotherapy that targets dividing cells. Thus, a tight regulation of TIEG1 expression in specific stages of B cell lymphopoiesis is necessary. The absence of TIEG1 in our knock out line may interfere with B cell development and be responsible for our findings in antibody levels. Furthermore, Rian et al. implicated in this process the transcription factor Id3 whose mRNA induction parallels that of TIEG1⁶⁹. Id3 is one of 4 members of the Id proteins (Inhibitors of Differentiation) which are part of the basic-helix-loop-helix (bHLH) family of transcription factors. Id proteins dimerize with the other family members (bHLH and E-proteins) and prevent them from acting as transcription activators⁷⁰. Id3 is indeed linked to TIEG1 by the fact that a minor variant of TIEG1 called EGR α (or EGR1), a gene that uses an alternative exon 1 via a different promoter⁷¹ induces expression of Id3⁷². Interestingly, a study of Id3^{-/-} mice by Zhuang et al. revealed defects in B cell response similar to our observations in the TIEG1^{-/-} line⁷³. Thus, although Id3^{-/-} mice had normal B cell numbers (as did TIEG1^{-/-} mice),

they showed reduced levels of IgG1 and IgG2a in naïve sera. These mice had a severely impaired response to a Type 2 T-independent antigen in all antibody classes and IgG subclasses. More importantly to our project *Id3*^{-/-} mice showed impaired ability to switch to IgG2a (another version of IgG2c) and IgG3 in response to a T-dependent antigen. *In vitro*, their B cells showed proliferation defects in response to BCR stimulation (unless it was delivered in conjunction with IL-4) but they had normal proliferation with PMA + Ionomycin and other generic non-BCR stimulants as did TIEG1^{-/-} B cells. This indicates that defects in the TIEG1/EGR1-*Id3* pathway could be responsible for the abnormal antibody responses we observed.

The fact that absence of TIEG1 did not necessarily cause a defective antibody response in the two cell-based immunizations seems to contradict the above argument. One explanation could be that these mice were immunized with rather large doses of allogeneic spleen cells or Her-2 expressing tumor cells ($3\text{-}5 \times 10^6$ /mouse). Differences in strong antibody responses may not be detectable with our flow cytometric method and may require analysis of further diluted serum samples with other techniques such as ELISA. Another explanation could be that defective B cell responses in Her-2 DNA-vaccinated TIEG1^{-/-} mice are due to factors intrinsic to DNA vaccination. A DNA-induced immune response requires cross-presentation by a special kind of antigen presenting cell among other features typical for DNA vaccination and TIEG1 could be involved in pathways governing this mechanism.

A direct effect of TIEG1 in T cells, independent of iTreg suppression, should not be discounted also. Specifically *Id3*, supposedly a downstream protein of TIEG1, is an inhibitor of GATA3, the Th2 transcription factor. Indeed, *Id3*^{-/-} T cells have much higher

levels of GATA3 mRNA⁷⁴. Th2 cytokines favor antibody over T cell responses, however this is inconsistent with our finding of lower antibody levels. The TIEG1/EGR1-Id3 pathway was also shown to be important in the generation of $\gamma\delta$ T cells⁷⁵ (a special kind of TCR), however we did not observe any differences in $\gamma\delta$ T cells in naïve mice (data not shown). Lastly, Id3 was also shown to be important in the generation of a Th17 response⁷⁴, however we did not test for these cells. Addressing these issues will require testing of TIEG1-/- B or T cells *in vitro* in response to various stimuli. Mice from the selected sub strain that have a severely impaired immune response should be used for these studies.

The fact that we did not observe a superior antitumor immune response in TIEG1-/- mice challenged with E0771/Her-2 cancer cells is perhaps not surprising considering the apparent pleiotropic and sometimes contradictory effects of absence of TIEG1 in immunity. The potent antitumor effects of the stronger T cell response we showed in the knockout mice could have been obviated by a weaker antibody response as well as higher levels of immunosuppressive MDSC, which we observed in these mice *in vitro*. In conclusion, targeting the transcription factor TIEG1 in cancer immunotherapy showed some encouraging features, but these were accompanied by other undesirable effects.

APPENDIX

WAYNE STATE
UNIVERSITY


INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
101 E. Alexandrine St.
Detroit, MI 48201-2011
Telephone: (313) 577-1629
Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A 3310-01

PROTOCOL # A 03-06-09

AMENDMENT # 3

TO: Dr. Wei-Zen Wei
Cancer Institute (Immunology and Microbiology)
4129.2 Prentis Building, Karmanos Cancer Institute

FROM: Lisa Anne Polin, Ph.D. 
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Amendment # 3 to Protocol # A 03-06-09
"Development of ERBB-2 based breast cancer vaccines"

DATE: July 23, 2009

The following requested changes to the above protocol have been reviewed and approved for immediate effect:

Request dated July 16, 2009:

The following animals will be transferred and bred as described is the amendment request:

MICE, female, C57BL/6 TIEG-1 (-/-) knockout, 12, "B"
*Transferred from Dr. Venuprasad Poojary (A 06-06-09)

MICE, male/female, C57BL/6 Her-2 (+/-) /TIEG-1 (-/-), 1200, "D"
MICE, male/female, C57BL/6 Her-2 (-/-) /TIEG-1 (-/-), 1200, "D"
*Bred in-house

This protocol, as amended, will be subject to annual review on the anniversary date of the initial IACUC review. This protocol was last reviewed on April 16, 2009.

cc: Patricia Denison, DLAR
Pat Sims, DLAR



INSTITUTIONAL ANIMAL
CARE AND USE COMMITTEE
101 E. Alexandrine St.
Detroit, MI 48201-2011
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Fax Number: (313) 577-1941

ANIMAL WELFARE ASSURANCE # A 3310-01

PROTOCOL # A 05-13-10

Protocol Effective Period: June 17, 2010 – May 31, 2013

TO: Dr. Wei-Zen Wei
Cancer Institute (Immunology and Microbiology)
4129.2 Prentis Building, Karmanos Cancer Institute

FROM: Lisa Anne Polin, Ph.D. *Lisa Anne Polin*
Chairperson
Institutional Animal Care and Use Committee

SUBJECT: Approval of Protocol # A 05-13-10
"Systemic and local vaccination against breast cancer with minimum autoimmune sequelae"

DATE: June 17, 2010

Your animal research protocol has been reviewed by the Wayne State University Institutional Animal Care and Use Committee, and given final approval for the period effective **June 17, 2010** through **May 31, 2013**. The listed source of funding for the protocol is **DOD**. The species and number of animals approved for the duration of this protocol are listed below.

<u>Species</u>	<u>Strain</u>	<u>USDA</u>	
		<u>Qty.</u>	<u>Cat.</u>
MICE.....	C57BL/6, female, 6-8 wks old	40	B
MICE.....	C57BL/6, female, 6-8 wks old	180	D
MICE.....	BALB/c, female, 6-8 wks old	540	B
*To be purchased			
MICE.....	C57BL/6 TIEG-1 ^{-/-} , male	30	B
MICE.....	C57BL/6 TIEG-1 ^{-/-} , female	160	B
MICE.....	C57BL/6 Her2 ⁺ TIEG1 ^{-/-} , male	50	B
MICE.....	C57BL/6 Her2 ⁺ , male	20	B
MICE.....	BALB Her2 ⁺ , male	135	B
**To be transferred from WSU protocol #A 03-06-09			
MICE.....	C57BL/6, female	60	B
MICE.....	C57BL/6, female	30	D
MICE.....	C57BL/6 Her2 ⁺ , male	90	B
MICE.....	C57BL/6 Her2 ⁺ , female	90	D
MICE.....	C57BL/6 Her2 ⁺ TIEG1 ^{-/-} , male	150	B
MICE.....	C57BL/6 Her2 ⁺ TIEG1 ^{-/-} , female	150	D
MICE.....	BALB/c, male	810	
MICE.....	BALB/c, female	810	D
MICE.....	BALB Her2 ⁺ , male	810	B
MICE.....	BALB Her2 ⁺ , female	810	D
MICE.....	C57BL/6 TIEG ^{-/-} , male.....	180	B
MICE.....	C57BL/6 TIEG ^{-/-} , female.....	180	D
MICE.....	C57BL/6 Her2 ⁻ TIEG ^{-/-} , male.....	150	B
MICE.....	C57BL/6 Her2 ⁻ TIEG ^{-/-} , female.....	150	D
***To be bred in-house			

Be advised that this protocol must be reviewed by the IACUC on an annual basis to remain active. Any change in procedures, change in lab personnel, change in species, or additional numbers of animals requires prior approval by the IACUC. Any animal work on this research protocol beyond the expiration date will require the submission of a new IACUC protocol form and full committee review.

The Guide for the Care and Use of Laboratory Animals is the primary reference used for standards of animal care at Wayne State University. The University has submitted an appropriate assurance statement to the Office for Laboratory Animal Welfare (OLAW) of the National Institutes of Health. The animal care program at Wayne State University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

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ABSTRACT**MODULATION OF ANTI-TUMOR IMMUNE RESPONSE BY TGF- β -INDUCIBLE EARLY GENE 1 (TIEG1)**

by

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Cancer immunotherapy has had limited clinical efficacy partly because regulatory T cells (Treg) suppress the immune response to tumor-associated antigens. Inducible regulatory T cells (iTreg), which are converted from naïve CD4 T cells by TGF- β , an abundant cytokine in the tumor microenvironment, may contribute to this immune suppression. Induction of Foxp3 by TGF- β is mediated by the transcription factor TIEG1 and abrogation of this protein prevents Foxp3 expression. We are testing the hypothesis that blockade of TIEG1 to prevent iTreg conversion will enhance immune response in DNA vaccination to the tumor associated antigen Her-2. Wild type and TIEG1 knockout mice in C57BL/6 background were immunized with three different antigens, then immune responses were compared. In TIEG1^{-/-} mice, immunization with allogeneic spleen cells resulted in a stronger T cell response with comparable levels of antibody. Similarly, Her-2-expressing irradiated tumor cells induced a stronger anti-Her-2 T cell response in TIEG1^{-/-} mice, without a significant difference in antibody levels. DNA vaccine encoding human Her-2 also induced a stronger T cell response but with

reduced IgG antibody titers specifically in IgG1 and deficient switching to IgG2c. B cells in TIEG1^{-/-} mice had adequate counts *in vivo* and they proliferated normally *in vitro* in response to PMA + Ionomycin stimulation. Foxp3⁺ cells had adequate numbers in TIEG1^{-/-} mice whereas Myeloid Derived Suppressor Cells had stronger proliferation to PMA + Ionomycin stimulation. Initially tumors grew more slowly in TIEG1^{-/-} mice but the difference disappeared later. A new sub strain of TIEG1^{-/-} mice was derived that has similar T cell response but more severely impaired antibody response. Together, these data suggest an amplified T (but not B) cell response in TIEG1^{-/-} mice, indicating pleiotropic immune modulating effects of TIEG1.

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