Michelle Sallin

University of Maryland, School of Medicine
Department of Otorhinolaryngology
800 W Baltimore St, rm 404
Baltimore, MD 21201
(410) 706-6161 (lab) (443) 742-5091 (cell)
michellesallin@hotmail.com

EDUCATION

PhD, Molecular Medicine (Cancer Biology) **2007-2013**
University of Maryland School of Medicine
Baltimore, MD

BS, Microbiology and BS Biochemistry **2001-2005**
North Carolina State University, Raleigh, NC

PROFESSIONAL EXPERIENCE

University of Maryland School of Medicine, Department of Otorhinolaryngology
Graduate Research Assistant **August 2007- 2013**
Mentor: Dr. Scott E. Strome, MD

Thesis: Defining the role of Fc-FcγR in the anti-tumor function of anti-CD137 monoclonal antibody (mAb) therapy

- Identify the FcγR responsible for the modulation of the anti-CD137 mAb anti-tumor immune response using techniques such as surface plasmon resonance, flow cytometry, and an *in vivo* EL4E7 murine tumor model.
- Evaluate the mechanisms through which FcγRs mediate the therapeutic efficacy of anti-CD137 monoclonal antibodies by processing mouse spleen, lymph node, liver, and tumors from the EL4E7 murine tumor model to use for *in vitro* functional assays (ex. qPCR, ELISA) and multi-color flow cytometry.

Burleson Research Technologies, INC, Morrisville, NC
Scientist I **January 2007- August 2007**
Worked as a project leader utilizing basic immunology assays (ex. Chromium release assay, local lymph node assay, and oxidative burst), in accordance to Good Laboratory Procedures (GLP), to determine off-target drug effects on the immune response. Provided assistance to the study director in the completion of final reports for clients.

Burleson Research Technologies, INC, Morrisville, NC
Archivist **April 2006- August 2007**
Organized and bound completed research notebooks for archival according to GLP standards. Verified all documentation, and insured complete study records maintained with final report.

Burleson Research Technologies, INC, Morrisville, NC
Research Technician **May 2005- January 2007**
Worked as the lead technician for the local lymph node assay. Assisted other lab members with mouse dosing, organ harvest and processing, and functional assays. Assisted study director with final reports for clients.

PUBLICATIONS

Anti-lymphoma activity of CD137 monoclonal antibodies are enhanced in FcγRIII$^{-/-}$ mice
Sallin MA, Zhang X, So EC, Zhao Y, Burch E, Cai, L,Wei L, Chapoval AI, and Strome SE
Submitted to Cancer Immunology, Immunotherapy in September 2013

A high throughput method for enrichment of natural killer cells and lymphocytes and assessment of *in vitro* cytotoxicity
So EC, **Sallin MA,** Zhang X, Chan SL, Sahni L, Schulze DH, Davila E, Strome SE, Jain A
Journal of Immunological Methods. 2013 Aug 30; 394(1-2):40-48. doi: 10.1016/j.jim.2013.05.001

CD137 promotes proliferation and survival of human B cells.
Zhang X, Voskens CJ, **Sallin M**, Maniar A, Montes CL, Zhang Y, Lin W, Li G, Burch E, Tan M, Hertzano R, Chapoval AI, Tamada K, Gastman BR, Schulze DH, Strome SE
Journal of Immunology. 2010 Jan 15; 184(2):787-95

Cell type-specific transcriptome analysis reveals a major role for Zeb1 and miR-200b in mouse inner ear morphogenesis.
Hertzano R, Elkon R, Kurima K, Morrisson A, Chan SL, **Sallin M**, Biedlingmaier A, Darling DS, Griffith AJ, Eisenman DJ, Strome SE
PLoS Genet. 2011 Sep;7(9):e1002309. doi: 10.1371/journal.pgen.1002309

TECHNICAL SKILLS

- Cell Culture
    - Mammalian cell culture techniques
    - Multi-color flow cytometry
    - Lymphocyte isolation (human and mouse)
    - Hybridoma culturing and antibody purification
- Molecular biology
    - Quantitative real-time PCR (qPCR)
    - Expression vector construction
    - Transient and stable transfections
- *In vivo* Skills

- Mouse breeding and breeding colony maintenance
    - Subcutaneous, intravenous, and intraperitoneal injections in mice
    - Saphenous vein, tail vein, ear vein, facial vein and cardiac bleeding in mice, rats, and rabbits
    - Transplantable tumor cell models
    - Collagen induce arthritis model
    - Mouse influenza model
- Presentation and Analysis
    - Microsoft office
    - Prism Graphpad
    - Flowjo
    - NCBI databases

OTHER SKILLS

- Team Leader
    - Lead eight team members in large harvests (50+ mice) to produce samples for chromium release assays, local lymph node assays, and respiratory burst.
    - Oversee the research and help execute experimental plans with two rotating otorhinolaryngology residents and two summer students.
- Collaboration
    - Worked with study director and director of laboratory operations by providing details of experimental data for the completion of study reports.
    - Supported other lab members by providing my expertise in flow cytometry and cell separation to improve upon a mouse ear cell enrichment protocol.
- Organization
    - Assisted with planning and execution of the first cancer research retreat at the University of Maryland, Baltimore.
    - Assisted with Medical student research summer symposium by helping with planning, moderating oral presentations and student registration
- Teaching
    - Trained rotating medical students, undergraduate students, and otorhinolaryngology residents in the completion of experimental projects.
    - Taught an FcγR biology lecture to second year graduate students.
    - Community outreach teaching middle school children math and literature

POSTER AND ORAL PRESENTATIONS

ORAL

"Activating FcγRs role in the function of anti-CD137"
Promise Research Symposium, College Park, MD **January 2010**

"Anti-tumor effects of anti-CD137 mAb are influenced by Fc:FcγR interactions"
Second Annual Cancer Research Symposium
University of Maryland, Baltimore MD **May 2011**

“Defining the role of Fc:FcγR interactions in the biology of anti-CD137 mAb”
Graduate Research Symposium
University of Maryland, Baltimore MD **April 2012**

POSTER
“Anti-tumor effects of anti-CD137 mAb are independent of Fc:FcγR interactions”
American Association of Immunologists Annual Meeting
San Francisco, CA **May 2011**

“Defining the role of Fc:FcγR interactions in the biology of anti-CD137 mAb”
Third Annual Cancer Research Symposium
University of Maryland, Baltimore MD **May 2012**

HONOR AND CERTIFICATION

“Outstanding Abstract Selected for Oral Presentation” at the Second Annual Cancer Biology Research Retreat, University of Maryland
School of Medicine, Baltimore **May 2011**

Good Laboratory Practices (GLP) Training **Aug 2005**

COMMUNITY OUTREACH

Higher Achievement, Baltimore, Maryland
Mentor **2010-present**
Taught math and literature to middle school children from under-privileged neighborhoods in Baltimore

Literacy Council of Wake County, Raleigh, North Carolina
Literacy tutor **2006-2007**
Taught English as a second language to immigrant workers and illiterate people to read

Abstract

Agonistic monoclonal antibodies (mAbs) directed against the co signaling molecule CD137 (4-1BB) elicit potent anti-tumor immunity in mice. This enhanced anti-tumor immunity has traditionally been thought to result from the ability of the Fab portion of anti-CD137 mAb to function as a CD137L analogue. Although engagement of CD137 by anti-CD137 mAbs has the potential to cross-link the Fc fragments, mediating Fc engagement of low to moderate affinity Fc gamma receptors (FcγR), the relative import of such Fc-FcγR interactions in mediating CD137 associated anti-tumor immunity is unknown. In order to address this knowledge gap, we studied the ability of rat anti-mouse CD137 mAb (2A) to mediate the anti-tumor response against the EL4E7 lymphoma in WT and FcγR$^{-/-}$ strains. We hypothesized that Fc interactions through activating FcγRs would enhance the anti-tumor immune response elicited by anti-CD137 mAb. 2A treated Fcγ chain deficient mice have an improved anti-tumor immune response against EL4E7 lymphoma relative to WT mice, which can be completely recapitulated in FcγRIII$^{-/-}$ mice. We tested the hypothesis using the MC38 colon carcinoma, but did not find the same enhanced anti-tumor immune response in the FcγRIII$^{-/-}$ mice as in the EL4E7 lymphoma. Kinetic experiments to analyze the immune response in the tumor bearing 2A treated FcγRIII$^{-/-}$ mice identified an increase in splenic CD8β^{+} T cell and dendritic cell (DC) populations compared to WT mice and IgG controls. There was a significant increase in the number of DCs expressing CD40, CD80, and CD86 molecules in the 2A treated FcγRIII$^{-/-}$ mice suggesting the potential for more effective antigen presentation compared to WT mice. The increased numbers of splenic CD8β^{+} T cell and dendritic cell (DC) populations in the 2A treated FcγRIII$^{-/-}$ mice significantly correlated with the tumor volume on Day 16. Collectively, these data suggest FcγRIII ligation negatively regulates anti-CD137 mAb stimulation of DCs with a secondary increase in CD8β^{+} T cells involved in the anti-tumor immune response. Our results demonstrate an unexpected inhibitory role for FcγRIII in the anti-tumor function of anti-CD137 mAb in the EL4E7

lymphoma, and underscore the need to consider antibody isotype when engineering therapeutic mAbs.

Defining the Role of Fc-Fc Gamma Receptor Interactions in the Anti-Tumor Function of Anti-CD137 Monoclonal Antibody Therapy

by
Michelle Sallin

Dissertation submitted to the faculty of the Graduate School
of the University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2013

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List of Abbreviations

ADCC antibody mediated cellular cytotoxicity

AICD activation induced cell death

AID activation induced cytodine deaminase

AKT protein kinase B

APCs antigen presenting cells

APRIL a proliferation inducing ligand

BAFF B cell activating factor

BCR B cell receptor

BcL-2 B cell lymphoma 2

Bcl-xL B-cell lymphoma-extra-large

Bfl-1 Bcl-2-related protein A1

Bim B-cell lymphoma 2 interacting mediator of cell death

CAR chimeric antigen receptors

CD137L CD137 ligand

CDC complement mediated cellular cytotoxicity

c-FLIP cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein

CH constant heavy

cIAP1 cellular inhibitor of apoptosis protein 1

COPD chronic obstructive pulmonary disease

CTLA-4 cytotoxic lymphocyte antigen 4

DAP12 DNAX activation protein of 12 kDa

DC dendritic cell

DC-SIGN dendritic cell-specific ICAM-3-grabbing non-integrin 1

ERK extracellular regulated kinase

$FcR\gamma^{-/-}$ Fc common γ–chain deficient

FcγR Fcγ receptors

GM-CSF Granulocyte-macrophage colony-stimulating factor

Hck hemopoietic cell kinase

HER2 Human epidermal growth factor receptor 2

HLA human leukocyte antigen

HPV human papillomavirus

ICAM-1 intercellular adhesion molecule 1

IDO indoleamine 2,3-dioxygenase

IFNγ interferon gamma

IgG immunoglobulin G

IL interleukin

ITAM immunoreceptor tyrosine-based activation motifs

ITAMi inhibitory ITAM

ITIM immunoreceptor tyrosine-based inhibition motif

IVIG intravenous immunoglobulin

JNK JUN N-terminal kinase

LAKs lymphokine-activated cells

Lck lymphocyte-specific protein kinase

LSP1 leukocyte-specific protein 1

mAb Monoclonal antibody

MAPK mitogen activated protein kinase

MCA methylcholanthrene

MDSCs myeloid derived suppressor cells

MHC major histocompatibility complex

MICA major histocompatibility complex class I related chain A

Mcl-1 Myeloid cell leukemia sequence 1

MCP-1 monocyte chemoattractant protein 1

NA neutrophil antigen

NADPH nicotinamide adenine dinucleotide phosphate

NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells

NIK NF-κB inducing kinase

NK natural killer

NKG2D natural killer group two member D

PD-1 programmed cell death 1

PDCA-1 plasmacytoid dendritic cell antigen 1

PD-L1 programmed cell death ligand 1

PGE2 prostaglandin E2

PI3K phosphatidylinositol 3-kinase

PKC protein kinase C

PtdIns(3,4,5)P_3 phosphatidylinositol (3,4,5)-triphosphate

PtdIns(4,5)P_2 phosphatidylinositol-4,5-bisphosphate

RAG recombinase activating gene

RTK receptor tyrosine kinases

sCD137 soluble CD137

SHIP SH2 domain-containing inositol 5'-phosphatase

SHP-1 Src homology 2 (SH2)-containing tyrosine phosphatase-1

STAT signal transducers and activators of transcription

Syk spleen tyrosine kinase

TAA tumor associated antigens

TAMs tumor associated macrophages

TCR T cell receptor

TGF transforming growth factor

TNFR tumor necrosis factor receptor

TRAF TNF receptor associated factors

TRAIL TNF-related apoptosis inducing ligand

Tregs T regulatory cells

VCAM-1 vascular cellular adhesion molecule 1

VEGF vascular endothelial growth factor

WT wild type

Chapter 1- Introduction

Cancer is the second leading cause of death in the United States, with an estimated 300,000 cancer related deaths in men and 270,000 cancer related deaths in women in 2013 (1). An estimated 800,000 new cancer diagnoses for both men and women in 2013 warrants the development of improved therapeutic options to arrest tumor growth and extend life expectancy (1).

Cancer is defined by the National Institutes of Health as "a term used for diseases in which abnormal cells divide without control and are able to invade other tissues"(2). The abnormal growth of cells is through both intrinsic and extrinsic mechanisms. *Intrinsic* events leading to tumor development include the acquisition of mutations resulting in the silencing of cell cycle inhibitory proteins, the overexpression of cell cycle progression proteins, accumulation of growth factors and receptors, and an altered metabolism (3). Tumor cell *extrinsic* elements include development of a lymphatic and/or vascular structure for nutrient and waste transport, factors to create a permissive microenvironment for growth, and finally elements to influence the anti-tumor immune response (3, 4).

In order to put our thesis in context, we first discuss the relationship between the immune system and tumorigenesis, and how knowledge of these interactions guides the development of cancer immunotherapy.

Cancer and the immune system

The immune system can infiltrate tumors, resulting in promotion and/or elimination of tumor progression (5, 6). The hallmark for the creation of a permissive tumor microenvironment is chronic inflammation (4). The role of inflammation is incorporated into a global scheme for immune-tumor interactions termed cancer immunoediting (5). The cancer immunoediting theory is defined by three phases: 1. elimination - the immune system eliminates tumor cells, 2. equilibrium - anti-tumor immunity and immune evasion are at equilibrium maintaining tumor dormancy, and 3. escape – immune evasion leading to tumor growth and metastasis (5). The goal of tumor immunotherapy is to reverse tumor escape, returning to a state of elimination and/or equilibrium. Lessons from detailed study of each of these three phases serve to guide current strategic efforts. In this section, we will first focus on the mechanisms of tumor immune evasion, and subsequently detail how the immune system normally checks tumor growth in order to define targets for therapeutic intervention.

Chronic inflammation- a form of immune escape

The involvement of the immune system in cancer development was proposed by Rudolph Virchow in the 1860s, after his observation of infiltrating leukocytes in tumors (7). In the 1950s, associations were made between chronic inflammation and tumorigenesis (8, 9). In the 1960s, these associations were solidified by experimental studies in mice, where spontaneous generation of reticuloendothelial tumors in aging C3H mice were associated with prior mucosal ulceration with lymphocyte infiltration (10). More recent studies now clearly indicate that patients with chronic inflammation e.g. Crohns's disease and chronic obstructive pulmonary disease (COPD) are at a higher risk of tumor development (11, 12). Collectively, these observations implicate inflammation as a trigger for tumorigenesis.

Cancers are described as "wounds that do not heal" due to the presence of inflammatory cells, growth factors, and wound healing components associated with the tumor microenvironment (4). A normal response to a wound is the recruitment of neutrophils, followed by monocytes that differentiate into macrophages, and finally the recruitment of mast cells (6).

Neutrophils are recruited to the site of inflammation through pro-inflammatory cytokines and chemokines, which promote the classical leukocyte transmigration through endothelial cells (13). Neutrophils produce metalloproteinases and extracellular matrix components such as actin, tubulin, and annexin 1, which are involved in tissue remodeling and angiogenesis (13). Tissue remodeling and angiogenesis are important processes for tumor development by remodeling the microenvironment with the creation of a vascular system to support growth and metastasis. Additionally, neutrophils recruit monocyte/macrophages through release of immune attractants in the granules, such as cathepsin G, and via apoptosis (13). The recruitment of monocytes/macrophages further enhances the tumor microenvironment by tissue remodeling, angiogenesis, and immune suppression.

Macrophages recruited for wound healing are responsible for tissue remodeling, angiogenesis, and immune regulation. Macrophages produce metalloproteinases involved in tissue remodeling, and are important to vascular development by guiding the fusion of endothelial cells (14). Additionally, macrophages' interactions with other immune cells induce production of proangiogenic molecules such as vascular endothelial growth factor (VEGF) and interleukin (IL)-8 (14). Immune regulatory function is associated with the M2 and M2-like phenotype through the expression of immunosuppressive cytokines, such as IL-10, and the production of chemokines, CCL17, CCL22, and CCL24, which attract T regulatory cells (Tregs) to induce an immune suppressive microenvironment (14).

Mast cells are recruited to the site of inflammation through monocyte chemoattractant protein 1 (MCP-1) (15). Mast cells are responsible for tissue remodeling, angiogenesis, and immune suppression. Mast cells produce matrix remodeling factors, extracellular matrix components, VEGF, and angiopoietin-1 supporting tissue remodeling and angiogenesis (16). Suppression of the immune response is mediated by the production of anti-inflammatory cytokines, such as IL-4 and IL-10; chemokines, attracting myeloid cells; prostaglandin E, important for the development of myeloid derived suppressor cells; and stimulation of Tregs through OX40-OX40L interactions (16, 17).

While wound healing both serves as a model of the tumor microenvironment and plays a role in tumor development, chronic inflammation only contributes to approximately 15% of tumor incidence (6). In fact, the majority of tumor development involves immune evasion through immune-tumor interactions, which reduce immunogenicity. The next section describes the mechanisms used by tumors to achieve an immunosuppressive tumor microenvironment.

Cancer immunoediting- Escape phase

While chronic inflammation creates a permissive environment for tumor growth, intrinsic changes within the tumor facilitate immune escape. Tumors attempt to actively escape the immune response through multiple mechanisms including loss of antigen, the down regulation of natural killer (NK) cell activation receptor ligands, and/or the expression of inhibitory ligands; and/or inducing an immunosuppressive tumor microenvironment by producing immunosuppressive factors, and the recruitment of immunosuppressive cells (Figure 1) (7).

The creation of a poorly immunogenic tumor is through the loss of tumor associated antigens (TAA). Tumors reduce TAA expression, and replace TAA with neoantigens because of genomic instability (7, 18). Reduction in TAA exposure is mediated by mutations in antigen presentation machinery and interferon gamma (IFNγ) responses (7). Antigen presentation machinery is silenced by loss of major histocompatibility complex (MHC), β2 microglobulin, and/or the immunoproteosome (7). IFNγ stimulation enhances expression of MHCI and/or MHCII, and tumor cells become desensitized to IFNγ resulting in reduced expression of MHC and TAA (19-21). The reduction in TAA reduced camouflages the tumor from the antigen specific immune response.

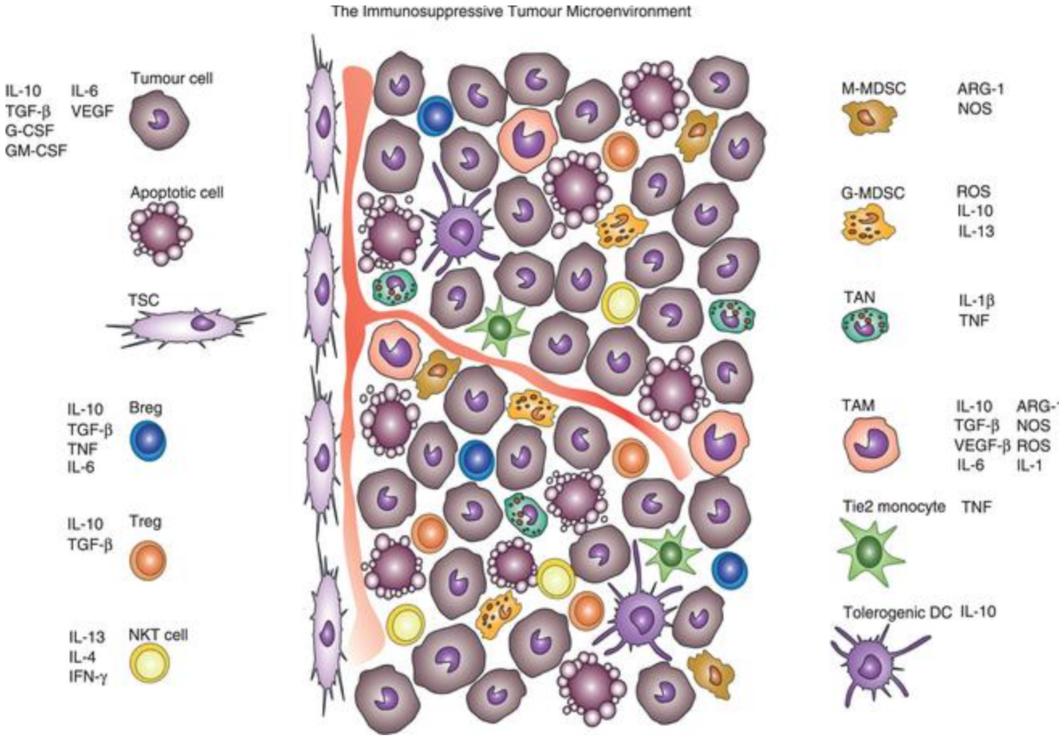

Figure 1: Major component of immune escape is the development of the immunosuppressive tumor microenvironment. Immunosuppressive tumor microenvironment illustrating the many immunosuppressive cells attracted to the tumor microenvironment to suppress the anti-tumor immune response through cell to cell contact or secretion of immune modulators. Reproduced with kind permission of Blackwell Publishing LTD from reference (22).

NK cells are named for the ability to kill transformed cells (23). NK cells recognize transformed cells by the lack of expression of MHCI and/or the expression of activating NK receptor ligands e.g. major histocompatibility complex class I related chain A (MICA)1/2 (24). Tumors avoid NK cell activation by loss of expression and/or secretion of NK activating receptor ligands, and the up regulation of NK inhibitory ligands e.g. human leukocyte antigen (HLA)-G, and HLA-E (24). The reduced expression of natural killer group two member D (NKG2D) ligands on tumors and/or the increased expression of soluble NKG2D ligands correlate with higher grade tumors indicating a reduction in NK cell mediated cellular cytotoxicity (25-28).

Tumors modulate the expression of cytokines and molecules to induce an immunosuppressive tumor microenvironment. Tumors reduce expression of pro-inflammatory cytokines and immunostimulatory chaperones resulting in a diminution of anti-tumor immunity (29, 30). Tumors produce anti-inflammatory cytokines, such as IL-6 and transforming growth factor (TGF)-β, which lower expression of costimulatory molecules and/or directly inhibit cytotoxic lymphocytes (31-33). TGF-β is an important cytokine in tumor immune evasion because TGF-β is responsible for the inhibition of T-cell dependent humoral responses, induction of Tregs, and induction of immunosuppressive cell populations (34-37). Tumors secrete anti-inflammatory cytokines, VEGF, and prostaglandin E2 (PGE2), that support infiltration of myeloid derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs); while suppressing dendritic cell (DC) antigen presentation and inhibiting T cells and/or NK cells (7, 38-40).

Tumor infiltrating leukocytes produce immunosuppressive molecules that support immune evasion. For example, Tregs produce IL-10 and TGF-β, inhibiting DC maturation and secretion of pro-inflammatory cytokines (41). Similarly, suppressive tumor infiltrating plasmacytoid DCs, induced through TGF-β, produce IL-10 to suppress anti-tumor T cells (42). Furthermore, suppressive DCs and MDSCs produce indoleamine 2,3-dioxygenase (IDO), the enzyme for tryptophan metabolism, which inhibits anti-tumor T cell responses by toxicity of metabolic products, tryptophan starvation in T cells, and the induction of Tregs (7, 43). These data provide clear evidence that tumor-immune interactions have the potential to create a permissive microenvironment for tumor growth.

Tumor cells that remain immunogenic avoid immunological assault by increasing anti-apoptotic protein expression, loss of caspase proteins, and loss of sensitivity to cell-mediated death (7). The B cell lymphoma 2 (BcL-2) family of anti-apoptotic proteins is overexpressed in many types of cancer with overexpression being tissue specific. For example, in solid tumors, myeloid cell leukemia sequence 1 (Mcl-1) is highly overexpressed, but in lymphomas/leukemias levels of Bcl-2 and Bcl-2-related protein A1 (Bfl-1) are enhanced (44). An alternative to enhanced expression of the anti-apoptotic proteins used by tumors is to alter caspase expression. The alteration of caspase expression occurs by silencing the caspase genes or overexpressing caspase inhibitors providing tumors with the ability to avoid receptor-mediated apoptosis (45, 46). A final tactic to evade apoptosis is to become desensitized to cell death mediators through tumor cell-immune cell contact. For example, tumors prevent cell-mediated cytotoxicity by granzyme inhibition and/or mutations in TNF-related apoptosis inducing ligand (TRAIL) receptors (47, 48).

The escape phase of cancer immunoediting results from the anti-tumor pressures of the immune system on the tumor. Understanding the multiple factors involved in the development of tumor immune evasion provides opportunities for therapeutic intervention. The next section describes the anti-tumor immune response to prevent tumor development.

Natural anti-tumor immune response: Cancer immunoediting- Elimination and Equilibrium

In the early 1900's Paul Ehrlich proposed that the immune system recognizes and eliminates malignant cells prior to tumor formation (49). A failure of the immune system to clear these potential tumor cells results in malignant growth (5, 49). In the 1950's Burnett and Thomas formally termed this model as "the cancer immune surveillance hypothesis", and implicated the adaptive immune response as responsible for the surveillance and clearance of the potential cancer cells (5, 50). In the 1970's, the introduction of the nude mouse model allowed for the cancer immune surveillance hypothesis to be tested because athymic nude mice partially lack an adaptive immunity due to the loss of T cells (5, 51). Unfortunately, tumor growth was comparable between the nude mice and wild type mice treated with methylcholanthrene (MCA), instead of the anticipated accelerated tumor growth in the nude mice lacking T cells (51). These results suggested the immune system is not involved in tumor development, and convinced scientists to disregard the cancer immune surveillance hypothesis.

The failure of experimental evidence for the cancer immune surveillance hypothesis was in part due to the gaps in knowledge of the immune system and mouse genetics. Nude mice are athymic and are devoid of mature T cells, but still produce NK cells and a small population of T cells, which could be responsible for cancer immune surveillance (52). The other caveat is that different strains of mice have different susceptibility to MCA induced tumors, and the experiment used CBA mice, which have high susceptibility to high concentrations of MCA (5, 53). The high susceptibility to MCA induced tumors may have produced a strong stimulation for tumorigenesis to overcome the lack of mature T cells involved in cancer immune surveillance.

The introduction of mice deficient for recombinase activating gene (RAG) 2, which lack T and B cells due to the inability to rearrange the lymphocyte receptors, provided a better model to test the cancer immune surveillance hypothesis (54). The $RAG2^{-/-}$ mice had earlier and increased tumor development in response to MCA tumor induction compared to wild type mice (55). These data provide the experimental evidence supporting the cancer immune surveillance hypothesis, which later become known as the elimination phase of cancer immunoediting.

Elimination phase

The generation of mice, genetically deficient for specific immune cell populations and key immune modulators, facilitated the characterization of immune cells and components responsible for tumor elimination. Mice deficient in CD8 T cells, NK cells, NKT cells, and γδ T cells have increased susceptibility to carcinogen induced or transplantable tumors (5). Recent data using $RAG2^{-/-}\gamma c^{-/-}$ mice, suggest that inflammatory macrophages also play a role in immunosurveillance (55). In addition to cell populations, mice deficient in key modulators of the immune response, IFNγ and perforin, have increased carcinogen induced tumor development, indicating that cells producing these factors are required for tumor elimination (5). While these data suggest a role for the immune system in eliminating early tumors cells, they do not provide cause and effect data to support the presence of an elimination phase.

The experiments described above indirectly support the elimination phase immune interaction with the tumor without provide direct tumor evidence of the process. A recent study identified a nascent protein from a RAG2$^{-/-}$ MCA derived cell line, which provides the first tumor derived evidence for the elimination phase. The study identified a mutant spectrin-β2 peptide from the RAG2$^{-/-}$ MCA derived cell line through cancer exosome sequencing, and *in silico* methods to identify potential MHCI binding peptides (56). An anti-tumor immune response against the mutant spectrin-β2 peptide was only elicited when RAG2$^{-/-}$ mice were challenged with the tumor cells as indicated by the identification of tetramer positive CD8^{+} T cells (56). WT mice challenged with the tumor cells did not elicit an anti-tumor immune response signifying the loss of expression of the mutant spectrin-β2 peptide after exposure to immune surveillance (56). These data support the existence of the elimination phase through immune-tumor interactions.

Equilibrium phase

Equilibrium is a stalemate between the tumors desire to proliferate/metastasize and the immune system's capacity to keep this growth in check. The existence of an equilibrium phase is supported by a series of experiments in which MCA induced tumors were initially established in RAG2$^{-/-}$γc$^{-/-}$ mice, RAG2$^{-/-}$ mice and WT mice, and subsequently transplanted into WT mice (55, 57). Sixty percent of tumors transplanted from RAG2$^{-/-}$γc$^{-/-}$ mice into WT mice were rejected (55). Analogous rejection rates from tumors initially established in RAG2$^{-/-}$ or WT mice were 40% and 0% respectively (55, 57). These data indicate the immune-tumor interaction influences the immunogenicity of a tumor, and the interaction controls tumor outgrowth.

Direct evidence for an equilibrium phase is the ability to detect an anti-tumor immune response. Antibodies targeting tumor antigens are detected in cancer patients. For example, antibodies against cancer-testis antigens e.g. NY-ESO-1, are commonly present in the serum of cancer patients, indicating a humoral response to the tumor (58). Similarly, anti-idiotype antibodies are found in patients with lymphoma and, if administered as a therapy, keep lymphoma patients in remission for 4 to 10 years despite detectable disease (59). Antigen specific T cell responses have been detected in murine models and cancer patients (60, 61). For example, in a murine model of acute myeloid leukemia a cytotoxic lymphocyte response is identified with evidence of detectable disease (62). Similarly, mixed lymphocyte reactions using patient lymphocytes and tumor biopsies resulted in tumor cell death indicating an antigen specific T cell response despite detectable disease in the patients (61). The presence of an anti-tumor response with detectable disease supports the existence of an equilibrium state.

Another piece of evidence supporting the existence of the equilibrium state is the increased frequency of malignant tumor development in organ transplant recipients. Solid organ recipients are at higher risk for the development of lymphomas and non-melanoma skin cancers due to inhibition of the adaptive immune response from immunosuppressive drug therapy (63). These findings suggest the donor’s immune system was maintaining the tumor at equilibrium, and that the initiation of immunosuppression altered the homeostatic balance in favor of tumor growth. The role of the immune system in defining this balance is confirmed by case reports that demonstrate tumor resolution upon discontinuation of immunosuppressive therapy (64).

A final argument for the existence of the equilibrium phase is tumor relapse after therapeutic intervention (65). Anti-tumor therapy induces an anti-tumor immune response reducing the tumor and redefining the tumor microenvironment. The remaining tumor is now selected as less immunogenic resulting in tumor outgrowth. For example, a metastatic melanoma patient receiving anti-programmed cell death 1 (PD-1) mAb resulted in a partial response that lasted for approximately one year (66). The tumor expressed programmed cell death ligand 1 (PD-LI/B7-H1) suggesting the anti-tumor response was through blockade of PD-1-B7-H1 interactions, which inhibit T cell responses (66). The relapse could be due to changes in the tumor microenvironment in response to therapy. Another example of therapeutic equilibrium is a case involving a patient with acute myelogenous leukemia who relapsed fifteen years after a complete response to therapy suggesting equilibrium between immune-tumor had been breached (67). These examples support a therapeutic equilibrium state.

The understanding of the interaction between the cancer cell and the immune system identifies targets to enhance the anti-tumor immune response, and suppress the pro-tumor immune response. Anti-tumor immune responses, such as tumor associated antigens and antibody responses, found in patients provide feasible strategies to elicit strong anti-tumor immunity to overcome the immunosuppressive microenvironment. These strategies in combination with therapeutics driven to reverse the immunosuppressive microenvironment, create an opportunity to diminish pro-tumor immunity, and enhance anti-tumor immunity. Cancer immunotherapy combines the understanding of pro- and anti-tumor immunity to provide a therapeutic approach to enhance anti-tumor immunity while overcoming pro-tumor immunity.

Cancer immunotherapy

William Coley is the “Father of Cancer immunotherapy.” His administration of “Coley’s Toxins,” a mixture of bacterial toxins, to cancer patients in the 1890s, induced an inflammatory response resulting in tumor rejection in some patients (68). At the time, Dr. Coley did not appreciate the cellular and molecular components required to elicit an effective anti-tumor immune response. However, it is now appreciated that the initiation of an antigen specific immune response requires careful selection of a tumor antigen(s) capable of inducing T cell specific immunity (Figure 2). Selection of appropriate tumor antigens is complicated by the fact that normal cells express most tumor antigens, and the tumors ability to alter expression of proteins due to the increased level of genomic instability (69).

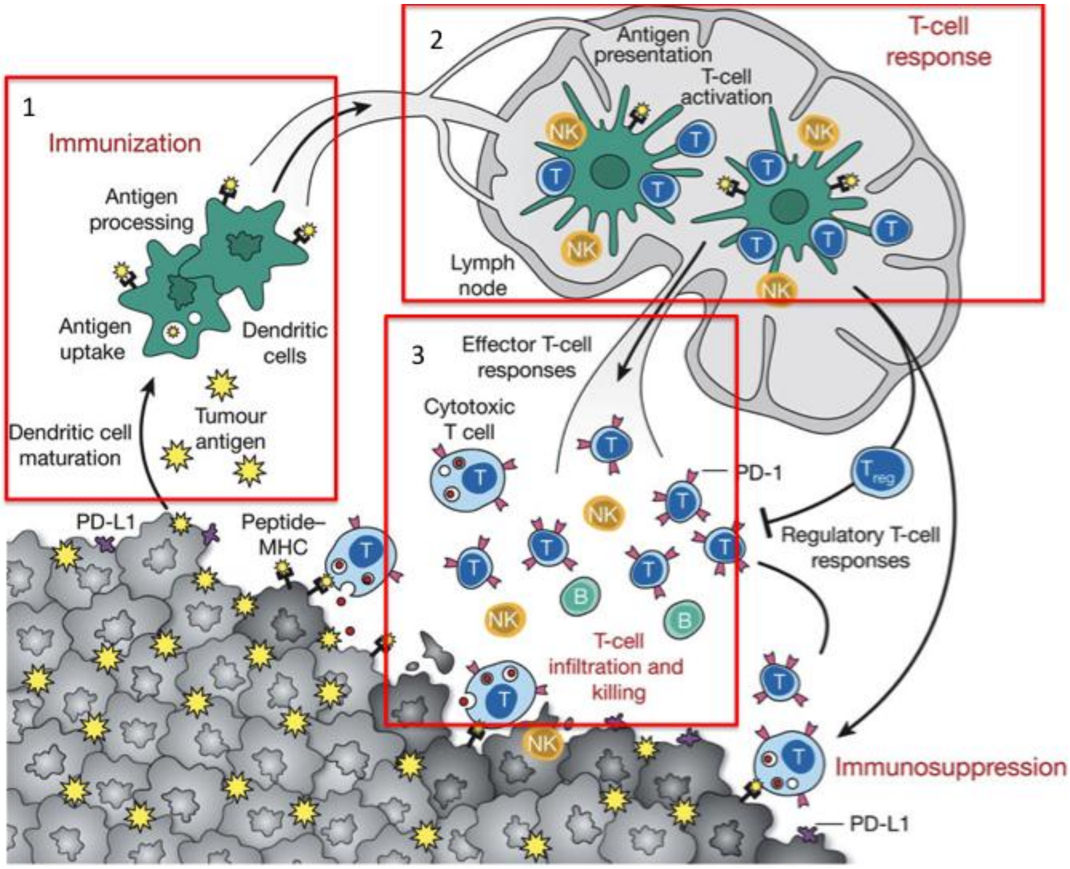

Figure 2: A demonstration of the immune response elicited by active immunity using vaccines and immune stimulants.

(1) Immunization with antigen to stimulate antigen uptake by dendritic cells. (2) Dendritic cells traffic to the tumor draining lymph node to present the antigen to tumor reactive T cells. (3) Tumor reactive T cells exit the lymph node and infiltrate the tumor. Tumor infiltrating T cells result in tumor killing. Reproduced with kind permission from Nature Publishing Group from (69).

In addition to the antigen, a DC is required to present the antigen, but the DC must receive an appropriate maturation signal to propagate an immunological response instead of a tolerogenic response (69).

The functional direction of antigen primed DCs can be manipulated by adjuvants and/or other immune stimulants that provide the appropriate maturation signal for the DC to produce an immunogenic response (69). The immunogenic DC presents the antigen to the T cell in the lymph node, generating cytotoxic lymphocytes capable of mediating tumor regression (69). This understanding of the generation of an antigen specific immune response drives the development of cancer immunotherapies.

Active cancer immunotherapy

Active immunization is the direct stimulation of a patients' immune system with one or more antigens +/- adjuvant(s) designed to elicit a cellular and/or humoral response to the vaccine (69). Cancer vaccines rely on the administration of a peptide tumor antigen, tumor cells or tumor destruction in the presence of an adjuvant to generate an anti-tumor immune response (69). Historically, active vaccination for cancer has largely failed due to lack of an effective adjuvant, and the lack of antigen knowledge to target a single T cell clone, however several recent studies suggest therapeutic utility in selected patients (69).

Peptide vaccines are a universal approach to cancer immunotherapy, but major limitations to peptide vaccines include incomplete data to generate potential antigens, and the lack of knowledge about immunogenicity of the chosen antigen (69). A recent clinical trial demonstrated the use of longer peptides for human papillomavirus (HPV) to generate $CD4^+$ and $CD8^+$ T cell responses (70). Patients receiving the vaccine with longer peptides saw improvements in $CD4^+$ and $CD8^+$ T cell responses corresponding to patients with complete remission at three months (70). Another method to improve the globe effect of peptide vaccination is to treat patients with allogeneic tumor cells modified to secrete granulocyte macrophage colony stimulating factor (GM-CSF) providing DC stimulation (71). Prostate cancer patients receiving the GM-CSF secreting tumor cells in combination with anti-cytotoxic lymphocyte antigen 4 (CTLA-4/CD152) mAb (Ipilimumab) had a decrease in serum levels of prostate specific antigen (71). A final method to improve peptide vaccines is to include multiple tumor associated antigens in the vaccine. Recently, clinical trials in renal cancer patients demonstrated the efficacy of including multiple tumor associated antigens which resulted in improved disease control and reductions in Tregs (72). These current studies provide new optimism in peptide vaccination as a potential cancer therapy.

Passive immunotherapy, adoptive T cell transfer and monoclonal antibodies

Passive anti-tumor immunity is the administration of molecules and/or cells that can directly mediate tumor regression and/or support and the intrinsic anti-tumor immune response (69). Adoptive T cell transfer and monoclonal antibody infusion are some of the most common examples of passive immunotherapy.

In the 1980's, Dr. Steven Rosenberg developed the *in vitro* culture system to generate lymphokine-activated cells (LAKs), and demonstrated in a murine model that adoptive transfer of LAKs results in tumor resolution (60, 73). A few years later, Rosenberg and colleagues found that tumor infiltrating lymphocytes are more effective at killing tumors (74). After successful demonstrations in murine models, adoptive cell therapy success was seen in melanoma patients indicated by increased necrosis and lymphocyte infiltration (75, 76). Limitation to adoptive T cell therapy is the requirement to expand antigen specific T cells *ex vivo*, and the variability of the antigen specific immune response between patients.

Improvements to adoptive T cell therapy to avoid the limitations on the number of antigen specific T cells is to use gene therapy. The first genetically engineered T cells were transfected with T cell receptors specific for tumor antigens (77). Melanoma patients receiving the genetically engineered lymphocytes had tumor regression (77). Further improvement in genetically engineered T cells is the introduction of chimeric antigen receptors (CAR). CARs are single chain antibodies linked to CD3, CD28, and CD137 signaling domains that bypass antigen recognition through T cell receptor (TCR)-MHC complexes, and allow direct T cell stimulation by recognition of the tumor antigen on the tumor cell (78). In early clinical trials, adverse outcomes occurred after administration of CARs to patients, and these outcomes have led to the improvement of these CARs, such as anti-tag CARs and Bi-CARs (78-80). Anti-tag CARs recognize a specific molecule conjugated to a therapeutic antibody, which allows for the therapy to accommodate to the changes in the expression of tumor antigens (79). Bi-CARs have the signaling domains, the TCR and costimulatory motifs, split between two CARs, that recognize two different tumor antigens (80). These improvements to the CAR structure to minimize off target effects should to result in an improved cancer immunotherapy by reducing interactions with healthy cells.

Monoclonal antibody (mAb) therapy provides a targeted therapy that is directed against a molecule that is overexpressed by tumors. The success of mAb therapy is due to the multiple modes of action including the blocking of growth factors or receptors to inhibit tumor growth; and/or the engagement of the immune system through complement mediated cellular cytotoxicity (CDC) or antibody mediated cellular cytotoxicity (ADCC) (81). The availability of mAb therapy is due to the introduction of hybridoma technology (82). The introduction of mice expressing human immunoglobulin has revolutionized the production of humanized monoclonal antibodies by generating completely human monoclonal antibodies with improved clinical efficacy (83).

Tumor antigens recognized by monoclonal antibody therapy include growth receptors and factors, hematopoietic or cell surface differentiation markers, molecules associated with angiogenesis, and tumor stromal antigens, that creates a targeted therapeutic approach (84). Human epidermal growth factor receptor 2 $(HER2)^+$ breast cancer is treated with Herceptin® (trastuzumab) a mAb directed against HER2 (85). The mechanism of action for Herceptin® is blocking growth factors, preventing receptor dimerization, receptor endocytosis, and/or the induction of ADCC or CDC (85). B cell lymphoma is treated with Rituxan® (rituximab), a mAb directed against CD20, which depletes malignant B cells through the engagement of ADCC, CDC, and/or the programmed cell death through Fcγ receptors (FcγR) (86-88). Anti-angiogenic treatment is achieved with Avastin® (bevacizumab), an anti-VEGF mAb therapy (89, 90).

Monoclonal antibodies directed to immune signaling molecules are another approach to alter the anti-tumor immune response. Immune signaling mAbs are directed against co-stimulatory molecules to provide a stimulatory or block an inhibitory signal to an immune cell (Figure 3) (84). For example, recently the FDA approved the use of anti-

CD152 (CTLA-4) mAb (Ipilimumab) for the treatment of metastatic melanoma, which helps to exemplify the use of this class of monoclonal antibodies (91).

The function of immune signaling targeted antibodies is attributed to interactions with the Fab of the antibody without consideration of the potential contribution of the Fc-FcγR interactions. Recently, publications have highlighted the importance of the Fc-FcγR interaction in the function of anti-CD40 mAbs. These reports indicated engagement with FcγRIIB was required for therapeutic efficacy (92, 93). FcγRIV engagement is important for the function of anti-CD152 mAb resulting in the depletion of Tregs at the tumor site (94). These data highlight the functional contribution of Fc-FcγRs in the efficacy of immune modulatory monoclonal antibodies, and support the careful consideration of antibody isotype to provide the best therapeutic outcome.

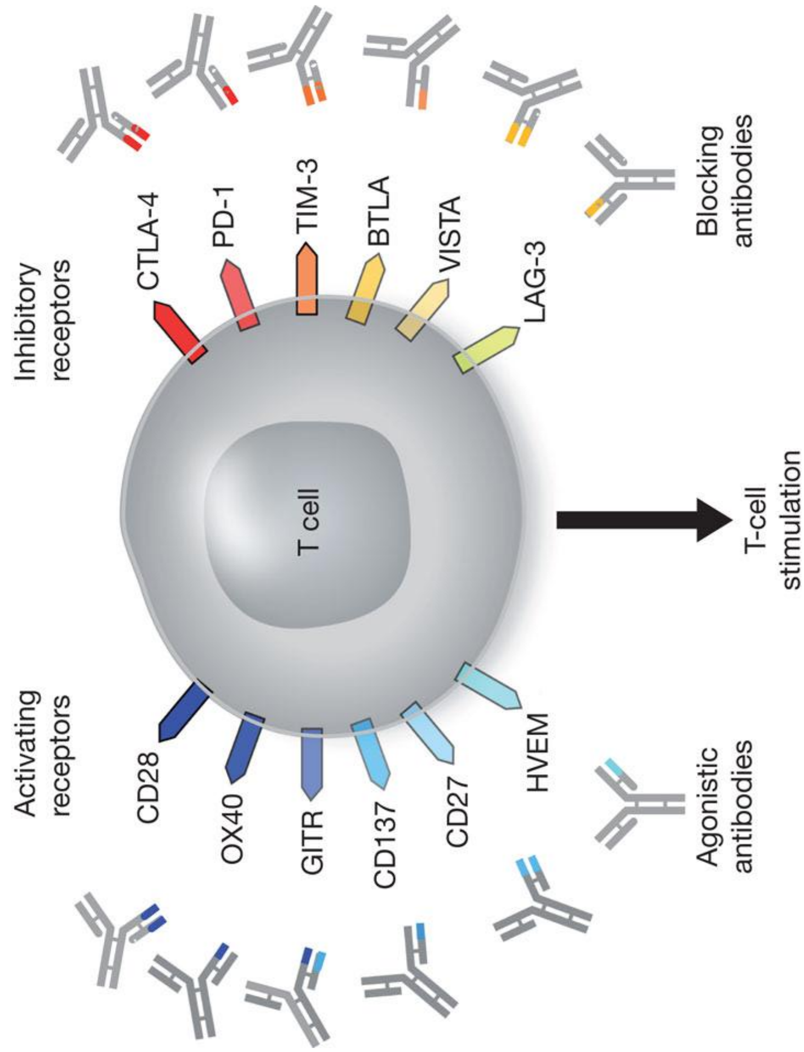

Figure 3: Immune signaling monoclonal antibody targets to modulate the T cell anti-tumor mediated immunity.

The left side of the image depicts activating receptors that are stimulated by agonistic antibodies. The right side of the image depicts inhibitory receptors that are blocked by antibodies. Reproduced with kind permission of the Nature publishing group from reference (69).

Thesis outline

As indicated in the background, the role of Fc-FcγR interactions in monoclonal antibody therapy is important in the field of targeted antibody therapy. However, there are few studies of the contributions of Fc-FcγR interactions in the function of immune modulatory antibody therapy. There is a large body of work dedicated to understanding the molecular interactions, the functional interactions, and methods to modify the Fc-FcγR interactions to improve the efficacy of monoclonal antibody therapy. The few examples characterizing the contribution of Fc-FcγR interactions in immune modulatory antibody therapy have found unanticipated FcγR interactions and target cell populations in the function of these antibodies. For example, in anti-CD152 mAb therapy, the original hypothesis for the function of this antibody was to inhibit the immune regulatory function of CD152 on effector T cells, thus releasing an immunological brake (95). A study determining the contribution of Fc-FcγR interactions in anti-CD152 mAb anti-tumor function found that the therapeutic mechanism included the depletion of Tregs through Fc-FcγRIV interactions locally at the tumor (94). Counterintuitively, Fc-FcγRIIB is important in the function of anti-CD40 mAb (92, 93). These two examples highlight the unique Fc-FcγR interactions for each immune modulatory targeted mAb therapy, and underscore the need to apply effort to understanding the contribution of these interactions to the function of each antibody. This study involves characterizing the Fc-FcγR interactions in the function of the anti-tumor immunity elicited by anti-CD137 mAb therapy. The function of the Fc-FcγR interaction is the recruitment of innate immune cells to elicit ADCC, release of pro-inflammatory cytokines, fixation of complement, phagocytosis of tumor cells, and/or antigen presentation (81, 96). Based on these described inflammatory responses in tumor antigen targeted monoclonal antibody therapy, we hypothesized that Fc interactions through activating FcγRs would enhance the anti-tumor immune response elicited by anti-CD137 mAb. If our hypothesis were correct, then engagement of the inhibitory FcγR, FcγRIIB, would dampen anti-CD137 mAb mediated anti-tumor activity while engagement of the canonical activating FcγRs would augment its therapeutic efficacy (96). In order to test this hypothesis, we employed an anti-CD137 mAb sensitive EL4E7 tumor model in mice deficient for FcγRs (97). Our work will help to improve the understanding of anti-CD137 mAb therapy in the anti-tumor response. Importantly, this work will help to define potential isotype selections in order to enhance the therapeutic value in the clinic.

We performed the following specific aims:

Aim I: Identify the FcγR responsible for the modulation of the anti-CD137 mAb anti-tumor immune response using the EL4E7 tumor model in FcγR deficient mice

The goal of this aim is to identify the FcγR(s) involved in the regulation of the anti-tumor immune response elicited by anti-CD137 mAb. Established EL4E7 tumors are susceptible to anti-CD137 mAb treatment in wild type mice resulting in measurable differences in tumor growth (97). Utilizing the EL4E7 tumor model, the contribution of individual FcγRs in the function of anti-CD137 mAb is assessed by challenging FcγRIIB$^{-/-}$, FcγRIII$^{-/-}$, and FcγRIV$^{-/-}$ with EL4E7 tumor cells. The therapeutic effect of anti-CD137 mAb treatment will result in measurable differences in tumor growth compared to wild type mice. Successful completion of this aim will define the FcγR(s) involved in modulating the anti-tumor immune response elicited by anti-CD137 mAb.

Aim II: Evaluate the mechanisms through which FcγRs mediate the therapeutic efficacy of anti-CD137 mAbs

This aim will identify a cell population(s) in wild type and FcγR deficient mice responsible for the modulation of the anti-tumor immune response after anti-CD137 mAb therapy. A kinetic experiment of the anti-tumor immune response will be used to identify potential $Fc\gamma R^{+}$ cell populations modulating the anti-tumor response of anti-CD137 mAb therapy. After the identification of the relevant $Fc\gamma R^{+}$ cell population(s), the candidate cell(s) will be characterized by phenotype and function to understand potential mechanisms for the differences in the anti-tumor response between WT and $Fc\gamma R^{-/-}$ mice. Successful completion of this aim will define the cell population(s) responsible for the modulation of the anti-tumor immunity elicited by anti-CD137 mAb.

Potential study limitations

There are four limitations of our study design. First, this study will not include the potential role of Fc interactions with complement or other non-canonical Fc receptors (98, 99). This decision is based on data indicating Fc-FcγR interactions are of primary import in monoclonal antibody therapy mediated antitumor immunity (100). Based on the study design, the study cannot define the role of non-canonical Fc receptors on the function of anti-CD137 mAb therapy.

Second, based on the fact that the study uses knockout mice, it is possible that some of our observations are related to non-specific differences in tumor viability/growth between the genetically modified and wild type mice. We controlled for potential non-specific differences by using tumors with similar sizes at the onset of treatment. However, our study results must be interpreted in this context.

Third, the study uses a single Fc isotype of antibody that is rat IgG2a. The decision to use rat IgG2a mAbs was based on the fact that all prior mouse tumor studies using anti-CD137 mAb, employed antibodies of rat origin. Therefore, use of rat mAbs enabled us to place these data in context with published studies.

Finally, the model employed cannot be directly translated into human therapeutics due to differences in human and mouse biology. First, there are differences in human and mouse FcγR expression, and the identified receptors in murine models do not always have a human homologue (101). Likewise, not all human FcγRs have murine homologues (101). The expression pattern in humans and mice for CD137 is different. For example, only a small subset of mouse B cells express CD137, whereas human B cells are able to express CD137 following appropriate stimulation (102, 103). These differences in expression will likely influence antibody function.

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Chapter 2- Literature Reviews

T cell activation

T cell activation requires two signals: 1. antigenic stimulation through MHC-TCR interactions, and 2. co signaling, providing a signal to enhance or reduce the T cell response (104). In fact, antigen signaling in the absence of co signaling results in T cell tolerance (105). In the 1980s, CD28 was identified as a prototypic second signal and interactions of CD28 with B7 receptors were shown to induce T cell proliferation (106-108). T cell stimulation was inhibited by CTLA-4 (CD152), a homologue of CD28, indicating costimulation regulates the T cell response (109). These important papers provided an early understanding of the potential for co-signaling to enhance or inhibit T cell function and opened a gateway for further study.

In the last two decades, there have been great strides made in the understanding of potential receptor and ligand interactions that are able to modulate an immune response (110). The current understanding of costimulation in T cell activation includes a variety of receptor families beyond the B7 family and the tumor necrosis factor receptor (TNFR) family (Figure 4) (105). Our work involves a classic costimulatory molecule, CD137 (4-1BB), which has, paradoxically, been shown to mediate antitumor immunity and ameliorate select autoimmune diseases (111). An important attribute of CD137 stimulation is the eradication of tumors after administration of mAbs, which is the focus of this review (112).

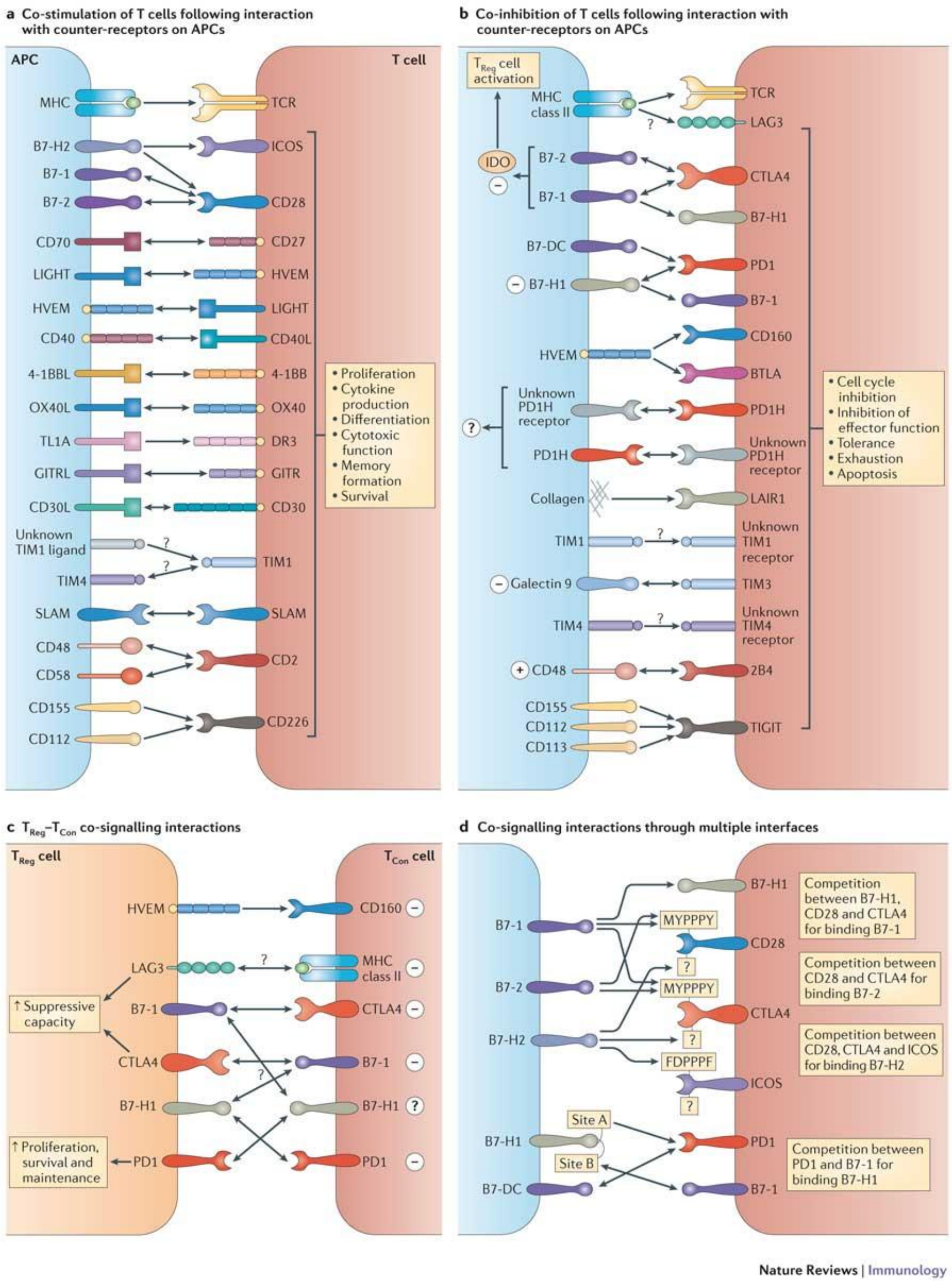


Figure 4: Co-signaling molecules expressed by T cells and antigen presenting cells **A.** A depiction of the molecules providing a stimulatory signal. **B.** Depiction of the molecules providing an inhibitory signal. **C.** Treg cell mediated suppression of T cells. **D.** Cross talk between co-stimulatory molecules. Reproduced with kind permission of Nature Publishing Group from (105).

CD137 genetic structure

In 1989, CD137 was cloned from mRNA of human cytotoxic lymphocytes (113). The gene for CD137 is located on chromosome 1p36 in humans, and chromosome four in mice, which is the location of other TNFR family members (114-116). Ten exons encode the two transcripts of CD137, a membrane bound and soluble form (115, 117). The first two exons contain the signaling sequence, with a partial transcript for one of the extracellular domains in exon two (115). Exons three through seven encode for the rest of the extracellular domains (115). The eighth exon encodes for the transmembrane region, and the ninth exon encodes for the cytoplasmic tail (115, 117, 118). The extracellular domains of CD137 consist of cysteine-rich regions, characteristic of TNFR family members (119, 120). Expression of the membrane bound and soluble forms of the protein is regulated by NF-κB and/or AP-1 transcription factors that bind to DNA motifs located in the promoter (115, 117, 118). Combined NF-κB and AP-1 activity is required for the up regulation of CD137, but only NF-κB activity is involved in the production of soluble CD137 (sCD137) (117, 118).

CD137 expression

CD137 is expressed on T cells, NK cells, NKT cells, B cells, macrophages, DCs, mast cells, Tregs, bone marrow cells and non-hematopoietic cells such as endothelial cells, lung epithelium, neurons, placental cells and astrocytes (103, 113, 121-134). Similarly, the ligand for CD137 (CD137L) is also ubiquitously expressed. CD137L is expressed on macrophages, DCs, mast cells, eosinophils, and non-hematopoietic cells such as epithelial cells, endothelial cells, and fibroblasts (135). On the majority of cell populations, CD137 expression is activation induced. However, Tregs, human monocytes and endothelial cells constitutively express basal levels of CD137 (125, 128, 136).

T cells require TCR engagement and costimulation through CD28 to induce CD137 transcription (137-139). CD137 is expressed early in T cell stimulation, with a similar temporal expression to CD69, an early activation marker, but prior to CD44hi, a late marker of T cell activation (140). The CD137 expression is specifically enhanced by IL-2 and IL-4, while the pro-inflammatory cytokines, IFNγ and IL-1β do not effect expression (138). These data indicate that CD137 expression on T cells is inducible and is tightly regulated by the cytokine microenvironment.

CD137 is induced on other lymphoid cells by analogous initial pathways and differing secondary cytokine requirements. For example, B cell receptor (BCR) engagement induces CD137 expression on B cells (103, 121). However, unlike the requirements for CD137 expression on T cells, CD40 and IFNγ promote and IL-4, IL-10, and IL-21 inhibit expression on B cells (103). Similarly, CD137 expression on innate immune cells, such as NK and mast cells, is induced after engagement of activating Fc receptors, FcγRIII and FcεRI, respectively (124, 130). Finally, growth factor or cytokine signaling induces CD137 expression on non-hematopoietic cells, such as human brain cells and epithelium (129, 132).

CD137 signaling

CD137 signaling involves the collaboration between the canonical and non-canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)/ AKT/protein kinase B signaling pathways (Figure 5) (141-144). Similar to other TNFR receptors, CD137 signaling is mediated through the scaffold proteins, TNF receptor associated factors (TRAF) 1, 2 and 3 (142, 143, 145). TRAF-1 is required for the maximum activation of canonical NF-κB signaling after CD137 stimulation, and prevents non-canonical NF-κB signaling by inhibiting NF-κB inducing kinase (NIK) after TCR stimulation (146). TRAF-1 and leukocyte-specific protein 1 (LSP1) interactions induce MAPK signaling resulting in the activation of extracellular regulated kinase (ERK) (147, 148). TRAF-2 is required for CD137 stimulation of the non-canonical NF-κB pathway, and TRAF-3 regulates TRAF-2 signaling (149). TRAF-3 is ubiquitinated after CD137 signaling by cellular inhibitor of apoptosis protein 1 (cIAP1) resulting in degradation (146). In addition to TRAF binding sites, the cytoplasmic tail of CD137 contains a site for lymphocyte-specific protein kinase (Lck), a protein tyrosine kinase involved in TCR signaling, providing an additional docking site to amplify TCR signaling (150-152).

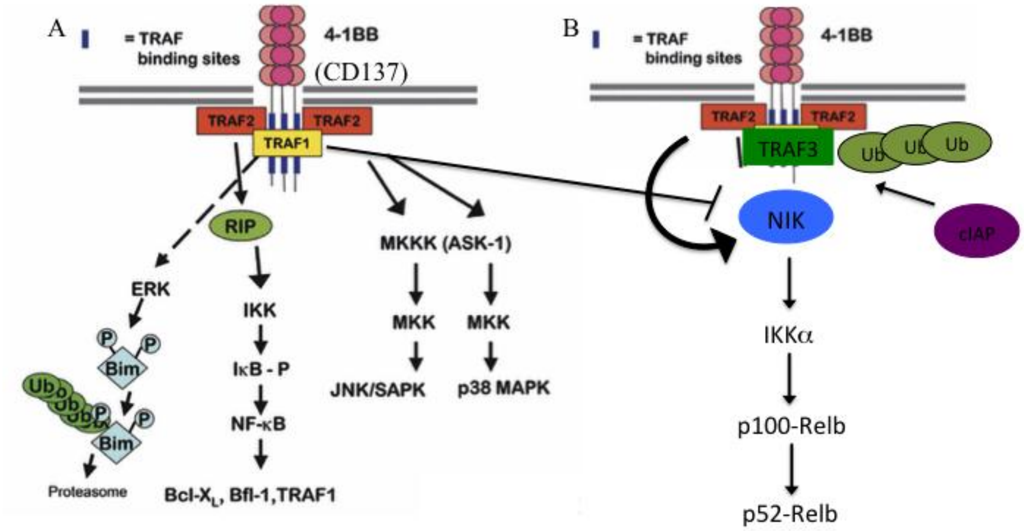


Figure 5: CD137 signaling

A. TRAF1 and TRAF2 mediated induction of canonical NF-κB and MAPK pathways to induce the anti-apoptotic proteins Bcl-xl and Bfl-1, and Erk activation leads to the degradation of the pro-apoptotic protein, Bim, respectively. **B.** TRAF2 mediated induction of the non-canonical NF-κB pathway. TRAF1 and TRAF3 inhibit non-canonical NF-κB signaling by directly inhibiting NIK and by the direct inhibition of TRAF2, respectively. TRAF3 is ubiquitinated by cIAP resulting in TRAF3 degradation. TRAF2 phosphorylates NIK, which phosphorylates IKKα. IKKα phosphorylates p100 tagging p100 for ubiquitination. The final signaling protein is the p52-Relb transcription factor. This image reproduced and adapted with kind permission of John Wiley & Sons A/S from reference (153).

Each of the three signaling pathways activated through CD137 ligation modulate the expression of both pro- and anti-apoptotic proteins responsible for the inhibition of activation induced cell death (AICD), a hallmark of CD137 stimulation. The canonical and non-canonical NF-κB signaling increase the anti-apoptotic proteins, B-cell lymphoma-extra-large (Bcl-xL) and Bfl-1, and down regulate the pro-apoptotic protein B-cell lymphoma 2 interacting mediator of cell death (Bim) (146, 154). MAPK signaling results in ERK activation causing the up regulation of the anti-apoptotic protein, Bcl-xL and the down regulation of the pro-apoptotic protein, Bim (147). PI3K signaling up regulates the anti-apoptotic proteins, cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) and Bcl-xL (144).

CD137 function

CD137 ligation results in different functional outcomes dependent upon the cell population involved in disease progression and/or resolution. In $CD8^+$ T cell mediated immune responses, such as an anti-tumor response, CD137 provides a stimulatory signal leading to tumor eradication (153). In $CD4^+$ T cell mediated immune responses, such as autoimmunity, CD137 stimulation abolishes autoimmune pathologies (153). Additionally, CD137 stimulation on innate immune cells also contributes to the outcome of the T cell response (153). The following discussion describes the contribution of CD137 stimulation to the function of innate and adaptive immune cells.

CD137 stimulation of innate immune cells

CD137 stimulation modulates the function of various innate immune cells such as monocytes, mast cells, NKT cells, NK cells, and DCs. All of these cells are important in shaping an adaptive immune response. This section briefly highlights the effects of

CD137 stimulation on monocytes, NKT cells and mast cells with a fuller description of its impact on NK and DCs, due to the intricate role of these cell populations in shaping T and B cell responses.

Monocytes/macrophages

CD137 stimulation of monocytes/macrophages can enhance inflammatory responses that mediate bacterial clearance and inflammatory diseases; but also, has the capacity to induce suppressive monocytes in autoimmunity. As a result of CD137 engagement, monocytes are activated and produce pro-inflammatory cytokines and have improved antigen presentation capability compared to DCs (136, 155). For example, in *Listeria monocytogenes* infection, CD137 stimulation of monocytes increases liver infiltration, production of reactive oxygen species, and phagocytosis (156). Similarly, in inflammatory diseases, such as type II diabetes, CD137 stimulation induces pro-inflammatory cytokine production in tissue infiltrating macrophages (157). In contrast, anti-CD137 mAb treatment of collagen-induced arthritis induces IDO producing monocytes, which are important for the suppression of antigen-specific $CD4^+$ T cells (158). The dual function of CD137 signaling in monocyte function is a property of CD137 signaling that is only beginning to be understood.

Mast Cells

CD137 stimulation of mast cells enhances their response to allergic reactions and wound healing. Combined mast cell stimulation with CD137 and IgE exacerbates the allergic response by enhancing pro-inflammatory cytokine production (159). Additionally, gene expression analysis of tumor infiltrating mast cells indicate an up regulation of the gene for CD137 and genes involved in angiogenesis. These findings suggest that CD137 stimulation of mast cells may promote tumor growth (160). Finally, mast cells present antigen to $CD8^+$ T cells, a process which induces CD137 expression on mast cells, supporting the development of a cytotoxic T cell response (161).

NKT cells

CD137 stimulation regulates the number of NKT cells, and enhances their cytokine secretion. During homeostatic proliferation, CD137 stimulation suppresses the repopulation of NKT cells indicating a functional role in NKT cell regulation (162). Additionally, NKT cells initiate immune responses by the rapid production of IFNγ or IL-4 skewing towards a Th_1 or Th_2 immune response, respectively (163). CD137 stimulation enhances NKT cell production of IFNγ and IL-4, suggesting CD137 stimulation may enhance either type of immune response (164).

NK cells

The effects of CD137 stimulation of NK cells is somewhat controversial and may be species dependent. In murine cells, CD137 stimulation enhances pro-inflammatory cytokine, specifically IFNγ, and cytotoxicity (164, 165). The IFNγ produced by NK cells enhances the $CD8^+$ T cell response (166). In human NK cells, CD137 stimulation does not enhance production of IFNγ or cytotoxicity (165). The differences in CD137 stimulation in NK biology, between mice and humans, potentially complicate the translation of anti-CD137 mAb therapy concerned with NK function.

DCs

CD137 stimulation of DCs promotes survival and antigen presentation. CD137 induces expression of the anti-apoptotic proteins, Bcl-2, and Bcl-xL (123, 167). In low antigen scenarios, CD137 signal provides optimal stimulation of DCs by enhancing costimulatory molecule expression and cytokine production to promote an antigen specific T cell response (122, 123, 158, 176-178). Importantly, DC maturation is independent of CD137 stimulation indicating CD137 signaling enhances DC function, but is not necessary for DC maturation (167). In fact, when antigen is prevalent, CD137 stimulation does not increase proliferation of antigen specific cells (168).

CD137 stimulation of innate immune cells is dependent upon the immune microenvironment. In most cases, CD137 supports a pro-inflammatory immune response, but induces suppressive cell populations in models of autoimmunity. This dual outcome of CD137 stimulation implies a regulatory role in an immune response dependent upon the pathophysiological condition. Understanding the nuances of CD137 stimulation on all cell types influences the inclusion of agonist antibody therapy to treat disease.

CD137 stimulation adaptive Immune Responses

CD137 in involved in adaptive immune responses by providing stimulation to T cells, regulation of $CD4^+$ T cells, induction of suppressive T cells, and inhibition of the humoral immune response.

T cells

CD137 stimulation of T cells is important for proliferation and cytokine production (155, 169, 170). CD137 mediated MAPK signaling enhances proliferation and cytokine production by effector T cells (141, 171, 172). Additionally, MAPK and PI3K pathways induced by CD137, control proliferation by modulating expression of cell cycle proteins. Both pathways lead to the up regulation of cyclins D2 and D3, which promote the transition from G_0 to G_1 for entry into the cell cycle, and reduce p27, which inhibits the transition to G_1 (171, 172).

A hallmark of CD137 stimulation is the generation of immunological memory. The enhanced survival of T cells through the inhibition of AICD mediated by CD137 signaling is responsible for the enhanced generation of immunological memory (140, 173, 174). Early characterization of CD137 stimulation suggested $CD4^+$ and $CD8^+$ T cell memory is generated, but influenced by antigen levels and cell numbers during the primary exposure (140). In fact, CD137 stimulation results in $CD4^+$ and $CD8^+$ memory T cell proliferation independent of antigenic stimulation, IFNγ, and IL-15, suggesting the importance of the CD137 signal in generating and sustaining a memory T cell population (140, 174). Furthermore, CD137 signaling actively maintains the reservoir of memory $CD8^+$ T cells in the bone marrow in a readily active state (173, 175).

$CD4^+$ T cells are regulated by CD137 stimulation. CD137 stimulation of $CD4^+$ T cells induces anergy (176, 177). In fact, after antigen exposure, $CD4^+$ T cell function is not further enhanced by CD137 stimulation (168). The regulatory role of CD137 stimulation in CD4+ T cell biology is indicated by the hyperresponsiveness of $CD137^{-/-}$ $CD4^+$ T cells to TCR stimulation (178). Interestingly, overexpression of CD137 also enhances $CD4^+$ T cells responses and reduces $CD4^+$ T cell apoptosis (179). Taken together these transgenic models support the anergic state of $CD4^+$ T cells induced by CD137 stimulation.

Finally, CD137 stimulation modulates $CD4^+$ and $CD8^+$ regulatory T cell populations. Similar to effector lymphocytes, CD137 stimulation of Tregs induces proliferation, enhances IFNγ production, and supports Treg mediated suppression (180, 181). Additionally, CD137 signaling induces suppressive $CD8^+$ T cells expressing CD103, a TGF-β1 marker, and CD11c (182-184). The suppressive $CD8^+$ T cells produce IFNγ that induces TGF-β1 or IDO suppressing $CD4^+$ T cells (182-184).

B cells

Similar to NK cells, CD137 expression on B cells is different between mice and humans. Until recently, CD137 was not detected on murine B cells, but recently CD137 expression has been described on a small plasmacytoid dendritic cell antigen 1 (PDCA-1/CD317)$^+$ B cell subset (102). CD137 stimulation of the PDCA-1$^+$ B cells enhances expression of MHCII and costimulatory ligands, but also induces IDO production involved in suppression of lymphocytes (102). Despite the enhanced expression of MHCII and antigen uptake, the CD137 stimulated PDCA-1$^+$ B cells inhibit $CD4^+$ T cell proliferation (102). In contrast to the mouse, human B cells express CD137 (103). Stimulation of CD137 on human B cells induces proliferation, inhibition of AICD, and cytokine secretion (103).

Despite the isolated expression of CD137 on murine B cells, anti-CD137 mAb stimulation reduces humoral responses. Indirectly, interaction of anti-CD137 mAb with $CD8^+$ T cells induces IFNγ, which leads to the depletion of B cells (162, 185, 186). In addition to the generation of IFNγ$^+$CD8$^+$ T cells, the anergic state of $CD4^+$ T cells induced by anti-CD137 mAb is responsible for the inhibition of T-cell dependent humoral responses (177, 185)**.** Finally, anti-CD137 mAb inhibits germinal center formation, required for the generation of antigen-specific antibodies (187). Anti-CD137 mAb mediated inhibition of germinal center formation through stimulation of T cells and follicular dendritic cells, which prevents B cells from interacting with follicular dendritic cells (191).

CD137 stimulation provides support for an adaptive immune response by stimulating T cells and B cells early in the immune response. In contrast, CD137 stimulation provided later in the immune response supports the immune response resolution by stimulating regulatory T cell populations and inhibiting humoral immune responses. This dual function of CD137 stimulation provides a therapeutic target, which can be utilized to modulate an immune response to obtain the desired outcome. This makes the use of anti-CD137 mAb a strong candidate for immunotherapy in many diseases.

CD137 role in the anti-tumor response

The therapeutic potential of CD137 stimulation was first characterized in tumor models. The ability of anti-CD137 mAb treatment to elicit an anti-tumor immune response has been demonstrated in many laboratories and models. Anti-CD137 mAb therapy is mediated through $CD8^+$ T cells, with NK cells and DCs involvement dependent upon the tumor model (112, 188-190). Anti-CD137 mAb stimulates each of these immune cell populations to producing anti-tumor immunity and toxicities, specifically hepatitis. The liver toxicities and alternatives are discussed in the appendix.

CD137 stimulation enhances the anti-tumor $CD8^+$ T cell response by reversing the anergic state of $CD8^+$ T cells induced by suboptimal antigen exposure found in the tumor microenvironment (191). The reversal of anergic anti-tumor $CD8^+$ T cells by CD137 stimulation enhances IFNγ production, tumor specific cytotoxicity, and memory $CD8^+$ T cells; ultimately, resulting in tumor reduction and/or resolution (97, 112, 190, 192). These data indicate the anti-CD137 mAb mediated anti-tumor immune response requires $CD8^+$ T cells.

NK cells are important in the anti-CD137 mAb mediated anti-tumor immune response through either enhanced cytotoxicity and/or the supportive role in the induction of tumor specific $CD8^+$ T cells. For example, CD137 stimulation induces the tumor infiltration of NK cells required for tumor resolution (193, 194). In human NK cells, CD137 stimulation up regulates activating receptors, such as NKG2D and the natural cytotoxicity receptors, which are responsible for the enhanced NK-mediated tumor cell cytotoxicity (193, 194). Additionally, NK cells support the anti-CD137 mAb anti-tumor immune response by producing IFNγ to support tumor specific $CD8^+$ T cells. The depletion of NK cells prior to anti-CD137 mAb stimulation reduces the anti-tumor immune response (112, 166). Additionally, IFNγ production by CD137 stimulated NK cells enhances adoptive T cell transfer therapy through the enhanced tumor specific cytotoxic lymphocytes and IFNγ producing cells (195, 196). These data demonstrate NK cells can provide direct killing of tumor targets and/or cytokine support to the $CD8^+$ T cell response after CD137 stimulation.

CD137 stimulated NK cell production of IFNγ polarizes immune responses through the modulation of DC function. Anti-CD137 mAb induces NK-DC interactions that lead to the polarization to a Th_1 immune response (197). In fact, the NK cells are required for tumor infiltration of DCs, and NK produced IFNγ increases CD137 expression on DCs (196-198). The increase in CD137 expression on DCs provides another target population for anti-CD137 mAb stimulation.

CD137 stimulation of DCs enhances antigen presentation. CD40 stimulation results in DC maturation, and is required for the anti-tumor immunity elicited by anti-CD137 mAb (199, 200). The combination of CD40 and CD137 stimulation of DCs is associated with an increase in the number of tumor infiltrating T cells (199, 200). Granulocyte-macrophage colony-stimulating factor (GM-CSF), a molecule involved in DC maturation, when combined with IL-12 and anti-CD137 mAb therapy enhances the anti-tumor immune response elicited by $CD8^+$ T cells (201, 202). These data underscore the import of DC maturation in combination with anti-CD137 mAb therapy to enhance antigen presentation, and amplify the tumor specific $CD8^+$ T cell response.

In the tumor microenvironment CD137 expression is induced on tumor infiltrating lymphocytes and tumor endothelial cells in hypoxic conditions (127, 203). The expression of CD137 on tumor endothelial cells provides another target for anti-CD137 mAb therapy. Treatment with anti-CD137 mAb stimulates the endothelial cells to up regulate adhesion molecules improving lymphocyte trafficking to the tumor (127). In fact, $CD137^{-/-}$ activated T cells adoptively transferred into $CD137^{+/+}$mice migrate to the tumor after anti-CD137 mAb treatment because of the increased expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule 1 (VCAM-1) (127). These data indicate the effects of anti-CD137 mAb on the tumor endothelial cells contribute to the enhanced anti-CD137 mAb elicited anti-tumor immune response.

CD137 Summary

The original description of CD137 on T cells lead to the characterization of CD137 in T cell biology. $CD4^+$ and $CD8^+$ T cells stimulated by CD137 have different activities such as induction of anergic $CD4^+$ T cells, and reversal of anergic $CD8^+$ T cells (97, 177). Further characterization of CD137 stimulation on T cells weakens the division between the outcome of CD137 stimulation on $CD4^+$ and $CD8^+$ T cells. For example, anti-CD137 mAb stimulation expands suppressive $CD8^+$ T cells in autoimmune models, whereas anti-CD137 mAb therapy in a melanoma model induces cytotoxic $CD4^+$ T cells (158, 204).

As discussed above there are many potential targets for anti-CD137 mAb therapy. Based on the stimulatory effects on $CD8^+$ T cells, reversal of anergy, inhibition of AICD, and induction of immunological $CD8^+$ memory, these stimulatory properties make CD137 an ideal candidate for cancer immunotherapy. Beyond $CD8^+$ T cells, NK cells and DCs are other potential cell populations that modulate the anti-CD137 mAb mediated anti-tumor immune response. NK cells and DCs express FcγRs, which may interact with the Fc of anti-CD137 mAb, and this interaction could potentially alter CD137 signaling. The role of anti-CD137 mAb is attributed to the Fab interactions with CD137, but potential improvements to this therapy may lie in an improved understanding of the Fc portion contribution to the anti-CD137 mAb elicited anti-tumor immune response.

Fcγ receptors and Immunoglobulin

FcγRs and immunoglobulin G (IgG) link the innate and adaptive immune systems. The link between the two phases of the immune response is through Fc-FcγR interactions, which makes monoclonal antibody therapy a strategy to modulate an immune response. Monoclonal antibody therapy is best known for its targeted Fab-antigen interactions, it also has the potential to provide a divergent set of interactions through FcR stimulation and/or blockade. The following contains a discussion about human and mouse IgG and FcγRs covering IgG genetics and structure, FcγR genetics and structure, and concludes with FcγR expression and function.

Immunoglobulin genes and somatic rearrangement

The immunoglobulin genes are located on three chromosomes for each chain: light chain κ, light chain λ, and the heavy chain (205). The three chromosomes in mice are 6, 16, and 12; and in humans are 2, 22, and 14 (205).

The variable regions of immunoglobulin consist of multiple genes classified as variable (V), diversity (D), and joining (J), which goes through RAG mediated gene rearrangement, independent of antigen recognition, to produce IgM natural antibodies (Figure 6)(206). VDJ recombination occurs somatically in developing B cells, and is the first process in antibody development (206). Upon antigen encounter, the activated B cell begins to enhance antibody specificity by somatic hypermutation, and effector function through class switching (207).

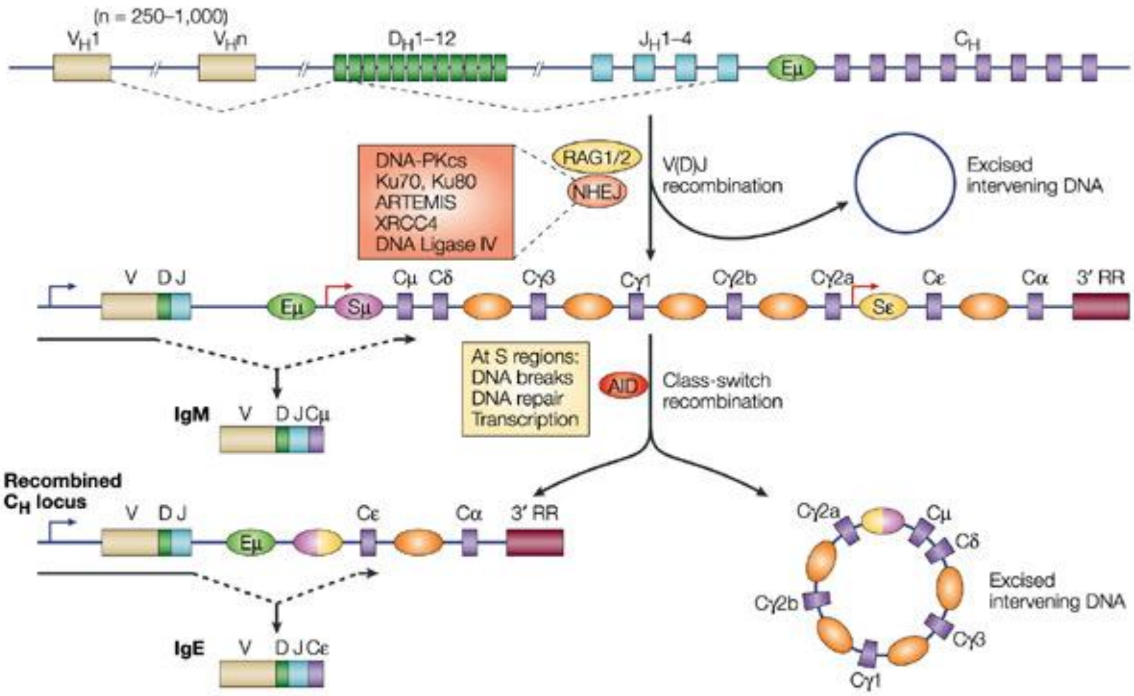


Figure 6: VDJ recombination and Class switching of the immunoglobulin heavy chain

From the top variable (V), diversity (D), and joining (J) are combined through recombination events initiated by DNA double strand breaks by RAG1/2 followed by non-homologous end joining (NHEJ) DNA repair mechanism called VDJ recombination. Class switching involves the induction of AID resulting in DS DNA breaks at a signal (S) region near the constant (C) gene of interest. The break at the μ gene and the desired C gene is excised and the remaining gene is repaired through the DNA repair system. Reproduced by kind permission of Nature Publishing Group from (206).

Antigen activated B cells produce activation induced cytodine deaminase (AID), which is the enzyme involved in somatic hypermutation and class switching (207). In somatic hypermutation, AID deaminates cytosine causing DNA double-strand breaks in the V gene (208). These DNA breaks lead to point mutations inserted during homologous recombination with a highly error prone polymerase, such as pol ζ (208). These point mutations in the V gene generate high affinity antibodies. Activated B cells will go through many rounds of somatic hypermutation to produce the highest affinity antibody, this process is termed "affinity maturation" (209).

Class switching is the change in the constant region from the μ chain of the natural antibody, to other constant genes, such as the γ chain (227). There are two steps in class switching: 1. the production of transcripts by cytokine inducible (I) promoters that form RNA-DNA hybrids; and 2. AID deamination directed by the RNA-DNA hybrids, which create double strand breaks that loop out the undesired constant genes (Figure 6)(210). Class switching is initiated by CD40 ligation, B cell activating factor (BAFF) or a proliferation inducing ligand (APRIL) stimulation, BCR cross-linking, or Toll-like receptor (TLR) stimulation, all of which result in NF-κB signaling (210). Cytokines induce specific transcription factors that engage with different I promoters resulting in an isotype switch for the desired effector function (Figure 6) (210). For example, class switching to IgG allows for the engagement of immune cells expressing FcγRs, which promotes superoxide production, phagocytosis, endocytosis, ADCC, CDC, and antigen presentation.

IgG structure and function

An antibody consists of two regions: the Fab region, which contains the variable domains that recognize the antigen, and the Fc region, which is the constant portion that is able to interact with FcγRs, complement receptors, the neonatal FcγR, and some noncanonical receptors such as dendritic cell-specific ICAM-3-grabbing non-integrin 1 (DC-SIGN, CD209) (Figure 7) (211, 212). The constant genes consisting of the hinge, constant heavy (CH)2, and CH3 are the Fc domain of the antibody, which are expressed as four sub-isotypes (Figure 6). The sub-isotypes in humans and mice contain: IgG1, IgG2, IgG3, and IgG4; and IgG1, IgG2a, IgG2b, and IgG3, respectively (213, 214).

Fc-FcγR interactions occur at a general consensus amino acid sequence in the CH_2 domain (215). The binding of the sub-isotypes to different FcγRs is determined by differences in key amino acids and glycosylation. High affinity IgG in human and mouse contain the following residues Leu^{234}, Leu^{235}, Gly^{236}, Gly^{237}, Pro^{238}, and Ser^{239} in the CH2 domain (215). Substitutions and deletions of these key amino acids modulate the binding of different IgG isotypes. For example, high affinity binding of human IgG1 and IgG3 to FcγRI is due to Leu^{234}, Leu^{235}, and Gly^{237} in the CH2 domain, whereas Val^{234}, Ala^{235}, and the lack of Gly^{237} inhibit binding of IgG2 to FcγRI (215, 216).

The range FcγR interactions of an IgG isotype is modulated by interactions with amino acids outside of the primary amino acid sequence located in the CH2 domain (Tables 1 and 2). For example, the interaction of Pro^{329} and Ala^{327} is important for IgG1-FcγRIIIA interactions as Ala substitution or Gly substitution, respectively, reduced binding (217). Furthermore, improved binding of IgG1 with FcγRIIA, FcγRIIB, and

FcγRIIIA is achieved with Ala substitutions at Thr^{256} and Lys^{290} in the CH2 domain (217).

The N-linked glycosylation at Asn^{297} in the CH2 domain is important for interactions with activating FcγRs and IgG stability (Figure 7) (218, 219). The minimal glycosylation is a hexasaccharide consisting of acetylglucosamine and mannose, $GlcNAc_2Man_3GlcNAc_2$, which is further modified by the addition of sugar moieties (218). In fact, loss of the N-linked glycosylation at Asn^{297} diminishes antibody function (220). The loss of Fc-FcγRIIB requires further deglycosylation by reducing the mannose residues at the core (219). Interestingly, defucosylation of Asn^{297} enhances IgG1-FcγRIIIA (221). These data indicate changes in sugar moieties influence Fc-FcγR interactions, and ultimately the effector response.

Table 1: Human FcγRs binding to Human IgG isotypes

	IgG1	IgG2	IgG3	IgG4	Reference
FcγRI	+	-	+	+	(215, 222)
FcγRIIA^{H131}	+	+	+	+	(222)
FcγRIIA^{R131}	+	+	+	+	(222-226)
FcγRIIB	+	-	+	+	(222, 224-227)
FcγRIIC	+	-	+	+	(222, 227)
FcγRIIIA^{F158}	+	-	+	-	(222)
FcγRIIIA^{V158}	+	+	+	+	(222)
FcγRIIIA^{NA1}	+	-	+	-	(222, 228)
FcγRIIIA^{NA2}	+	-	+	-	(222, 228)
FcγRIIIA^{SH}	+	-	+	-	(222, 228)

Table 2: Mouse FcγRs binding to mouse IgG isotypes

	IgG1	IgG2a	IgG2b	IgG3	Reference
FcγRI	-	+	-	-	(229, 230)
FcγRIIB	+	+	+	-	(223, 230, 231)
FcγRIII	+	+	+	-	(223, 230, 231)
FcγRIV	-	+	+	-	(223, 230, 232)

+ indicates binding, - indicates no binding

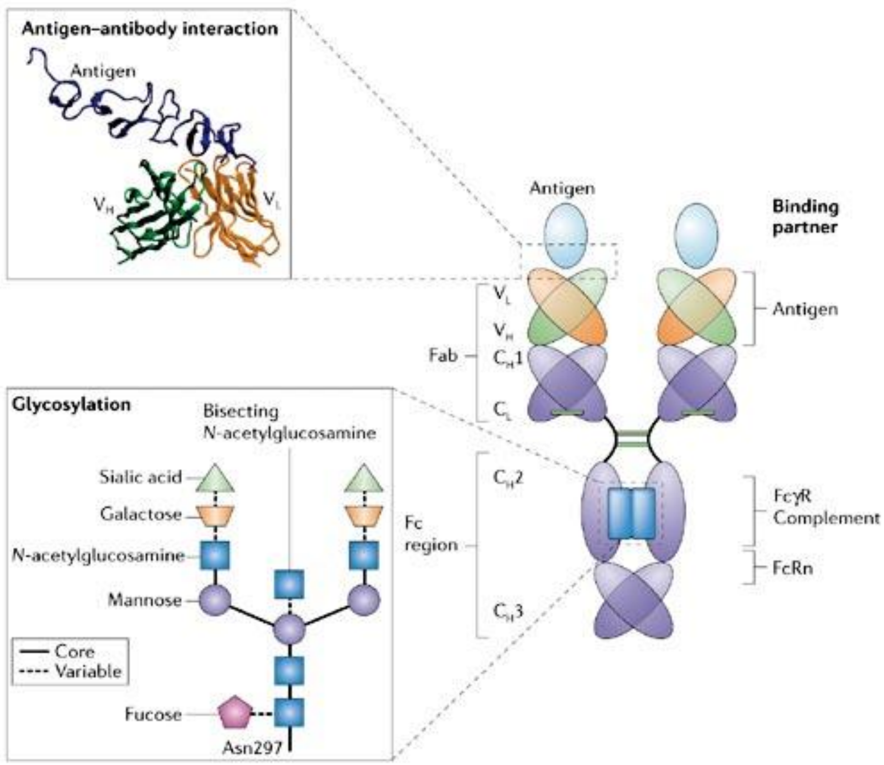


Figure 7: Antibody structure
Fab region includes the variable domain and the first constant heavy chain domain (C_H1). The variable domain recognizes the antigen. The Fc region contains the hinge, CH2, and CH3 domains. This region contains an N-linked glycosylation that modulates the interaction with Fc receptors. Complement also binds the Fc region. Reproduced by kind permission of Nature Publishing Group from (233).

FcγR Structure

The α-chain of FcγRs includes extracellular immunoglobulin domains bridged together through disulfide bonds formed between the cysteine residues, and the domains contain sites for N-glycosylation (234-236). Each α-chain contains a transmembrane region and a cytoplasmic tail, which lack a signaling motif, except for FcγRII (Figure 8) (234-236). The activating FcγRs co-localize with the Fcγ chain, which contains immunoreceptor tyrosine-based activation motifs (ITAM) (234-236). The human FcγRs are located on chromosome 1, and consist of FcγRI, FcγRII, and FcγRIII, with each member containing different variants (237). The murine FcγRs are located on chromosome 1 and chromosome 3, and consist of FcγRI, FcγRIIB, FcγRIII, and FcγRIV (236, 238-240).

Human FcγRs genetics and structure

FcγRI

There are three variants of *human FcγRI:* FcγRIA, FcγRIB, and FcγRIC encoded by six exons (241, 242). The first two exons contain the signaling sequence, exons three to five code for the three extracellular domains, and the final exon codes for the transmembrane and cytoplasmic tail (242). The extracellular region consists of three extracellular immunoglobulin domains with two N-glycosylation sites in the first domain, three N-glycosylation sites in the second domain, and a single N-glycosylation site in the third domain (243).

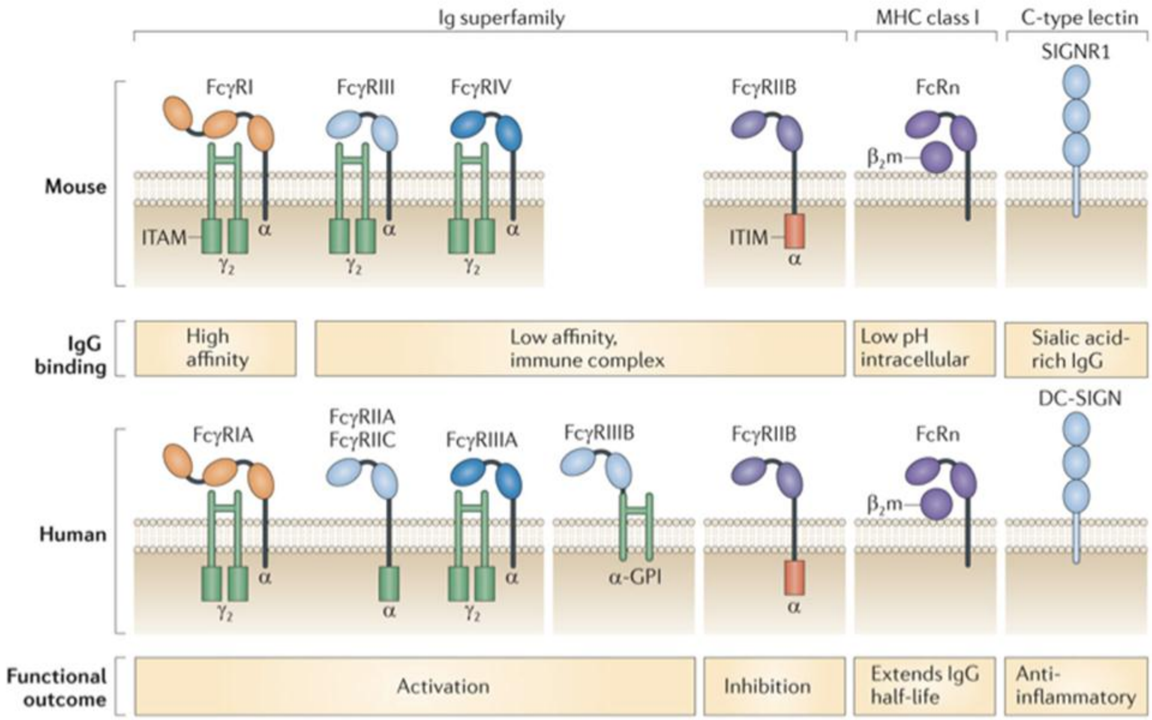


Figure 8: Mouse and Human families of FcγRs
The top row is a depiction of mouse FcγRs, which includes the Fcγ chain that contains the ITAM motif for the activation signal. FcγRIIB contains an ITIM motif, which induces an inhibitory signal. The bottom row is a depiction of human FcγRs. Human FcγRIIA and FcγRIIC contain ITAMs in the cytoplasmic tail. Similar to mouse, FcγRIIB contains an ITIM. Additionally, the neonatal receptor (FcRn) and non-canonical lectin receptors are included in this diagram. Reproduced and modified with kind permission of Nature Publishing Group from (99).

The glycosylation of the extracellular domains allow for the correct protein conformation (220). A premature stop codon in the exon for the third domain results in the production of the soluble forms of FcγRI, FcγRIB and FcγRIC (242, 244). FcγRI is the only receptor with three extracellular immunoglobulin domains. While the third domain was originally described as being involved in the high affinity binding to Fc, recent crystal structures suggests the interaction with Fc does not involve the third domain (243, 245). In fact, the high affinity binding is located in the FG loop of the first extracellular domain, which is lacking the Val^{172} that is found in the low affinity receptors (Figure 9) (243).

FcγRII

Human FcγRII consists of three variants FcγRIIA, FcγRIIB, and FcγRIIC, which are encoded by eight exons (246, 247). The first two exons contain the signaling sequences, exons three and four code for the two extracellular immunoglobulin domains, exon five codes the transmembrane, and the final exons code for the cytoplasmic tail (247). The glycosylation of the immunoglobulin domains are different between FcγRIIA and FcγRIIB. FcγRIIA contains two N-linked glycosylation sites in the extracellular IgG domains, whereas FcγRIIB contains three N-link glycosylation sites, which are important for protein structure (225, 246). FcγRII contain signaling motifs in the cytoplasmic tail. Specifically, FcγRIIA and FcγRIIC contain ITAMs whereas FcγRIIB has an immunoreceptor tyrosine-based inhibition motif (ITIM) (246, 248). Similar to FcγRI, a soluble form of FcγRIIA is produced by platelets (249, 250).

Fc-FcγRII interactions occur in the BC, C'E, and FG loops of the second immunoglobulin domain with eight amino acids in C'E loop determining specificity for different IgG isotypes (Table 1, Table 2, and Figure 9) (251, 252). For example, differences in Fc binding affinity between FcγRIIA and FcγRIIB, are due to two amino acids, Leu^{132} in the C'E loop and Phe^{160} in the FG loop (Figure 9) (252, 253). Additionally, binding of Fc is modulated by a single polymorphism at position 131 in human FcγRIIA (224, 254). The polymorphism at 131 encodes for either an arginine or histidine altering the confirmation of the dimerized receptor (224, 254). High affinity binding occurs with the arginine expression due to the conformational change allowing for interaction with two molecules of Fc compared to the single molecule interaction with the histidine expression (224, 254).

FcγRIII

Human FcγRIII has two variants, FcγRIIIA and FcγRIIIB, encoded by five exons (255, 256). Exons one and two contain the signaling sequence, exon three and exon four contain the extracellular domains, and exon five contains the transmembrane and cytoplasmic tail (247). FcγRIIIA contains a transmembrane region that interacts with the Fcγ chain or CD3ζ; whereas FcγRIIIB has a phosphatidylinositol linkage on the cell membrane (235, 257). The cytoplasmic tail is determined by the amino acid sequence from position 203 (255, 256). Phosphatidylinositol glycan-anchoring occurs with the following amino acid sequence; Ser^{203}, Thr, Tyr, Asn, Gly, Ala, Asp and Lys, and the transmembrane occurs with the following amino acid sequence; Phe^{203}, Ile, Leu, and Val (255, 256).

There are polymorphisms of both FcγRIIIA and FcγRIIIB. FcγRIIIA has a polymorphism, for Val or Phe at position 158 (258). $\text{Fc}\gamma\text{RIIIA}^{158F}$ has reduced binding affinity for IgG1, IgG3, and IgG4 than $\text{Fc}\gamma\text{RIIIA}^{158V}$ (258). FcγRIIIB has three different isoforms: neutrophil antigen (NA) 1, NA2, and SH (259, 260). There are four amino acid differences in FcγRIIIB NA1 and NA2, which cause changes in the number of N-glycosylation sites in the extracellular domains, four sites in NA1, and six sites in NA2 (261). The SH isoform is a duplication of NA2 with a single amino acid change from Ala to Asp at position sixty (260).

Fc interacts with FcγRIII in its second extracellular domain, and the Fc interactions are enhanced by glycosylation. Both FcγRIIIA and FcγRIIIB bind Fc in the second domain with specific residues found in the CC' and FG loops (Figure 9) (262, 263). The glycosylation of FcγRIIIA on different cell types, e.g. NK or monocytes, modulates the binding affinity for IgG (264). For example, highly mannosylated side chains of FcγRIIIA on NK cells can bind monomeric IgG with higher affinity than FcγRIIIA on monocytes, which do not have high mannose glycosylation (264). Similarly, FcγRIIIB on neutrophils also contain highly mannosylated side chains, allowing for IgG binding and lectin interactions (265).

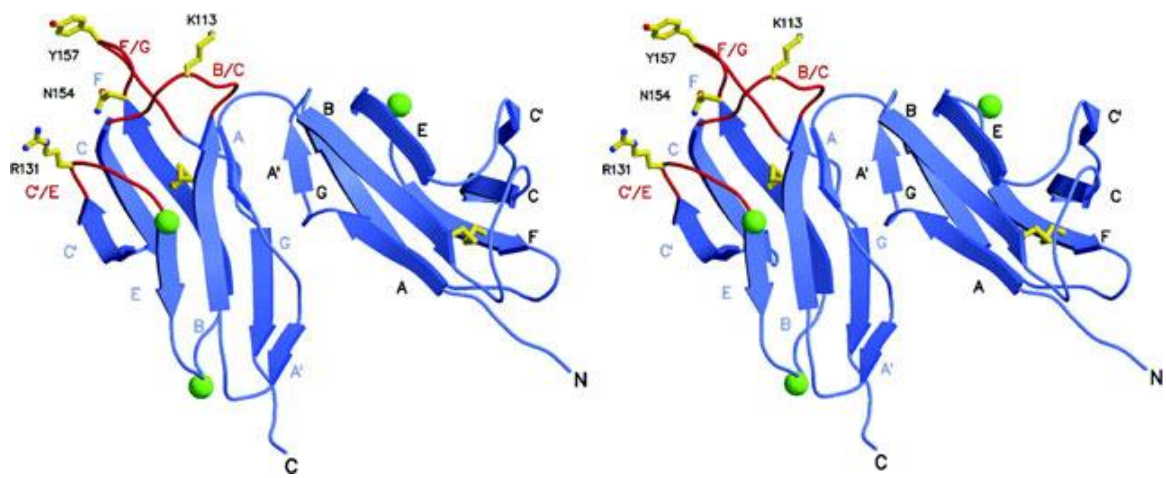


Figure 9: Immunoglobulin domain ribbon structure for sFcγRIIB
A ribbon structure of the two immunoglobulin domains from sFcγRIIB. The red loops depict potential binding locations with IgG. The green balls indicate the site for glycosylation. Reproduced by kind permission of Nature Publishing Group from (266).

Mouse FcγR genetics and structure

Murine FcγRI contains six exons: exons one and two encode the signal peptide, exons three through five encode the extracellular domains, and exon six encodes the transmembrane and cytoplasmic tail (238). Similar to human FcγRI, murine FcγRI contains three extracellular immunoglobulin domains containing five N-glycosylation sites (229). There are conserved amino acids in the second domain of both human and mouse FcγRI that may be involved the high affinity Fc binding (229, 243).

Murine FcγRII is expressed as four variants: FcγRIIB1, FcγRIIB1', FcγRIIB2, and FcγRIIB3 (267-269). The gene for FcγRII contains ten exons: exons one through four code for the 5' untranslated region and leader sequence, exons five and six code for the extracellular domains and transmembrane, and exons seven through ten code the cytoplasmic tail and 3' untranslated region (267). The eighth exon contains an alternative splicing site producing FcγRIIB1 and FcγRIIB2 (267). FcγRIIB1' lacks amino acids in the first domain of the cytoplasmic tail (268). FcγRIIB3, a soluble form of FcγRII, lacks the transmembrane and first intracellular domain (269).

Murine FcγRIII consists of a single transcript, encoded by five exons, the first two exons contains signaling peptides, exons three through five encode the extracellular domains, transmembrane, and cytoplasmic tail (239, 270). The murine FcγRIII gene does not encode for phosphatidylinositol linkage variant, like FcγRIIIB found in humans (239, 270).

Murine FcγRIV contains only one transcript that is encoded by five exons: exons one and two contain the signal peptide, and exons three through five encode for the extracellular domains and cytoplasmic tail (271).

Despite the variability between human and murine FcγRs, some homology does exist. First, the structure of FcγRI is similar between the species, with both having homologous sequences and three extracellular domains (243). Second, both species have only a single inhibitory receptor, FcγRIIB, which contains an ITIM signaling motif in the cytoplasmic tail (246). Finally, murine FcγRIV and human FcγRIIIA share 65% homology in the extracellular domain and a conserved transmembrane sequence (271). Based on these similarities between the human and mouse FcγRs, preclinical models in the mouse provide experimental support to justify Fc modifications for the treatment of different types of human disease.

FcγR Expression

FcγRs are widely expressed on leukocytes, and most leukocytes express multiple FcγR types. In humans, FcγRI is expressed on monocytes and DCs (272, 273). Human FcγRIIA is expressed on basophils, neutrophils, monocytes, and platelets; whereas FcγRIIB is expressed on neutrophils, monocytes, myeloid DCs, and B cells (246, 274-277). Human FcγRIIIA is expressed on monocytes and NK cells, while FcγRIIIB is only expressed on neutrophils (257).

Mouse FcγRI is expressed on resting macrophages, IFNγ stimulated macrophages, mast cells, bone marrow derived DCs, and lymph node DCs (278). Mouse FcγRIIB1 is expressed on lymphocytes, FcγRIIB1' is expressed on myeloid cells and lymphocytes, FcγRIIB2 is expressed on myeloid cells, and the soluble form, FcγRIIB3 is secreted by macrophages (267-269). Mouse FcγRIII is expressed on mast cells, basophils, neutrophils, dendritic cells, NK cells, and macrophages (101, 279, 280). Mouse FcγRIV is expressed on Ly6C^{lo} monocytes, macrophages, and neutrophils (281, 282).

Similar to immunoglobulin class switching, the expression of FcγRs is influenced by cytokine stimulation. IFNγ is the main cytokine involved in modulating the expression of all FcγRs and the Fcγ chain (231, 273, 283, 284). The promoters of the FcγRs have DNA elements that are responsive to IFNγ induced transcription. For example, signal transducers and activators of transcription (STAT)1, a transcription factor induced by IFNγ signaling, enhances expression of FcγRI (273). Upstream of the promoter for FcγRIIB are two IFNγ response elements, which modulate the expression of the receptor (244). In murine macrophages, IFNγ up regulates the mRNA expression of FcγRIII, and down regulates the expression of FcγRIIB (231).

Other cytokines are also able to modulate the expression of FcγRs. For example, IL-4 induces expression of FcγRIII on mouse mast cells through STAT6 (279). Similarly, IL-10 increases expression of FcγRI expression on human dendritic cells (285, 286). Furthermore, TNFα stimulation of monocytes decreases FcγRIIB expression, but if the TNFα stimulated monocytes are exposed to IL-10, this results in up regulation of FcγRIIA and FcγRIIB resulting in alternatively activated macrophages (285, 286). Finally, TGF-β reduces expression of FcγRs on myeloid cells through a reduction in Fcγ chain production (287). Based on these data, it is evident that the cytokine microenvironment can manipulate the FcγR expression pattern on cells, which alters the immune response.

Inhibitory FcγR function

FcγRIIB contains an ITIM signaling motif that inhibits ITAM signaling, inhibits mitogenic signaling and induces B cell apoptosis (248). The general signaling cascade induced by FcγRIIB stimulation is the activation of SH2 domain-containing inositol 5'-phosphatase (SHIP) leading to the hydrolysis of phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P_3) into phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2), which inhibits the recruitment of other kinases involved ITAM signaling(288). FcγRIIB signaling negatively regulates mitogenic signaling through receptor tyrosine kinases (RTK) (289). SHIP1 activation by FcγRIIB signaling abrogates Akt (protein kinase B) phosphorylation preventing PI3K signaling (289). The inhibition of PI3K signaling stops cyclin production induced by mitogenic signals (289). Finally, apoptosis is induced after the crosslinking of two FcγRIIB receptors by an immune complex (288). The crosslinking of the receptors activates JUN N-terminal kinase (JNK) apoptosis pathway (288).

FcγRIIB signaling induces cellular changes that modulate the immune response. For example, FcγRIIB stimulation reduces the expression of L-selectin on neutrophils thus limiting cellular adhesion at the inflammation site (290). FcγRIIB stimulation of DCs alters the ability to propagate an antigen specific immune response by inhibiting maturation and reversing TLR4 stimulation (291, 292). The outcome of the altered DC is the inhibition of T cell proliferation, induction of Tregs, and an increase in IL-13 producing T cells (291, 292). Overall ligation of FcγRIIB results in the dampening of an immune response by inhibiting ITAM signaling and the production of a pro-inflammatory response.

Activating FcγR Function

FcγR mediated effector functions include superoxide production, degranulation, cellular cytotoxicity, cytokine production, phagocytosis, and antigen presentation. These effector functions are mediated by the phosphorylation of the ITAMs either located in the cytoplasmic tail or the associated Fcγ chain (96). The phosphorylation of the ITAMs induces spleen tyrosine kinase (Syk) phosphorylation leading to signaling through the PI3K and protein kinase C (PKC) pathways (96). The following is the description of the effector functions of activating FcγRs contributing to a pro-inflammatory response.

Superoxide production

Exposure of peripheral blood to heat aggregated IgG results in the release of superoxide in the absence of phagocytosis (293). This early observation connected superoxide production to FcγR stimulation. FcγR stimulation induces PI3K signaling, which interacts with p40 (phox) linking it to nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity producing reactive oxygen species (294). The production of superoxide by neutrophils and macrophages is important for the clearance of bacterial pathogens (295). In fact, most activating receptors will induce superoxide production in macrophages and neutrophils after ligation with immune complexes (285, 294, 296, 297).

In addition to bacterial clearance, reactive oxygen species modulate T cell populations. For example, reactive oxygen species inhibit phagosomal acidification in

DCs, enhancing the quality of peptide presented to T cells, which maintains the T cell response (295). In contrast, reactive oxygen species can induce Tregs, which utilize reactive oxygen species to mediate immunosuppression (295). The production of reactive oxygen species by phagocytes can provide stimulation to the anti-tumor immune response, but may also support the immunosuppressive tumor microenvironment.

Degranulation and Cellular cytotoxicity

FcγR engagement on neutrophils and NK cells results in degranulation (96). In neutrophils, cross-linking of phosphatidylinositol-linked FcγRIIIB stimulation induces exocytosis of granules (298, 299). In NK cells, FcγRIII engagement induces degranulation through PI3K signaling (300, 301). In fact, the cytoplasmic tail of FcγRIIIA contains a phosphorylation site for PKC, which inhibits signaling through the PI3K pathway when phosphorylated (301). S100A4 prevents phosphorylation at the PKC site in the FcγRIIIA cytoplasmic tail allowing for PI3K signaling and enhanced degranulation (301).

Opsonization of pathogens or tumor cells results in the engagement of FcγRs on NK cells and macrophages leading to ADCC (285, 288, 302). In this setting, low affinity FcγRs interact with aggregated Fc on the surface of opsonized tumor cells mediating tumor death through granule release or phagocytosis (81). This mechanism is important to the therapeutic efficacy of targeted monoclonal antibody therapies.

Cytokine production and other immune mediators

Engagement of activating FcγRs induces pro-inflammatory cytokine production. Macrophages and monocytes produce M-CSF, macrophage inhibitory cytokine-1, LSP1, macrophage inflammatory protein 2, TNFα, IL-2, and IL-1R antagonist in response to FcγR stimulation (303-305). Additional stimulation by accessory receptors enhances the production of FcγR induced cytokines. For example, FcγR stimulated production of TNFα in monocytes is enhanced through CD8α signaling (304). FcγRIIA, which contains ITAMs in the cytoplasmic tail, induces IL-2 production that is enhanced by interactions with the Fcγ chain (305).

FcγR ligation up regulates expression of adhesion molecules sustaining immune cells at the site of inflammation. For example FcγRIIA and FcγRIIIA stimulation induces the expression CD11b/CD18 adhesion molecules (290). The enhanced expression of adhesion molecules provides opportunities for FcγR engagement. Mac-1 sustains cell adhesion, spreading, and migration allowing for enhanced FcγRIIA interactions with immune complexes (302). The FcγR signal creates a feedback loop to enhance a pro-inflammatory immune response.

Endocytosis and Phagocytosis

FcγR mediated endocytosis involves internalization into clathrin coated pits, vesicle formation, delivery to the endosome, and finally fusion to lysosomes (306). Endocytosis is mediated independently of ITAM signaling indicated by FcγRI mediated endocytosis in the absence of phosphorylation (307, 308). The α-chain of FcγRI interacts with periplakin, a protein involved in cellular junctions (333, 334). The interaction with periplakin enhances FcγRI ligand binding, endocytosis, and antigen presentation (309, 310). The interaction with periplakin may explain the high affinity for Fc.

FcγR mediated phagocytosis is described by four steps: receptor-ligand binding, pseudopod extension, internalization, and lysosomal fusion (311). The FcγR α-chain is needed for the initiation of phagocytosis by mediating actin polymerization for pseudopod extension, but for particulate internalization ITAM signaling is required (311, 312). The FcγR mediated ITAM signaling activates PI3K signaling, which reduces the concentration phosphoinositides aggregated at the phagosome allowing vesicle formation (313, 314). FcγRIIA mediated phagocytosis is faster than FcγRI due to direct interactions of ITAMs with Syk instead of with hemopoietic cell kinase (Hck), an intermediate tyrosine kinase (315).

Antigen Presentation

DCs express both activating and inhibitory FcγRs, which mediated endocytosis leading to antigen presentation. The endocytosis of immune complexes through activating FcγRs induces maturation, whereas antigen uptake through FcγRIIB maintains DCs in an immature state by down regulating CD40 and CD86 (316, 317). For example, FcγRI mediated endocytosis stimulates a $CD4^+$ or $CD8^+$ T cell response by directing antigen to MHCII or MHCI receptors, respectively (318). Furthermore, DC maturation by FcγRIIA signaling reduces FcγRIIB expression (319). Interestingly, FcγRIIA mediated maturation induces IL-10 production without influencing IL-12 (340). The induction of IL-10 suggests a potential negative regulation by an activating FcγR. (320).

As anticipated, FcγRIIB mediated endocytosis maintains peripheral tolerance by inhibiting the cross presentation of self-antigens through activating FcγRs (321). The inhibition of cross-presented self-antigen reduces the $CD8^+$ T cell and Th1 responses (321). Additionally, FcγRIIB mediated endocytosis by DCs can induce humoral responses to support an inflammatory response (322).

Antigen presentation is able to regulate antigen specific immune responses, and FcγRs mediate the endocytosis and antigen processing to elicit the response. DCs are key immune cells involved in antigen presentation, and have been utilized in cancer immunotherapy to induce an anti-tumor immune response.

Activating FcγRs regulating inflammatory responses

An emerging concept about activating FcγRs is the involvement in the regulation of inflammatory responses. FcγRIII mediates the regulation of an inflammatory response through the induction of cytokines. The FcγRIII mediated regulation of inflammatory macrophages occurs through the suppression of IL-12, and the induction of IL-10 (323-325). IL-12 is suppressed by the disruption of PU.1-c-Rel complex transcriptional activation at the p40 promoter after FcγRIII (323). IL-10 is induced by FcγRIII mediated PI3K signaling (325).

A potential mechanism for the switch to an anti-inflammatory response through FcγRIII is the initiation of inhibitory ITAM (ITAMi) signaling by associated phosphatases with the receptor (326). Phosphatases involved in FcγRIIB signaling, SHIP and Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1), associate with activating FcγRs, which can activate the phosphatases reducing activating FcγR signal

(327-329). For example, FcγRIII associated SHP-1, is able to reduce endocytosis and phagocytosis independently of FcγRIIB signaling (330). Additionally, intravenous immunoglobulin (IVIG) stimulation of FcγRIII or FcγRIIA induces SHP-1 mediated inhibition of inflammation (331-333).

FcγR biology Summary

The family of FcγRs consists of activating and inhibitory receptors that modulate an immune response. The gene structure for these receptors allows for many different transcripts to be produced. These various transcripts provide an array of receptors to mediate the various effector functions. The role of the inhibitory receptor, FcγRIIB, is to provide an immunological brake to counteract many cellular processes. Specifically, FcγRIIB suppresses the signal from the activating FcγR to limit the effector function.

Engagement of activating FcγRs results in effector functions dictated by the cellular population expressing the receptor, and the microenvironment. For example, in the tumor microenvironment, FcγR engagement by NK cells induces cellular activation resulting in tumor cell death through ADCC, whereas engagement of FcγRs in mast cells may induce cytokine production to promote angiogenesis. These different biological outcomes underscore the necessity to understand the details of the Fc-FcγR interactions to make appropriate isotype selections for monoclonal antibody therapy, to achieve the desired outcome.

Chapter 3- Methods

Mice, cell lines and therapeutic antibodies

C57BL/6 and B6.129P2-*Fcgr3*^{tm1Sjv}/SjvJ (FcγRIII$^{-/-}$) were purchased from Jackson Laboratory. Fcer1g (FcRγ$^{-/-}$) and Fcgr2b (FcγRIIB$^{-/-}$) were purchased from Taconic Farms. CD137$^{-/-}$ and FcγRIV$^{-/-}$ mice were generous gifts from Dr. Lieping Chen and Dr. Jeffery Ravetch, respectively. Breeding colonies for FcγRIII$^{-/-}$, FcγRIV$^{-/-}$, and CD137$^{-/-}$ were maintained in the animal facility at the University of Maryland School of Medicine. Female mice from eight to twelve weeks of age were used in the experiments. The Institutional Animal Care and Use Committee (IACUC) at the University of Maryland, School of Medicine approved all experiments.

The EL4E7 cell line, a gift from Dr. Chen, was passaged *in vivo* four times to generate a cell line for the tumor experiments. B16-F10 cell line was a gift from Dr. Paul Antony. MC38 cell line was a gift from Dr. Suzanne Ostrand-Rosenberg. All cells were maintained in complete RPMI 1640 medium supplemented with 10% FBS (Atlantic biologicals), 1% L-glutamine (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 1% HEPES buffer (Corning). B16-F10 medium additionally supplemented with 1% non-essential amino acids (Invitrogen). MC38 medium additionally supplemented with $5x10^{-5}$ M β-mercaptoethanol (Sigma).

Rat anti-mouse CD137 mAb clone 2A (IgG2a), was a gift from Dr. Lieping Chen, and rat anti-mouse CD137 mAb clone 3H3 (IgG2a), was a gift from Dr. Robert Mittler. Both antibodies were purified from hybridoma supernatant using affinity chromatography on a protein G sepharose column (GE Healthcare). Antibodies were verified to have <1 IU/ml of endotoxin using the Limulus Amebocyte Lysate (LAL) Test (Charles Rivers Laboratories). Rat IgG control was purchased from Sigma or Innovative Research. Antibodies against CD8β FITC (ebioH35-17.2), CD103 PE (2E7), CD40 APC (1C10), CD80 APC (16-10A1), CD86 APC (GL1), CD137L (TKS-1) PE, TCRβ FI (H57-597), F4/80 PE (BM8), Armenian hamster IgG PE, rat IgG2a PE, rat IgG2a Alexa Fluor 700, and rat IgG2a APC were purchased from eBioscience. Antibodies against CD11c PercP-Cy5.5 (HL3), NK1.1 APC (PK136), Ly-6C APC (AL-21), Ly-6G Alexa Fluor 700 (1A8), CD11b FI (M1/70), Gr-1 APC (RB6-8C5), hamster IgG PercP-Cy5.5, rat IgM APC and mouse IgG APC were purchased from BD Biosciences. Antibodies against MHCII/I-A^b APC (AF6-120.1), CD137 biotin (17B5), Syrian hamster biotin, and TruStain fcX were purchased from Biolegend. Anti-polyhistidine PE was purchased from R&D systems. Recombinant mouse CD137L containing a His tag was purchased from R&D systems. Anti-Bim were purchased from Cell signaling. Secondary antibodies anti-Rat IgG FITC and anti-Rabbit IgG Cy5 were purchased from Jackson Immunoresearch. Data were acquired using an LSRII instrument (BD Biosciences) and were analyzed using FlowJo software (Tree Star).

Tumor growth models

Mice were inoculated subcutaneously with $8x10^6$ EL4E7 tumor cells in the flank. Tumor volume was determined by measuring the width (w) and length (l) of the tumor and using the following formula: $(w^2 x\ l) \times 0.5$.

Day 7 treatment model: Mice bearing tumors with a length of at least 5mm on Day 7 were randomized into two groups receiving 100μg of either Rat IgG or 2A by intraperitoneal injection on Day 7 and 10.

Day 10 treatment model: Mice bearing tumors with a length of at least 8mm on Day 10 were randomized into two groups receiving 100μg of either Rat IgG or 2A by intraperitoneal injection on Day 10 and 13.

Tumor growth was monitored until mice appeared moribund or the diameter of the tumors reached 25 mm, the mice were sacrificed, and this was recorded as the date of death for survival studies. Mice with complete tumor resolution were rechallenged, at least 125 days after primary tumor resolution, with EL4E7 and B16-F10 tumor cells. Tumor growth was monitored until the combined tumor volume reached approximately 20% of the mouse's total body weight.

To evaluate the immune cell kinetics, the Day 7 treatment model was utilized. Two or three mice per group were euthanized on Day 7, Day 12, Day 16, and Day 21 and spleens were processed. Cell counts of individual spleens were performed manually or with the Countess automated cell counter (Invitrogen) using trypan blue exclusion to count live cells. Total cell numbers were calculated by multiplying the manual cell count by the lymphocyte gate and then by the appropriate gate of the cell population of interest.

To evaluate the tumor infiltrating lymphocytes, the Day 10 treatment tumor model was utilized. Three to five mice per group were euthanized on Day 14, and tumors harvested. Tumors were minced and incubated in a digestion buffer consisting of collagenase (1mg/ml, Sigma), hyaluronidase (2.5 units/ml, Sigma), and DNaseI (0.1mg/ml, Sigma) in PBS for 30 minutes with slight agitation. Digested tumors were pressed through a 70μm cell strainer to obtain a single cell suspension. Cell counts for individual spleens were performed manually or with the Countess automated cell counter (Invitrogen) using trypan blue exclusion to determine the number of live cells. One million cells per sample were analyzed by flow cytometry for tumor infiltrating lymphocytes.

IFNγ stimulation of EL4E7 tumor cells

EL4E7 cells were plated at $4x10^5$cells/ml in 48 well plate in complete RPMI supplemented with 0, 50, or 100U/ml of IFNγ (Biolegend). Cells incubated for 24 hours prior to FACS analysis for MHCII, MHCI, CD137L, and CD137.

Measurement of mouse FcγRs binding to rat IgG2a using Surface Plasmon Resonance (SPR)

Antibodies (2A, rat IgG2a (eBioscience), and mouse IgG2a (eBioscience)) at 10μg/mL in acetate buffer at pH4.5 were immobilized to a CM5 chip in a BIAcore 3000 (GE Healthcare). A response of 600RU was the immobilization target level. Actual immobilization levels were 680RU, 670RU and 640RU, respectively, on flow cell (FC)2, FC3 and FC4. FC1 was used as a blank reference. Mouse FcγRI, FcγRIIB, FcγRIII and FcγRIV were serially diluted from 1μM to less than 1nM with running buffer (HBS-EP) and injected for 120sec at 20μl/min over all FCs followed by a 120 sec dissociation step. Full regeneration was achieved using a 15-30sec injection of 1M MgCl at 100μl/min. FCs were washed with a 30sec injection of running buffer at 20μl/min. KDs generated using a 1:1 Langmuir binding model. Kinetics were run in duplicate.

CD137L binding competition assay

HEK-293 cells were stably transfected with mouse CD137 using lipofectamine (Invitrogen) transfection with a pcDNA3.3 vector containing CD137. The HEK-mCD137 cells were incubated with Rat IgG or anti-CD137 mAb (clone 2A or 3H3) at 0.1ug/ml, 1ug/ml, 10ug/ml or 100ug/ml; followed by incubation with 31ng/ml of His-tagged recombinant mouse CD137L (R&D systems). Finally cells were incubated with anti-polyhistidine PE (R&D systems) to determine CD137L binding. Data acquired on an LSRII instrument (BD Biosciences) and analyzed using FlowJo (TreeStar).

In vitro stimulation of CD8β$^+$ cells and CD11c$^+$ cells for assessment of CD137 and CD137L expression

CD8β$^+$ cells and CD11c$^+$ cells were isolated from the spleens of naïve WT or FcγRIII$^{-/-}$ mice using FITC microbeads and CD11c$^+$ microbeads, respectively. Briefly the spleens were digested in the following buffer: 25mM HEPES buffered RPMI 1640, 1mg/ml collagenase type IA (Sigma), 20μg/ml DNase I (Sigma), and 2% FBS, for one hour at 37°C. After the incubation, a single cell suspension was obtained by mechanical dissociation, and the lymphocytes isolated by an Opti-prep (Sigma) gradient. CD11c$^+$ cells were enriched by a second Opti-prep gradient. CD8β$^+$ cells were purified by incubation with anti-CD8β FITC, then isolated using FITC microbeads (Miltyeni Biotec) following manufacturer's instructions. CD11c$^+$ cells were purified with CD11c microbeads (Miltyeni Biotec) by incubating with beads for 30 minutes prior to magnet isolation. For cell activation, CD8β$^+$ cells were stimulated with anti-CD3 mAb and anti-

CD28 mAb at 1μg/ml for 48 hours. CD11c+ cells were stimulated with 5 μg/ml of lipid polysaccharides (LPS) for 48 hours. The expression of CD137 and CD137L was determined by flow cytometry.

Bim expression

$CD8^+$ T cells isolated from a C57BL/6 spleen by CD8 microbeads (Militenyi Biotec) following manufacturer's instructions. Cells were plated in a 96 well plate at a final concentration of $1x10^6$ cells/ml in complete RPMI containing 5μg/ml concanavalin A (Con A, Sigma) and incubated for 24hrs. After the ConA stimulation, cells were washed and rested for 2hrs prior to exposure to immobilized 2A or Rat IgG. Cells were collected after 24hr stimulation and were plated in complete RPMI for 24hrs. The cells were fixed by incubating for 10 minutes at 37°C with 3% formaldehyde (Sigma) followed by a one minute incubation. The cells were permeabilized using chilled 90% methanol solution and incubating the cells for 30 minutes on ice. Cells were washed with FACS buffer prior to staining with anti-Bim (Cell signaling technologies). A secondary anti-Rabbit IgG-Cy5 (Jackson Laboratories) was used to detect Bim expression. Samples were acquired on LSRII (BD Bioscience) and were analyzed with Flowjo software (TreeStar).

Statistical Analysis

All Kaplan-Meier survival curves were compared using the log rank test. The differences in the $CD11c^+$ $MHCII^+$ expressing CD40, CD80, and CD86 were compared using the Krustal-Wallis Test (H) and post-hoc Dunn's Test. All statistical analysis was performed using GraphPad (Prism). A p value < 0.05 was considered significant.

Chapter 4- Results

The anti-tumor activity of anti-CD137 mAb (clone 2A) requires host, but not tumor expression of CD137.

We first sought to validate the absolute requirement for host expression of CD137 on the anti-tumor activity of anti-CD137 mAb. As anticipated 2A failed to mediate tumor regression in CD137$^{-/-}$ mice, while retaining its therapeutic efficacy in wild type (WT) animals (Figure 10, WT 2A vs WT IgG $p<0.0001$; WT 2A vs CD137$^{-/-}$ 2A $p=0.0013$). Next, while it is known that the EL4E7 lymphoma model is sensitive to 2A the potential contribution of tumor associated CD137 to this anti-tumor efficacy is unknown (97). In order to rule out the possibility that EL4E7 might express CD137 and thus confound our findings, we determined the levels of CD137 expression both *ex vivo* and *in vitro,* in the presence or absence of the pro-inflammatory cytokine, IFNγ. The absence of CD137 expression in these assays indicates that EL4E7 tumors lack constitutive or inducible CD137 expression under the conditions tested (Figure 11). These data establish that host expression of CD137 is required for 2A mediated EL4E7 anti-tumor responses, and that the therapeutic effects of 2A in this model are not dependent upon direct interaction of 2A with CD137 on the tumor cell surface.

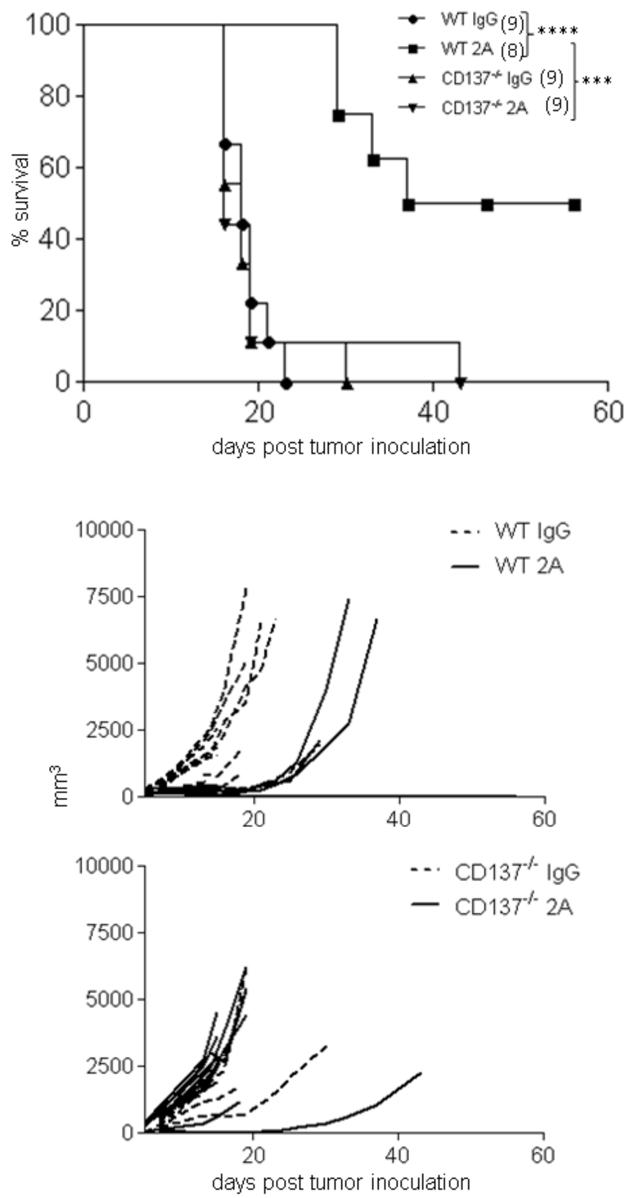
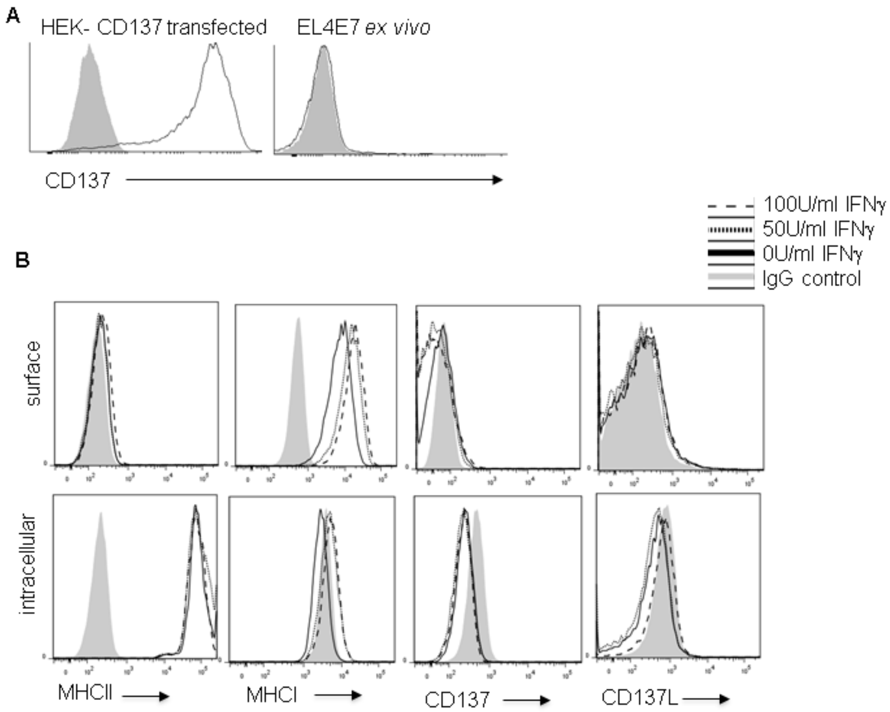


Figure 10: Host expression of CD137 is required for the anti-tumor effects of 2A
Mice inoculated with EL4E7 tumors on Day 0 and treated with 100µg of either Rat IgG or 2A on Day 7 and Day 10.
Statistical significance determined by Log-rank Test: * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤0.0001
Survival curve and growth curves the sum of two experiments.



C

surface

Sample	MFI MHCII	MFI MHCI	MFI CD137	MFI CD137L
IgG	167	534	357	53.5
0U/ml	211	8012	406	38.3
50U/ml	208	14900	466	-4.12
100U/ml	246	18800	470	5.02

intracellular

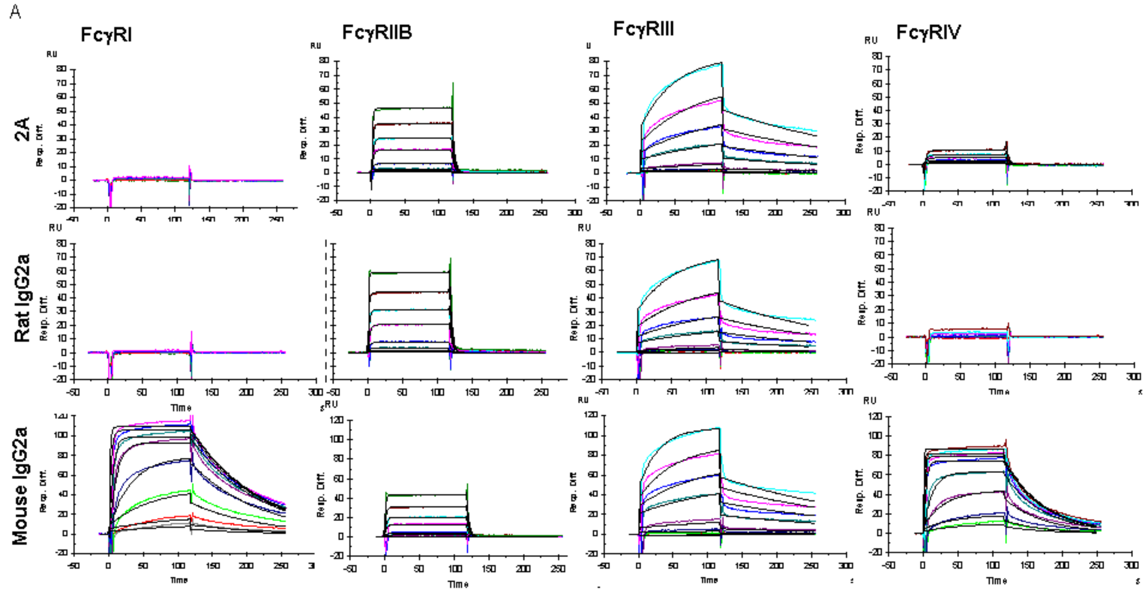
Sample (IC)	MFI MHCII	MFI MHCI	MFI CD137	MFI CD137L
IgG	269	4162	1172	534
0U/ml	88200	2910	534	287
50U/ml	96100	4897	494	242
100U/ml	79100	5337	770	236

Figure 11: *Ex vivo* EL4E7 tumor samples and IFNγ stimulated *in vitro* EL4E7 tumor cells do not express CD137
A. First panel, CD137 expression on HEK-CD137 transfected cells; second panel, CD137 expression on EL4E7 ex vivo. Gray histogram is the IgG control. **B.** Expression of MHCII, MHCI, CD137, and CD137L after 24hr stimulation with the indicated concentrations of IFNγ on EL4E7 cells *in vitro*. **C.** The MFI values which correspond to histograms

Rat anti-mouse CD137 mAb binds to mouse FcγRIIB, FcγRIII, and FcγRIV.

The majority of the anti-CD137 mAbs used as surrogates to study the impact of CD137 ligation on anti-tumor immunity, including 2A, are of rat origin (97, 334). Based on our desire to define the import of anti-CD137-FcγR interactions on anti-CD137 mAb function, we characterized the interactions between 2A and mouse FcγRs by surface plasmon resonance (SPR) using mouse IgG2a as a control. SPR characterizes protein interactions using quantifying the binding interactions between two proteins. A higher KD value (the equilibrium dissociation constant, expressed in molarity) indicates a weaker interaction, whereas a lower KD value indicates a stronger interaction between two proteins. FcγRIIB, FcγRIII, and FcγRIV, but not FcγRI, bound to immobilized rat 2A (Figure 12). FcγRIIB and FcγRIII bound comparably to both immobilized 2A and mouse IgG2a (2A: FcγRIIB KD= 528nM, FcγRIII KD= 238 nM; mouse IgG2a: FcγRIIB KD= 501nM, FcγRIII KD= 138 nM). In contrast, the interactions between FcγRIV and 2A (KD= 3180nM) were dramatically weaker than interactions between FcγRIV and mouse IgG2a (KD= 8.63nM). Our binding prolife for 2A to mouse FcγRs is similar to the binding profile for mouse IgG1 (92). This similarity in binding profiles between 2A and mouse IgG1 is anticipated because rat IgG2a and mouse IgG1 have structural homology (335). These data and the published data collectively suggest 2A has the potential to interact with mouse FcγRs in a similar manner as mouse IgG1.

A



B

	Immobilized to chip		
Mouse FcγR	**anti-CD137 mAb**	**Rat IgG2a**	**Mouse IgG2a**
FcγRI	N/A	N/A	14.7 (61)
FcγRIIB	528 (1.87)	353 (2.76)	501 (3.2)
FcγRIII	238 (8.06)	375 (17.5)	138 (44.7)
FcγRIV	3180(2.14)	N/A	8.63 (33.7)

Figure 12: Rat IgG2a binds to mouse FcγRIIB, FcγRIII, and FcγRIV but not to FcγRI
A. Mouse IgG2a, Rat IgG2a, and anti-CD137 mAb bound to SPR chip at 10μg/ml. Recombinant mouse FcγRs were serially diluted from 1μM to less than 1nM with running buffer (HBS-EP), injected for 120sec at 20μl/min over all FCs (flow cells), and followed by a 120sec dissociation step. **B.** The KD values (nM) and the Chi^2 in parenthesis.

Mice lacking the common Fcγ chain have enhanced 2A mediated anti-tumor immunity.

We employed Fc common γ–chain deficient (FcRγ$^{-/-}$) mice to define the potential role of Fc-FcγR interactions on the anti-tumor activity of 2A. These mice do not express activating FcγRs, which require the Fcγ chain to signal, yet maintain expression of FcγRIIB (336). This experiment tested the hypothesis that activating FcγRs are required for the anti-CD137 mAb mediated anti-tumor function. Unexpectedly, the EL4E7 bearing FcRγ$^{-/-}$ mice treated with 2A enjoyed improved survival versus IgG treated controls (Figure 13A, $p=0.01$). While 2A treated WT mice had a partial therapeutic response, it was less effective than that observed in FcRγ$^{-/-}$ animals (Figure 13A). Because these data were unexpected we sought to confirm our findings using a more aggressive tumor model, in which initiation of treatment was delayed until Day 10 post tumor inoculation. Consistent with our previous findings, 2A treated FcRγ$^{-/-}$ mice had a significant improvement in survival, while 2A treated WT mice or rat IgG control treated mice all experienced progressive disease (Figure 13B, FcRγ $^{-/-}$ 2A vs FcRγ $^{-/-}$ IgG $p= 0.0027$; FcRγ $^{-/-}$ 2A vs WT 2A $p= 0.0161$, WT 2A vs WT IgG $p= 0.0063$).

Importantly, FcRγ$^{-/-}$ mice, which enjoyed initial 2A mediated tumor rejection, were immune to second challenge with EL4E7 but not B16-F10 melanoma (Figure 14), demonstrating the development of tumor specific immunologic memory.

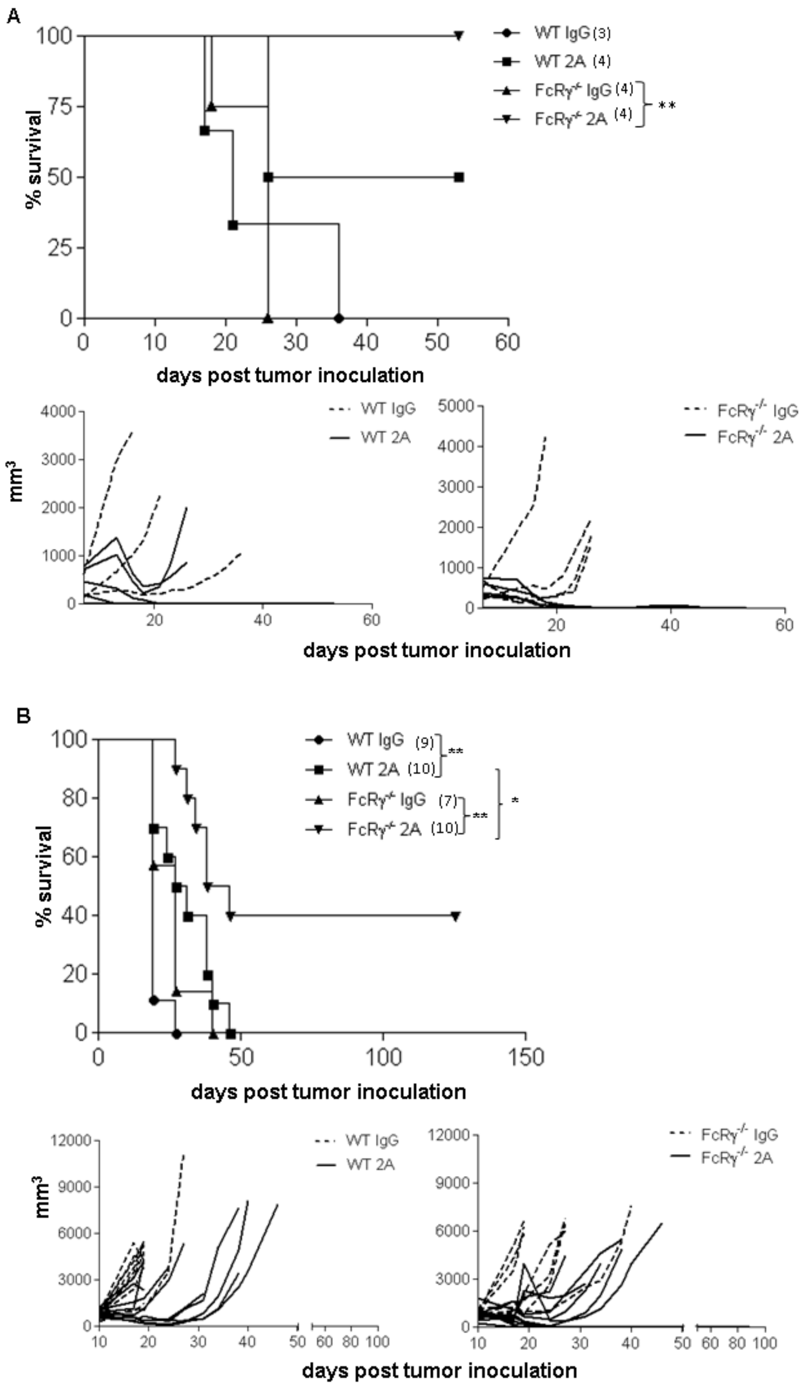


Figure 13: 2A treated FcRγ$^{-/-}$ mice have improved tumor survival

Mice inoculated with 8x10^6 EL4E7 tumor cells on Day 0. **A.** Survival and growth curves for mice treated with 100μg of either Rat IgG or 2A on Days 7 and 10. **B.** Survival and growth curves for mice treated with 100μg of either Rat IgG or 2A on Days 10 and 13. Number in parenthesis indicates the number of mice. Statistical significance determined by Log-rank Test: * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤0.0001

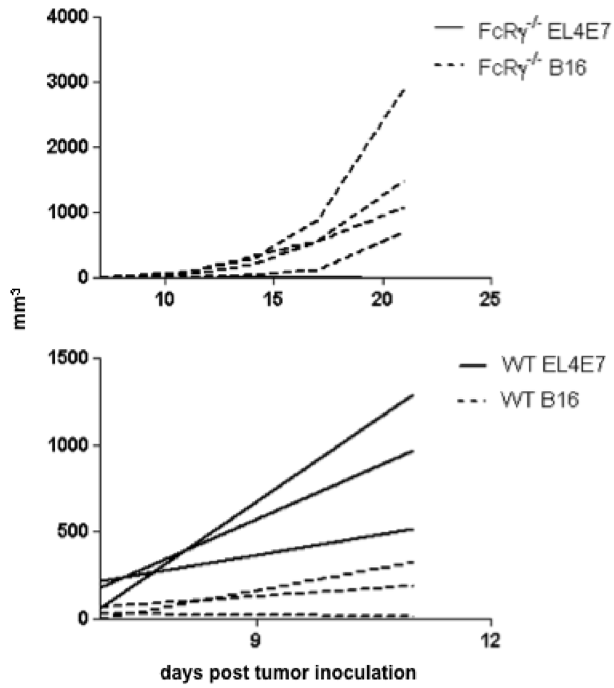


Figure 14: 2A treated FcRγ$^{-/-}$ mice develop a memory response to EL4E7 tumors 125 days after tumor resolution, mice with complete resolution of their primary tumors were re challenged with $8x10^6$ EL4E7 or B16-F10 tumor cells. Tumor volume determined by measuring the length (l) and width (w), and then using the following equation: $w^2*l*0.5$. Tumor growth monitored until experimental endpoint. WT naïve mice used as a control for tumor growth.

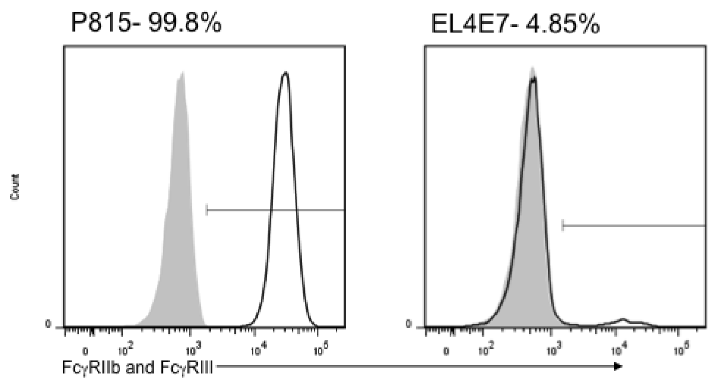


Figure 15: FcγRIIB and/or FcγRIII is low on EL4E7 tumor cells cultured *in vitro*
EL4E7 cells incubated with anti-FcγRIIB/FcγRIII, followed with a secondary antibody against Rat IgG. The gray shaded region is secondary antibody only.

Furthermore, these findings are likely not related to tumor expression of FcγRs, as flow cytometric analysis revealed low levels of FcγRIII and/or FcγRIIB on the tumor cell surface (Figure 15). These data suggest the activating FcγRs inhibit the 2A mediated anti-tumor response. However, these findings do not rule out the possibility of Fc-FcγRIIB interactions are enhancing the 2A mediated tumor rejection, especially considering the requirement for FcγRIIB interactions in the anti-tumor function of anti-CD40 mAbs (92, 93).

FcγRIII$^{-/-}$ mice have improved anti-CD137 mAb mediated anti-tumor immunity.

Next we sought to identify the individual FcγR contributing to the improved 2A mediated anti-tumor immunity in FcγR$^{-/-}$ mice. Based on the fact that 2A does not effectively bind FcγRI$^{-/-}$ (Figure 12), we restricted our studies of the activating receptors to FcγRIII and FcγRIV. 2A treatment significantly improved survival of tumor bearing FcγRIII$^{-/-}$, but not FcγRIV$^{-/-}$ mice versus WT mice (Figures 17, FcγRIII$^{-/-}$ 2A vs FcγRIII$^{-/-}$ IgG $p< 0.0001$; WT 2A vs WT IgG $p= 0.0023$; FcγRIII$^{-/-}$ IgG vs WT IgG $p= 0.0311$, and FcγRIII$^{-/-}$ 2A vs WT 2A $p= 0.0006$ and Figure 16A, FcγRIV$^{-/-}$ 2A vs FcγRIV$^{-/-}$ IgG $p=0.0217$; WT 2A vs WT IgG $p= 0.0484$; and FcγRIV$^{-/-}$ 2A vs WT 2A $p= 0.1062$).

The inhibitory receptor, FcγRIIB, did not enhance 2A mediated anti-tumor immunity as FcγRIIB$^{-/-}$ mice, treated in an analogous fashion, had similar survival rates as WT animals (Figure 16B, WT 2A vs WT IgG $p= 0.0078$).

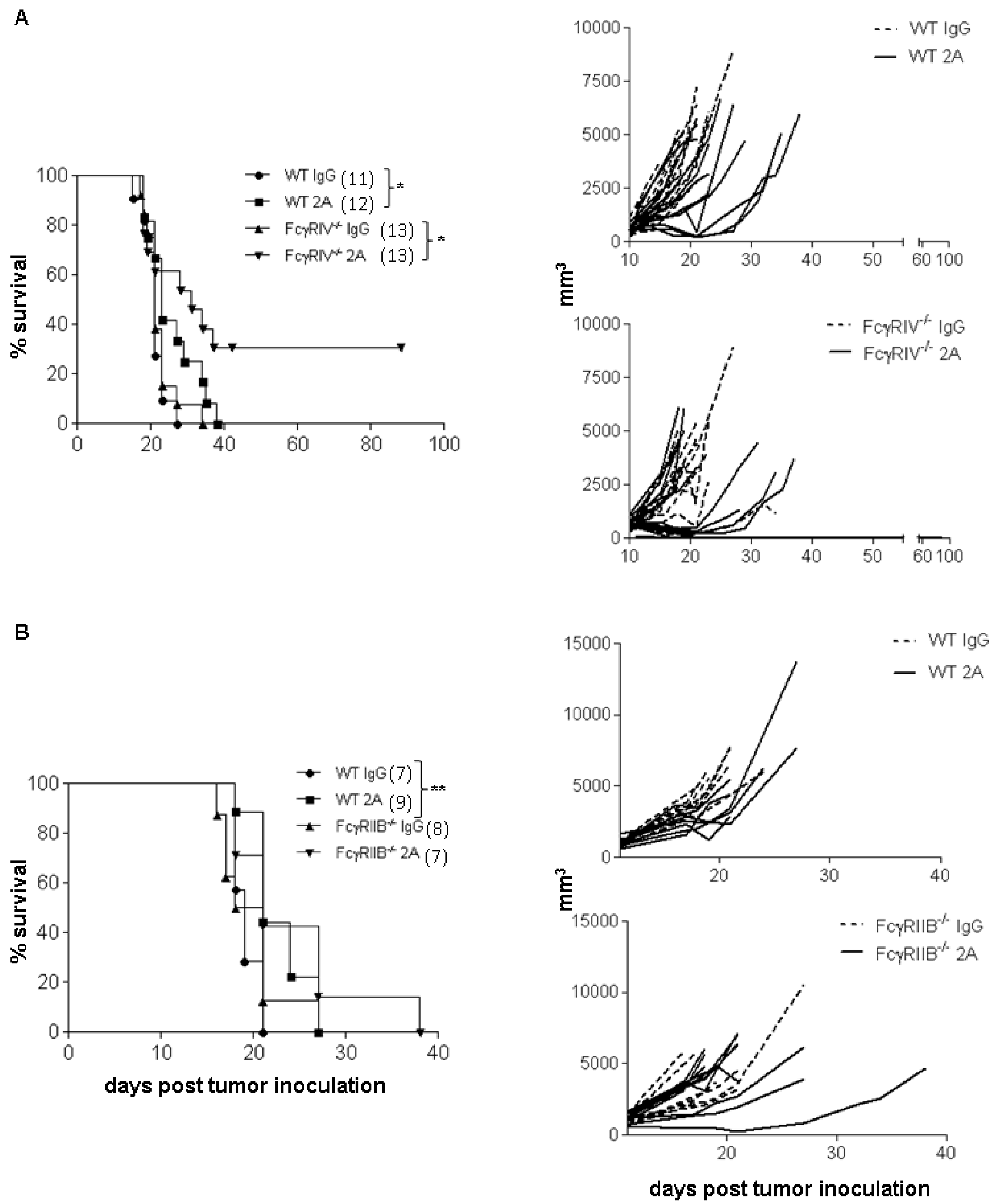


Figure 16: EL4E7 tumor bearing FcγRIIB$^{-/-}$ and FcγRIV$^{-/-}$ mice treated with 2A do not have a statistically significant difference in survival compared to WT mice

Mice inoculated with $8x10^6$ EL4E7 tumor cells on Day 0. On Day 10 and Day 13, mice treated with either 100μg of anti-CD137 mAb or Rat IgG. Results are combined from two separate experiments; **A.** FcγRIV$^{-/-}$ and **B.** FcγRIIB$^{-/-}$ mice. Number in parenthesis indicates the number of mice. Statistical significance determined by Log-rank Test: * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤0.0001. Results for A combined from two separate experiments. Results for B are from one experiment.

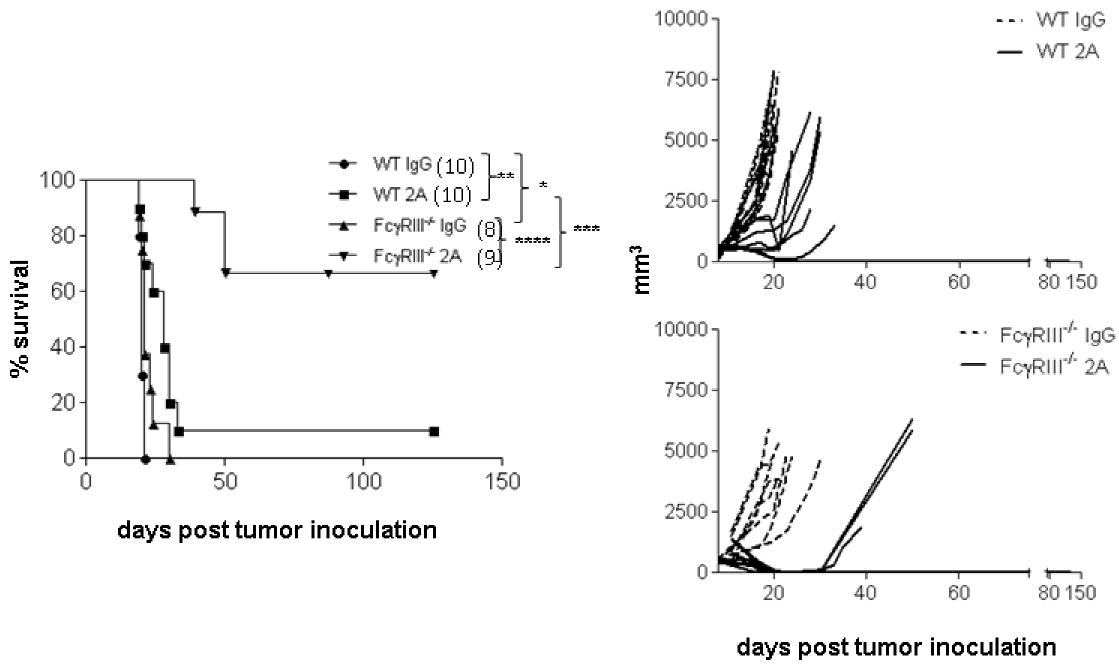


Figure 17: EL4E7 tumor bearing FcγRIII$^{-/-}$ mice treated with 2A results in enhanced survival.

Mice inoculated with 8x10^{6} EL4E7 tumor cells on Day 0. On Day 10 and Day 13, mice treated with either 100µg of anti-CD137 mAb or Rat IgG. Results are combined from two separate experiments. Number in parenthesis indicates the number of mice. Statistical significance determined by Log-rank Test: * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤0.0001.

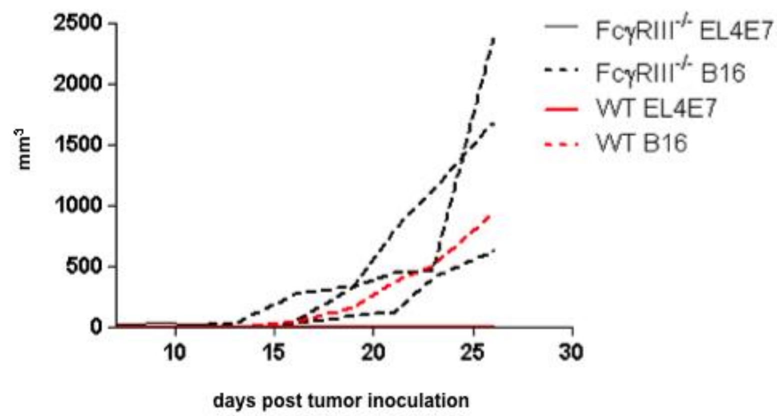


Figure 18: 2A treated FcγRIII$^{-/-}$ mice develop a memory response to EL4E7 tumors 125 days after tumor resolution, mice with complete resolution of their primary tumors, were re challenged with $8x10^6$ EL4E7 tumor cells or B16-F10 tumor cells. Tumor volume determined by measuring the length (l) and width (w), and then using the following equation: $w^2*l*0.5$. Tumor growth monitored until experimental endpoint end. Black group represents FcγRIII$^{-/-}$ mice and red group represents WT mouse rechallenged after tumor resolution.

Similar to the $FcR\gamma^{-/-}$ mice, $Fc\gamma RIII^{-/-}$ mice that experienced complete tumor regression were immune to EL4E7 re challenge yet were capable of establishing B16-F10 tumors, indicating the development of an antigen specific memory response (Figure 18). These data suggest FcγRIII is regulating the anti-tumor immune response elicited by 2A treatment.

To exclude the possibility that our observations were 2A specific we evaluated a second rat anti-mouse CD137 mAb, 3H3, in the $Fc\gamma RIII^{-/-}$ mice. Similar to 2A treated mice, 3H3 preferentially prolonged survival of EL4E7 tumor bearing $Fc\gamma RIII^{-/-}$ mice (Figure 19A, $Fc\gamma RIII^{-/-}$ 2A vs $Fc\gamma RIII^{-/-}$ IgG $p= 0.015$, and $Fc\gamma RIII^{-/-}$ 2A vs WT 2A $p= 0.0010$). Differences in the absolute efficacy of these individual mAbs may be related to the fact that at higher doses, 3H3, but not 2A, blocks CD137-CD137L interactions (Figures 19B and C). These data demonstrate that anti-CD137 mAb mediated anti-tumor immunity is enhanced in $Fc\gamma RIII^{-/-}$ mice.

To determine if the modulation of the 2A mediated anti-tumor response in the $Fc\gamma RIII^{-/-}$ mice could be recapitulated with other tumor type, we utilized the MC38 colon cancer model, which has a weak therapeutic response to 2A treatment (337). Interestingly, the enhanced anti-tumor response of 2A treated $Fc\gamma RIII^{-/-}$ was not observed in an MC38 tumor model (Figures 20A, WT IgG vs WT 2A $p=0.0017$, 20B WT IgG vs WT 2A $p=0.002$, $Fc\gamma RIII^{-/-}$ IgG vs $Fc\gamma RIII^{-/-}$ 2A $p=0.002$). These results suggest the FcγRIII deficiency is not sufficient to further enhance the 2A anti-tumor response in the MC38 model.

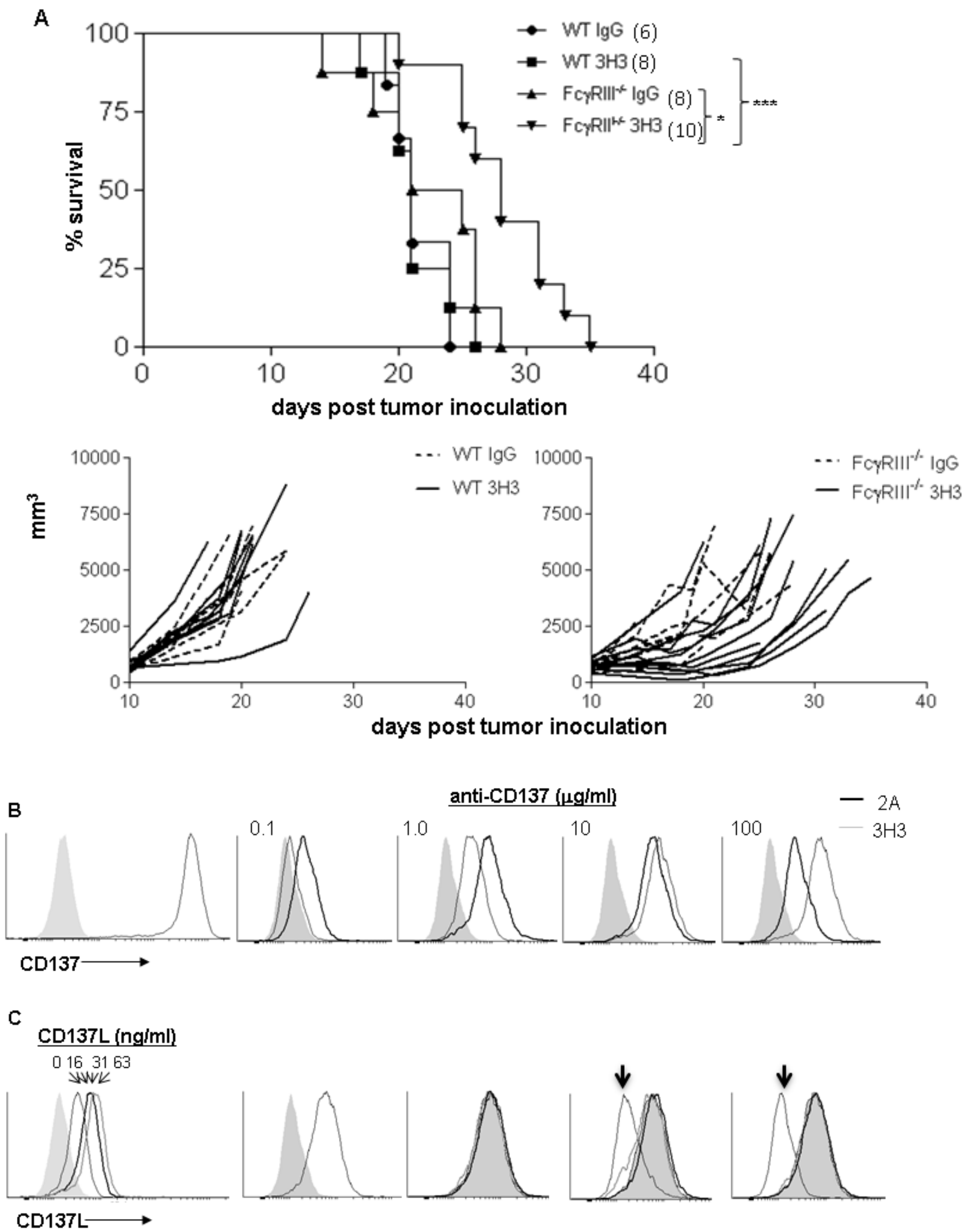


Figure 19: Treatment with anti-CD137 mAb clone 3H3 results in enhanced anti-tumor response in FcγRIII$^{-/-}$ mice

A. Mice inoculated with 8×10^6 EL4E7 tumor cells on Day 0. On Day 10 and Day 13 mice, treated with either 100μg of anti-CD137 mAb or Rat IgG. Number in parenthesis indicates the number of mice. Statistical significance determined by Log-rank Test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Survival curve and growth curves the sum of two experiments. **B.** HEK-mCD137L expressing cells incubated with anti-CD137 mAb clone 2A or 3H3. **C.** HEK-mCD137L expressing cells incubated with recombinant mCD137L at 31ng/ml (2nd panel). First cells incubated with anti-CD137 mAb at concentrations listed in A, then cells incubated with mCD137L at 31ng/ml.

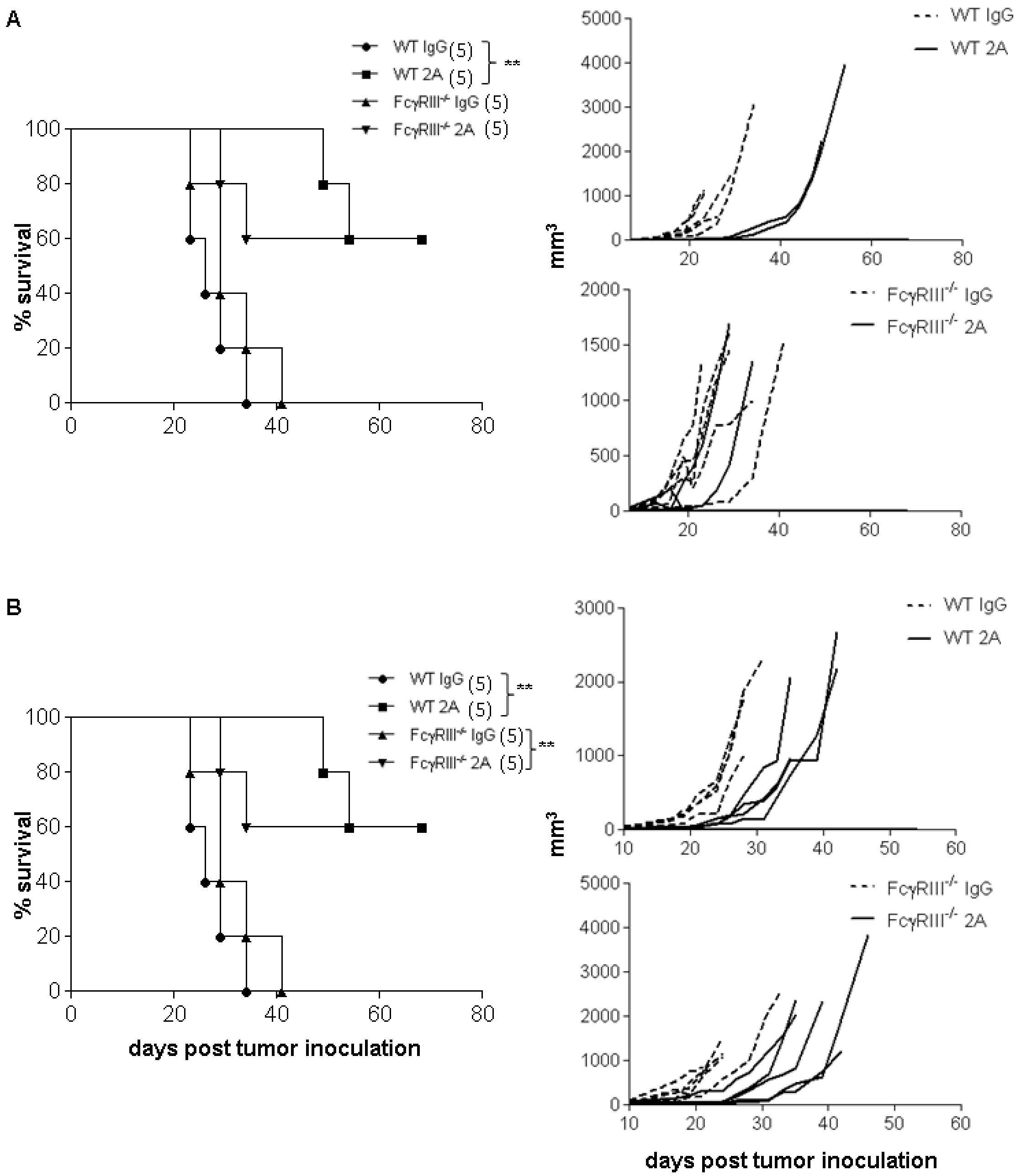


Figure 20: FcγRIII$^{-/-}$ is not sufficient to enhance the 2A mediated anti-tumor response in the MC38 colon cancer model

Mice were inoculated with MC38 tumors on Day 0. **A.** Mice were treated with 100μg of Rat IgG or 2A on Day 7 and Day 10. **B.** Mice treated with 100μg of Rat IgG or 2a on Day 10 and Day 13. Number in parenthesis indicates the number of mice. Statistical significance determined by Log-rank Test: * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001, **** p≤0.0001.

2A treatment preferentially enhances CD8β^{+} T cell and CD11c^{+} dendritic cell populations in FcγRIII$^{-/-}$ mice.

The expansion of CD8^{+} T cells is a hallmark of the CD137 stimulated anti-tumor response (97, 188, 189, 338-341). Therefore, we utilized a kinetic experiment to test the hypothesis that the CD8+ T cells were enhanced in the FcγRIII$^{-/-}$ mice compared to the WT mice. As anticipated, 2A treatment resulted in expansion of the CD8β^{+}CD11c^{+} T cells in WT and FcγRIII$^{-/-}$ mice, but the expansion of the CD8β^{+}CD11c^{+} cell occurred at an earlier time point in the spleens of FcγRIII$^{-/-}$ mice (Figure 21A). Next, based on reports that CD103 is induced on the CD8β^{+}CD11c^{+} cell after anti-CD137 mAb treatment and is associated with CD8^{+} regulatory T cells, we employed CD103 expression as a phenotypic marker of suppressor function to test the hypothesis that a change in the ratio of effector CD8+ T cells to regulatory CD8+ T cells is driving the enhance anti-tumor immunity in the FcγRIII$^{-/-}$ mice (184, 339, 342). We found the CD8β^{+}CD11c^{+}CD103^{-} cells to be increased by one log compared to the CD8β^{+}CD11c^{+}CD103^{+} cells in the 2A treated FcγRIII$^{-/-}$ mice (Figure 21B). We confirmed the expansion of the CD8β^{+} T cells is not due to differences in expression of CD137 in the WT and FcγRIII$^{-/-}$ mice (Figure 21C). These data indicate the FcγRIII$^{-/-}$ mice produce a more rapid and robust CD8β^{+}CD11c^{+} T cell response after 2A treatment.

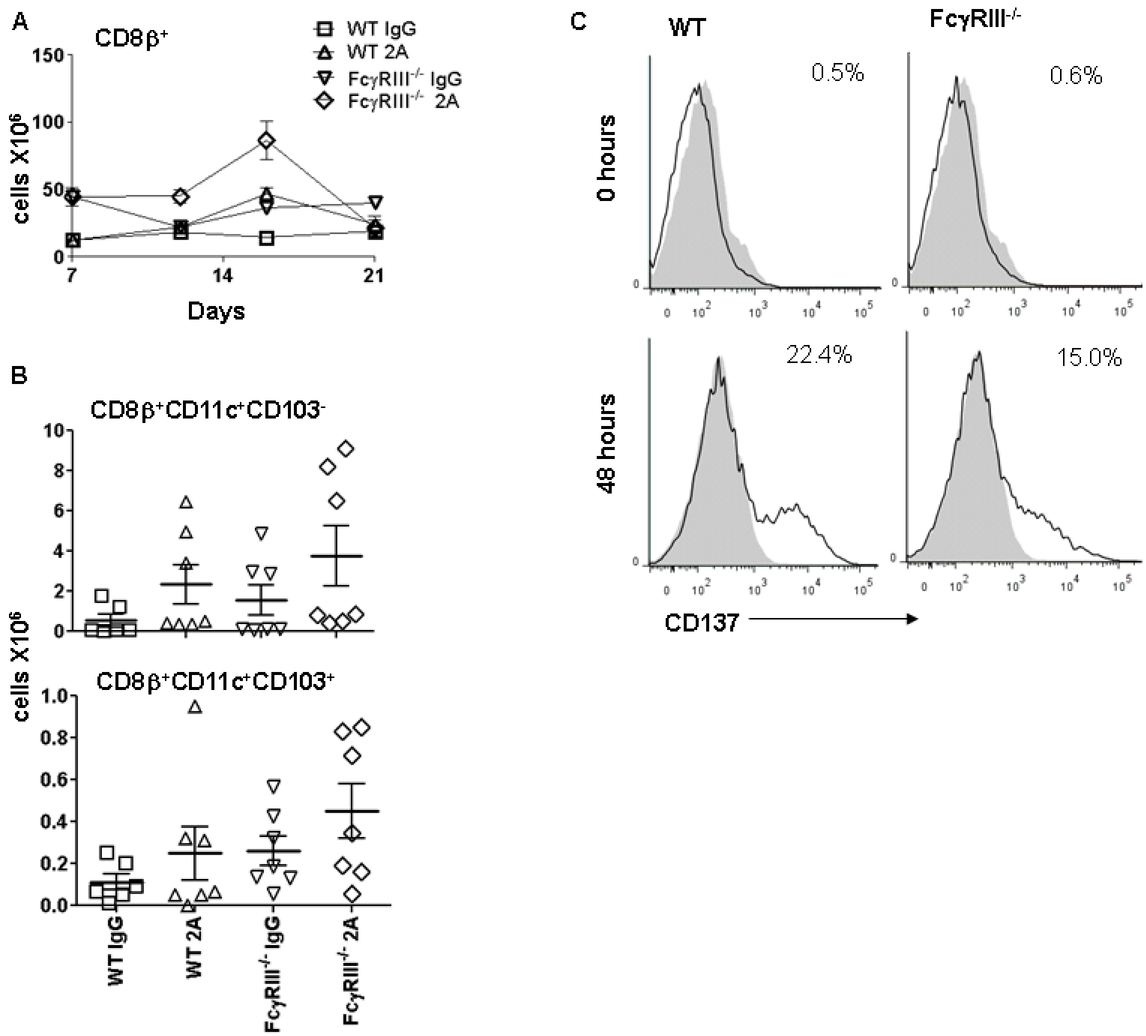


Figure 21: 2A treated tumor bearing FcγRIII$^{-/-}$ mice have an increase in CD8β^{+}CD11c^{+} T cells due to the FcγRIII deficiency, and not due to differences in CD137 expression.

A. Mice inoculated with 8x10^{6} EL4E7 tumor cells on Day 0. On Day 7 and Day 10 mice received either 100μg of 2A or Rat IgG. The spleens harvested from 2 or 3 mice per group on Days 7, 12, 16, and 21. Representative data from one experiment repeated three times. **B.** Cells isolated by positive selection from spleens of naïve WT and FcγRIII$^{-/-}$ mice, and stimulated with anti-CD3 mAb and anti-CD28 mAb at 1mg/ml for 48 hours. CD137 expression determined by flow cytometry. Representative data from one experiment repeated four times.

Because FcγRIII is not expressed on CD8β$^+$ T cells; it is likely that an FcγR$^+$ cell population is responsible in the regulation of the CD8β$^+$ T cell response (96). Therefore, we assessed the kinetics for MDSCs, macrophages, NK, NKT, and DCs, all of which express FcγRs (101). The 2A treated FcγRIII$^{-/-}$ mice have increases in all FcγR$^+$ populations on Day 12 and Day 16 in the spleen (Figure 22). MDSCs and macrophages are normally associated with immune suppression of the anti-tumor immune response; we posited that the changes in MDSCs and macrophages are unlikely to be associated with the enhanced anti-tumor response in the FcγRIII$^{-/-}$ mice. Our data does not rule out the potential of the NK and NKT cells mediating the enhanced anti-tumor response, but with the variability between experiments in the numbers of NK and NKT cells, and the enhanced CD8β$^+$ T cell response; we focused on determining the contributions of the DCs. The potential of enhanced DC-T cell interactions would improve the 2A mediated anti-tumor immune responses.

Additionally, a hallmark of CD137 signaling is a reduction in Bim expression, a pro-apoptotic protein, which is important to the inhibition of AICD (147, 153). With the overall increases in all cell populations in the 2A treated FcγRIII$^{-/-}$ mice, we hypothesized there would be a reduction in Bim expression in the CD8β$^+$ T cells and DCs. We analyzed Bim expression by flow cytometry. In the pilot experiments, we found the resting the cells had similar reductions in Bim expression as CD137 stimulation (Figure 23). Therefore we concluded the resting period from the activating stimuli obscures the effects of CD137 stimulation on Bim expression, and did not further analyze Bim expression.

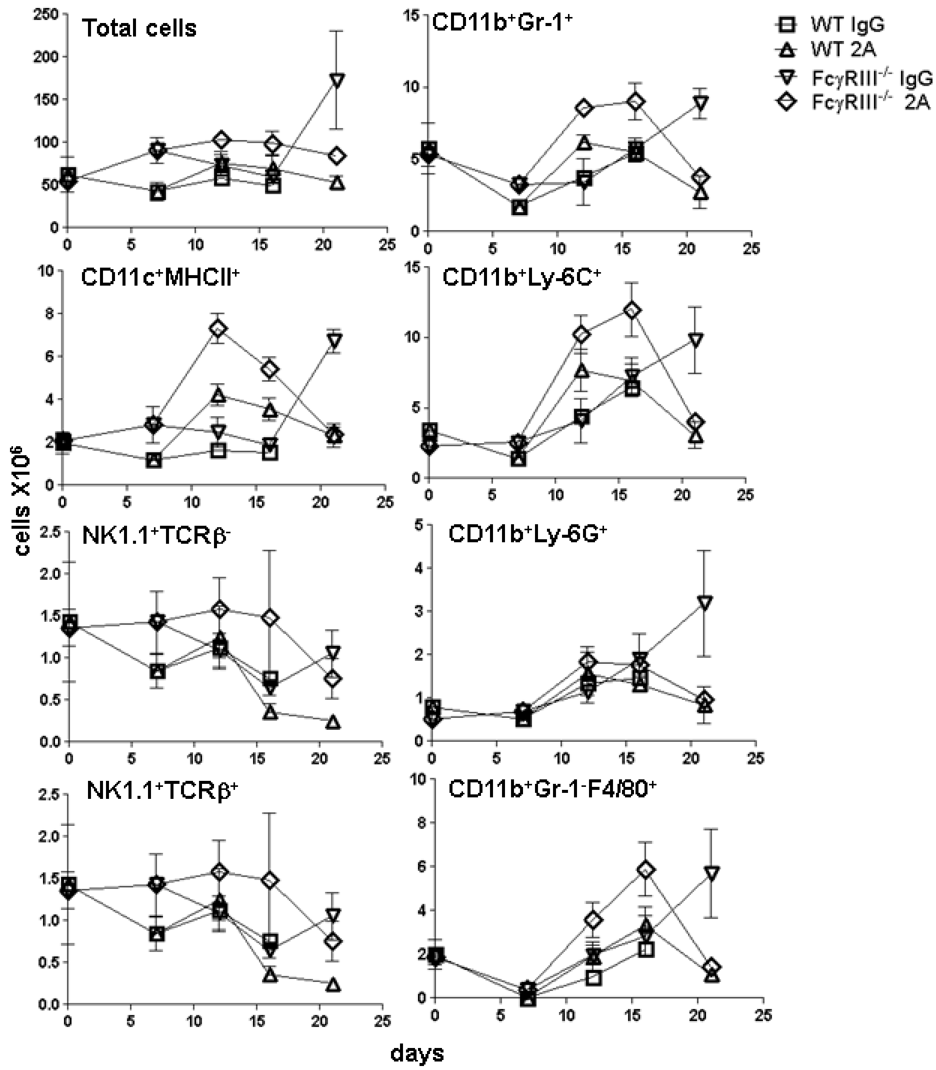


Figure 22: CD11c$^+$MHCII$^+$ preferentially increased in the 2A treated FcγRIII$^{-/-}$ mice

Mice inoculated with $8x10^6$ EL4E7 cells on Day 0. Mice treated with 100μg of Rat IgG or 2A on Day 7 and Day 10. Spleen harvested from 2 or 3 mice per group on Days 0, 7, 12, 16, and 21. Trypan blue dye exclusion used to determine live cell population. To determine cell number, (1) cell count multiplied by lymphocyte gate percentage, then (2) total lymphocytes multiplied by desired cell population gate percentage. Experiment determining CD11c$^+$MHCII$^+$ population repeated a total of three times to confirm results.

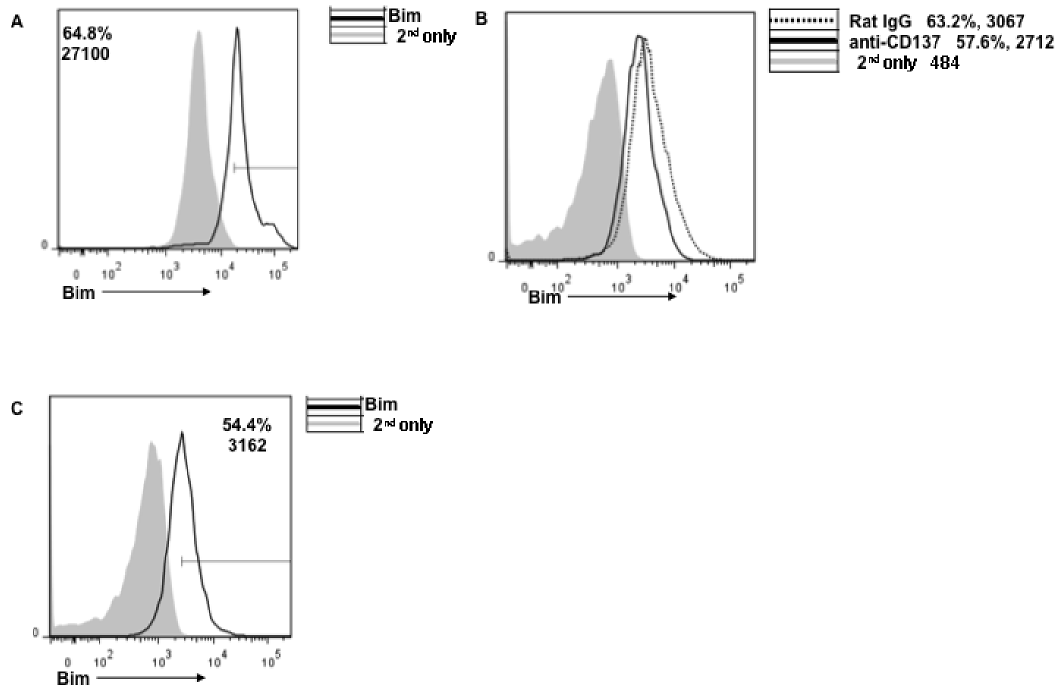


Figure 23: Bim expression is reduced after rest from ConA stimulation and not stimulation with 2A

$CD8^+$ T cells isolated from the spleen, and stimulated for 24hrs with 5μg/ml of Con A. Cells rested for 24hrs and then stimulated with immobilized 2A for 24hrs prior to assess Bim expression. **A.** Bim expression on $CD8^+$ T cells after Con A stimulation. **B** Bim expression on $CD8^+$ T cells after exposure to immobilized Rat IgG or 2A. **C.** Bim expression after 24hr rest period. Panels A and B from the same experiment. Panel C from a follow up experiment.

DCs are considered the most potent antigen presenting cells (APC) and their role is crucial to the development of robust antigen specific responses (343). Interestingly, we observed the $CD11c^{+}MHCII^{+}$ cell population is preferentially expanded in the spleen of 2A treated $Fc\gamma RIII^{-/-}$ mice (Figure 22). Further phenotypic analysis indicated a significant increase in the numbers of $Fc\gamma RIII^{-/-}$ $CD11c^{+}$ $MHCII^{+}$ cells expressing CD40, CD80, and CD86 compared to the WT mice after 2A treatment, suggesting that more antigen presenting cells (APCs) are present to interact with the $CD8\beta^{+}$ T cells (Figure 24A, Krustal-Wallis Test : $CD40^{+}$, H=12.91, p=0.0048; $CD86^{+}$, H=12.01, p=0.0074; and $CD80^{+}$, H=13.02, p=0.0046; post-hoc Dunn's test, WT 2A and $Fc\gamma RIII^{-/-}$ 2A: $CD40^{+}$, $CD86^{+}$, and $CD80^{+}$ $p \leq 0.01$). These increases in cell numbers are not due to differences in CD137 or CD137L expression on $CD11c^{+}MHCII^{+}$ cells in the WT and $Fc\gamma RIII^{-/-}$ mice (Figure 24B). In an attempt to functionally support the phenotypic data a tumor antigen presentation assay using tumor lysates was performed to test the hypothesis that the increased numbers of $CD11c^{+}MHCII^{+}$ cells in the $Fc\gamma RIII^{-/-}$ mice enhances the antigen specific $CD8^{+}$ T cell response. This experiment did not provide conclusive results (data not shown). These data indicate $Fc\gamma RIII^{-/-}$ mice produce more APCs, which may be driving the $CD8\beta^{+}$ T cell response.

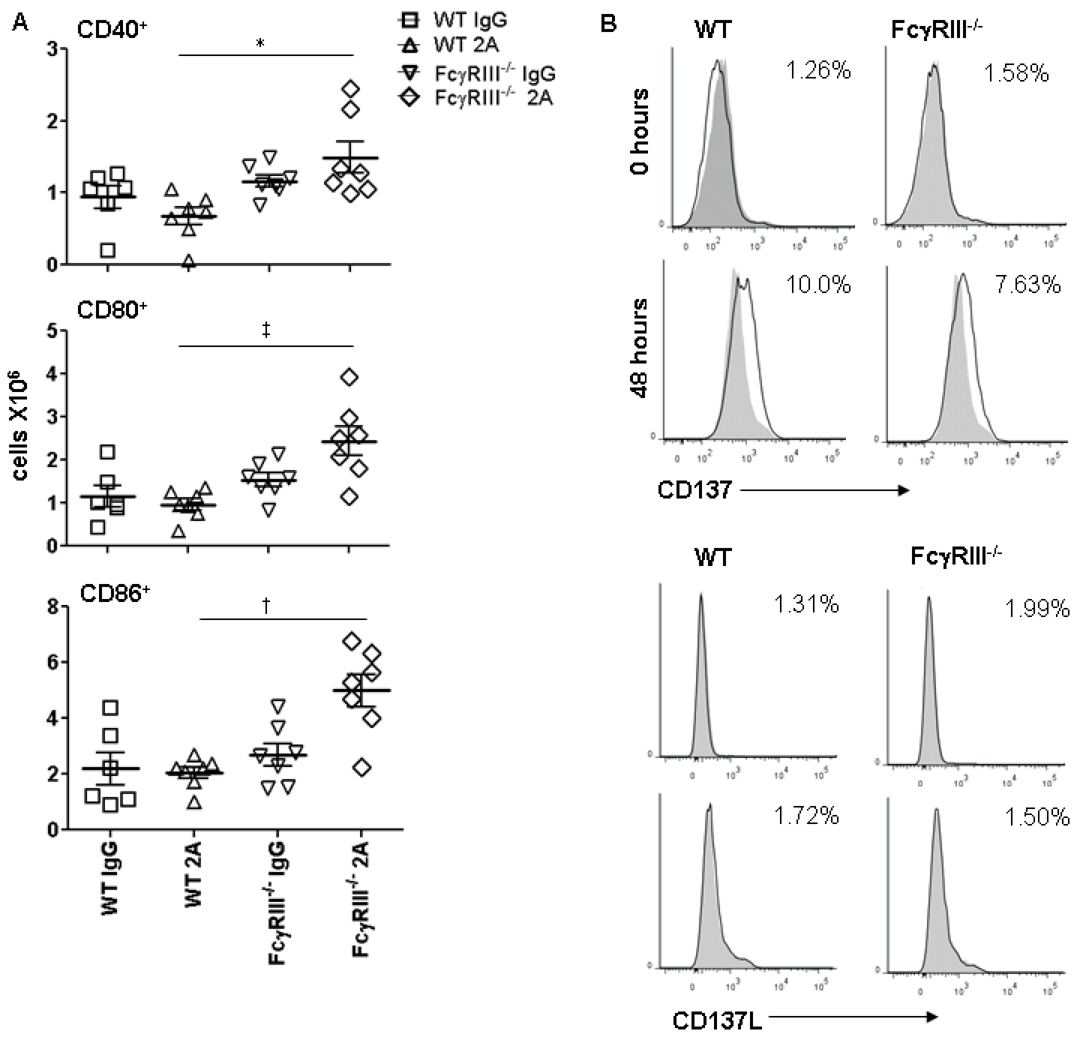


Figure 24: 2A treated tumor bearing FcγRIII$^{-/-}$ mice have an increase in CD11c^{+} MHCII^{+} cells with an APC phenotype due to the FcγRIII deficiency, and not due to differences in CD137 or CD137L expression.

A. Mice inoculated with $8x10^6$ EL4E7 tumor cells on Day 0. On Day 7 and Day 10 mice received either 100μg of 2A or Rat IgG. The expression of CD40, CD80, and CD86 on CD11c^{+} MHCII^{+} cells from spleen on Day 16. Data combined from two experiments for a final n=6 or 7. Using the post-hoc Dunn's test, a significant difference between WT 2A and FcγRIII$^{-/-}$ 2A (*CD40^{+}, †CD86^{+}, and ‡CD80^{+} $p \leq 0.01$). **B.** Cells isolated by positive selection from spleens of naïve WT and FcγRIII$^{-/-}$ mice. CD11c^{+} cells stimulated with 5 μg/ml of LPS for 48 hours. CD137 and CD137L expression determined by flow cytometry. Representative data of one experiment repeated four times.

$CD11c^{+}MHCII^{+}$ cells are involved in the enhanced 2A mediated anti-tumor response in the FcγRIII$^{-/-}$ mice

The cellular population data presented is from the spleen of tumor bearing mice, and may potentially not be representative of the anti-tumor immune response. A first step in associating the spleen data with the tumor resolution was to correlate the increased CD8β^{+} T cells and $CD11c^{+}MHCII^{+}$ cells with tumor volume. The Pearson's correlation coefficient comparing the number of CD8β^{+}CD11c^{+}CD103^{-} T cells to tumor volume is positively, statistically significant for the 2A treated mice, suggesting the CD8β^{+}CD11c^{+}CD103^{-} T cells are involved in 2A mediated tumor regression (Figure 25A).

A significant positive Pearson's correlation comparing the number of $CD11c^{+}MHCII^{+}$ cells to tumor volume was only found with the 2A treated FcγRIII$^{-/-}$ mice (Figure 25B). This result supports the hypothesis that the FcγRIII$^{-/-}$ $CD11c^{+}MHCII^{+}$ cells are involved in the enhanced 2A elicited anti-tumor response. These data suggest that 2A treatment of FcγRIII$^{-/-}$ DCs polarizes DCs to present tumor antigen better than WT DCs, which supports the enhanced anti-tumor immunity.

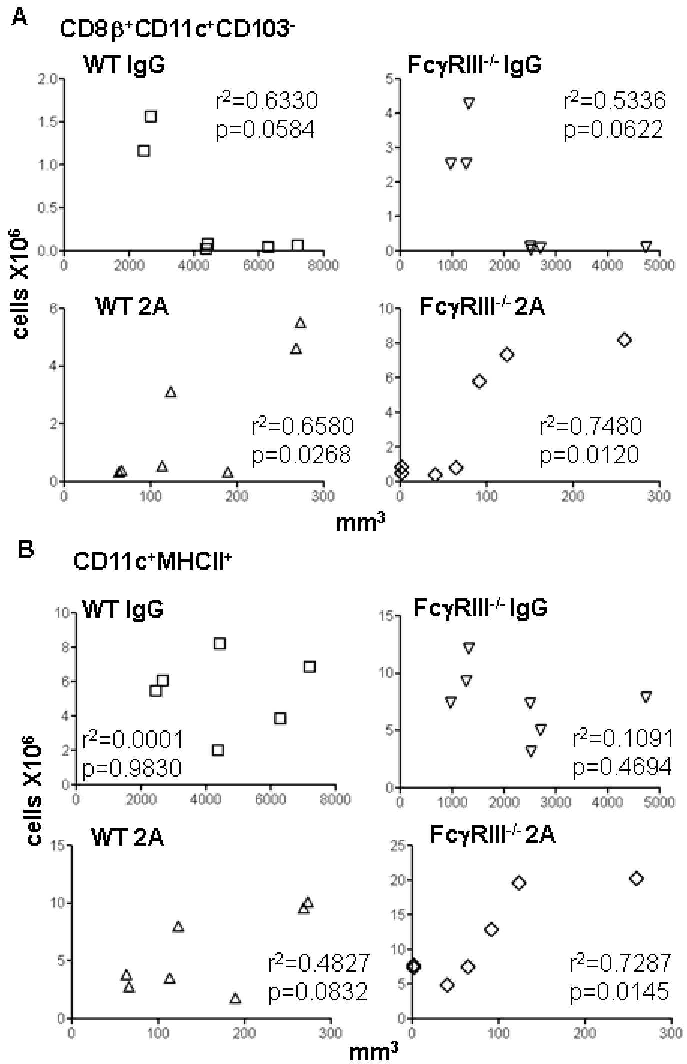


Figure 25: CD11c^{+}MHCII^{+} cells are involved in the enhanced 2A mediated anti-tumor response in the FcγRIII$^{-/-}$ mice.
Mice inoculated with 8x10^{6} EL4E7 tumor cells and treated on Day 7 and Day 10 with 100μg of either Rat IgG or 2A. **A**. Pearson correlation of CD8b^{+}CD11c^{+}CD103^{-} absolute cell number versus tumor volume. Data combined from two experiments for a final n=6 or 7. **B.** Pearson correlation of CD11c^{+}MHCII^{+} absolute cell number versus tumor volume. Data combined from two experiments for a final n=6 or 7.

Chapter 5- Discussion

Our data indicate that FcRγ $^{-/-}$ mice have improved anti-CD137 mAb mediated anti-tumor immunity, which is recapitulated in FcγRIII$^{-/-}$ mice. These findings suggest, for the first time, that Fc-FcγR interactions play an important role in defining the function of anti-CD137 mAbs in the EL4E7 tumor model. Furthermore, our data indicate that this enhanced anti-tumor immunity is associated with increases in the number of phenotypically activated DCs in the spleen. Because these DCs express FcγRIII, our results suggest a paradigm, at least in the EL4E7 tumor model, in which anti-CD137 mAb preferentially enhances the number of activated DCs in tumor bearing FcγRIII$^{-/-}$ mice, with a secondary increase in the number of effector T cells, resulting in improvement in the anti-tumor response (Figure 26).

To begin exploring our hypothesis, we first verified that anti-CD137 mAb requires CD137 to be present in the host using CD137$^{-/-}$ mice in the EL4E7 tumor model. We observed a loss of anti-CD137 mediated anti-tumor immunity in CD137$^{-/-}$ mice, implying that interactions between anti-CD137 mAb and host cells expressing CD137, but not anti-CD137 mAb interactions with tumor, are required for the anti-tumor response. We further confirmed the EL4E7 tumor cells do not express CD137 by assessing CD137 expression on EL4E7 tumor cells *ex vivo* and after IFNγ stimulation *in vitro*. The lack of CD137 expression in both assays confirmed that anti-CD137 mediated anti-tumor responses require host expression of CD137.

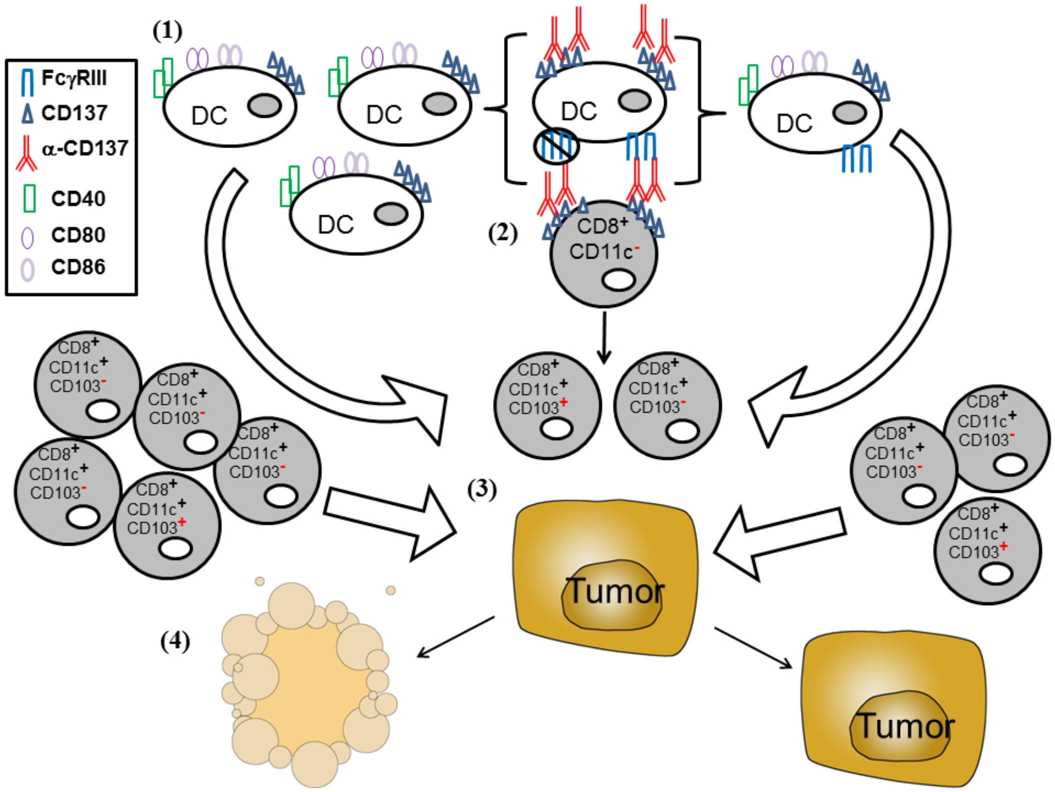


Figure 26: FcγRIII suppresses the anti-CD137 mAb stimulation of DCs reducing the CD8$^+$ T cell anti-tumor response

1. Anti-CD137 mAb stimulation of DCs enhances expression of CD40, CD80, and CD86, but FcγRIII ligation inhibits DC expansion. **2.** Anti-CD137 mAb stimulated CD8$^+$ T cells producing two populations: CD8$^+$CD11c$^+$CD103$^-$ effectors or CD8$^+$CD11c$^+$CD103$^+$ suppressors. **3.** The expanded DC population increases the number of CD8$^+$CD11c$^+$CD103$^-$ effectors, which interact with the tumor cells. **4.** The expanded CD8$^+$CD11c$^+$CD103$^-$ effectors results in tumor cell death. Whereas the FcγRIII+ DC produces fewer CD8$^+$CD11c$^+$CD103$^-$ effectors so the tumor persists.

These data confirm the EL4E7 model is appropriate for determination of the role of Fc-FcγR interactions in the anti-tumor function of anti-CD137 mAb, independent of direct tumor interactions.

The anti-tumor function of anti-CD137 mAbs was defined in preclinical mouse models using rat-anti mouse mAbs (131, 156, 181, 192, 199, 334, 338, 341, 344-346). Based on these studies, we elected to utilize these same anti-CD137 mAbs in our work. Due to species differences between rat and mouse Fc, we first determined how rat IgG2a, the isotype of anti-CD137 mAb, interacts with mouse FcγRs. Using mouse IgG2a as a control for binding to all mouse FcγRs, we found anti-CD137 mAb (2A) bound to mouse FcγRIIB and FcγRIII with similar affinity and kinetics. Interestingly, 2A weakly bound to mouse FcγRIV, and did not bind to mouse FcγRI, which are the two high affinity receptors (101). The FcγR binding profile for 2A is similar to the binding profile of mouse IgG1, which is perhaps not surprising, since rat IgG2a has structural homology to mouse IgG1 (92, 335). The previously published data combined with our findings suggest that the rat IgG2a isotype has the potential to interact with mouse FcγRs in a similar manner as mouse IgG1.

To define the import of the Fc-FcγR interactions on the anti-tumor immunity elicited by anti-CD137 mAb therapy, we used the EL4E7 tumor model in FcRγ$^{-/-}$ mice, which are deficient of all the activating FcγRs, but still express FcγRIIB, the inhibitory receptor (336). Based on the lack of activating FcγRs in these mice, we hypothesized that the anti-tumor immune response elicited by 2A treatment would be diminished, since these receptors are associated with pro-inflammatory effects. In contrast to our initial hypothesis, 2A treatment of EL4E7 in FcRγ$^{-/-}$ mice resulted in improved survival compared to WT controls.   To further test the robustness of the anti-tumor response in the FcRγ$^{-/-}$ mice, we employed a more aggressive tumor model, changing the timing of the first dose of 2A from Day 7 to Day 10 post tumor inoculation. FcRγ$^{-/-}$ mice with the later dosing schedule still experienced an improved anti-tumor response with 2A treatment compared to control groups. To confirm the generation of a memory response, a hallmark of CD137 stimulation, we rechallenged the cured mice with EL4E7 tumors, and found the mice did not develop tumors. These data suggest that there is an activating FcγR mediated regulation of the anti-tumor response elicited by anti-CD137 mAb therapy. However, these findings do not eliminate the possibility of FcγRIIB involvement in the anti-CD137 mAb mediated anti-tumor response.

In order to determine whether these findings were secondary to isolated expression of FcγRIIB or the absence of activating receptor expression, we performed an analogous series of studies in FcγRIIB, FcγRIII, and FcγRIV deficient mice. The anti-tumor activity of 2A was comparable in FcγRIIB$^{-/-}$ and WT mice, indicating FcγRIIB is not involved in the regulation of anti-CD137 mAb therapy.   This result was surprising as ligation of FcγRIIB results in inhibition of activating signaling pathways, and because two recent studies showed the dependence of anti-CD40 mAb function on FcγRIIB (92, 93, 288).   While there was a trend towards improved outcomes in 2A treated FcγRIV$^{-/-}$ mice, this failed to reach statistical significance.   In contrast to mice deficient in FcγRIIB or FcγRIV, FcγRIII$^{-/-}$ mice responded better than the WT mice to anti-CD137 mAb therapy. These data do not rule out the potential role of FcγRIV in modulating anti-CD137 mAb tumor responses. Our findings demonstrate that the improved survival of the 2A treated FcγR$^{-/-}$ mice can be fully reproduced in FcγRIII$^{-/-}$ mice, and suggest that the interplay between the Fc fragment on anti-CD137 mAb and host FcγRIII, impairs 2A mediated anti-tumor immunity.

Importantly, these results in FcγRIII$^{-/-}$ mice are not specific to an individual clone of anti-CD137 mAb based on the significantly improved survival in anti-CD137 mAb clone 3H3 (rat IgG2a) treated FcγRIII$^{-/-}$ mice. Though the survival was significantly improved in the 3H3 treated FcγRIII$^{-/-}$ mice, 3H3 treatment did not result in complete tumor resolution as seen with 2A treatment. The differences in the response between the two clones of anti-CD137 mAb may involve the blocking potential of CD137L by 3H3 or the difference in the epitope recognized by the antibody. The significant survival in the FcγRIII$^{-/-}$ mice treated with 3H3 therapy indicates our observations in 2A treated FcγRIII$^{-/-}$ mice are not limited to a single clone of antibody.

To confirm the enhanced anti-CD137 mAb mediated anti-tumor response was not limited to the EL4E7 lymphoma model, we used the MC38 colon cancer model based on the incomplete response with only 2A treatment (337). The anti-tumor response elicited by anti-CD137 mAb was not enhanced in MC38 tumor bearing FcγRIII$^{-/-}$ mice, as seen in the EL4E7 tumor model. The lack of response in the MC38 model implies that FcγRIII deficiency does not provide adequate stimulation to enhance the 2A mediated anti-tumor response in this model. The inability to recapitulate the improved anti-tumor response in the MC38 colon cancer model suggests additional tumor factors modulate the efficacy of anti-CD137 mAb. These data reflect the variability of responses in patients after the use of cancer immunotherapy.

We next sought to understand the mechanisms underlying the improved anti-tumor efficacy of anti-CD137 mAb in FcγRIII$^{-/-}$ mice. Specifically, we performed a detailed kinetic analysis of FcγRIII$^+$ cells and CD8β$^+$ T cells to identify the potential cell population(s) involved in the enhanced 2A mediated tumor rejection. The expansion of tumor specific CD8+ T cells is a hallmark of the anti-CD137 mAb elicited anti-tumor immune response (97, 155, 192). As anticipated, we observed a profound increase in CD8β$^+$ T cells following anti-CD137 mAb therapy, which was dramatically enhanced in the FcγRIII$^{-/-}$ mice. The increase of CD8β$^+$ T cells in the 2A treated mice occurred at Day 12 and peaked at Day 16 post tumor inoculation. The changes in the CD8β$^+$ T cells population did significantly correlate with tumor volume on Day 16, which suggests the CD8β$^+$ T cells are involved in the anti-tumor immune response. Since CD8β$^+$ T cells do not express FcγRIII, our data suggest that a FcγRIII$^+$ cell population regulates the secondary expansion of these CD8β$^+$ T cells (101).

FcγRIII is expressed by macrophages, DCs, NK cells, NKT cells, and MDSCs (101). We performed a kinetic experiment to determine changes in the cell numbers of these FcγRIII$^+$ cells, since these populations can potentially modulate the anti-tumor response. We found increases in all cell populations, except in NK and NKT cells. The changes in the DC populations corresponded with the kinetics of the CD8β$^+$ T cell populations with increases in DC cell numbers on Day 12 and peaked on Day 16 only in the 2A treated FcγRIII$^{-/-}$ mice. DCs are a good candidate cell population for the enhanced anti-tumor immune response elicited by anti-CD137 mAb in the FcγRIII$^{-/-}$ mice. First, FcγRIII$^{-/-}$ and WT DCs have equal ability to present OVA antigen (325). These data suggest that the enhanced anti-tumor immunity in the 2A treated FcγRIII$^{-/-}$ mice is due to a loss of the suppression of CD137 signaling by FcγRIII engagement. Furthermore, anti-

CD137 mAb enhances CD40 stimulation of DCs, which corresponds to an increase of tumor infiltrating T cells (199, 200). These findings combined with our data suggest CD137 stimulation of DCs in the absence of FcγRIII interactions results in enhanced antigen presentation capabilities that promote the secondary expansion of CD8β$^+$ T cells at least in the EL4E7 tumor model.

As a first step to characterize the APC function of DCs in the 2A treated FcγRIII$^{-/-}$ mice we defined the expression levels of CD40, CD80, and CD86 on splenic DCs. The 2A treated FcγRIII$^{-/-}$ mice had significantly more DCs expressing CD40, CD80, and CD86 compared to 2A treated WT mice. The increases in CD40, CD80, and CD86 expressing DCs in the FcγRIII$^{-/-}$ mice did significantly correlate to tumor sizes on Day 16, suggesting the changes in DCs are involved in the improved anti-tumor immune response. We attempted to confirm these phenotypic results using a tumor antigen presentation assay, but were unable to successfully induce T cell proliferation with the tumor antigen. These data suggest that FcγRIII engagement prevents CD137 enhancement of antigen presentation by DCs, ultimately limiting the EL4E7 specific CD8β$^+$ T cell response.

FcγRIII is associated with pro-inflammatory and anti-inflammatory responses (96, 330, 347-349). The anti-inflammatory mechanism of FcγRIII signaling involves tonic signaling through the receptor that is able to activate an associated phosphatase, SHP-1, which induces the "inhibitsome" that blocks activating signaling(326, 330, 350). Chu *et. al.* found DCs lacking ITAM signaling co receptors DNAX activation protein of 12 kDa (DAP12) and the Fcγ chain have improved TLR responses with increases in expression of CD80 and CD86 and pro-inflammatory cytokines (351). Further confirmation of FcγRIII on murine DCs regulating an immune response is the induction of a regulatory DC population by IVIG (348). Additionally, IVIG directly alters antigen presentation by decreasing expression of co-stimulatory molecules on DCs resulting in a loss of a T cell response (352). These data provide potential mechanisms for the regulation of the DC population by FcγRIII in the anti-tumor response against EL4E7.

Our data suggests FcγRIII regulates CD137 signaling that enhances the antigen presenting function of DCs, which ultimately influences the anti-tumor immune response in the EL4E7 tumor model. These findings add additional support to the inhibitory function of FcγRIII biology, and support the importance of the Fc-FcγR interactions in the function of monoclonal antibody therapy. Finally, in monoclonal antibody development careful consideration should be taken in isotype selection due to the two functional sides of the antibody, the Fab and Fc.

Future Directions

This study provides an important link between Fc-FcγR interactions and the anti-CD137 mAb mediated anti-tumor immune response. There are six experimental questions that still remain. 1. What is the role of FcγRIV in anti-CD137 mAb mediated anti-tumor response? 2. Are the findings of this study limited to only lymphomas? Or can using anti-CD137 mAb as an adjuvant to another anti-tumor therapy have enhanced anti-tumor immunity by inhibiting Fc-FcγR interactions? 3. What factor(s) causes the differences in the anti-tumor immunity elicited by the two clones of anti-CD137 mAb, 2A and 3H3? 4. Does blockade of Fc-FcγRIII interactions in WT mice enhanced the anti-tumor immune response? 5. Are FcγRIII$^{-/-}$ DCs cell population responsible for the modulation of the anti-tumor immunity mediated by anti-CD137 mAb? 6. How are the NK cells involved in the anti-tumor response elicited by anti-CD137 mAb in the FcγRIII$^{-/-}$ mice?

Our data suggest a trend towards an improved anti-tumor immune response despite the low binding interaction between 2A and FcγRIV. These data do not rule out the potential role of FcγRIV in modulating anti-CD137 mAb tumor responses. FcγRIV expression is limited to Ly6C^{lo} monocytes, macrophages, and neutrophils and these cells are common tumor infiltrating leukocytes (281, 282). CD137 expression is highest on tumor infiltrating lymphocytes limiting the potential interaction with FcγRs at the tumor site (203). The expression patterns of CD137 and FcγRIV *in vivo* may surmount the weaker interaction of FcγRIV to rat IgG2a by increasing the concentration of 2A at the tumor. In fact, anti-CD152 (CTLA-4) mAbs mechanism of action includes Fc-FcγRIV interactions between T regulatory cells (Tregs) and myeloid derived suppressor cells

(MDSCs), which results in Treg depletion at the tumor (94). These potential expression patterns of each receptor may explain the improvement of the anti-tumor response in the 2A treated FcγRIV$^{-/-}$ mice. A kinetic experiment in the FcγRIV$^{-/-}$ mice would provide a good starting point to determine potential cell populations involved in the changes in the anti-tumor response elicited by anti-CD137 mAb. Additionally, *in vitro* stimulation of cells expressing both CD137 and FcγRIV with CD137 stimulation and FcγRIV stimulation could be used to determine if a CD137 effector function is lost with FcγRIV stimulation.

Our data demonstrate the Fc-FcγRIII interaction is regulating the anti-CD137 mAb mediated anti-tumor immune response in a single tumor model. First, the specificity of the enhanced anti-CD137 mAb anti-tumor response may be limited to lymphoma. Testing the anti-tumor immune response elicited by anti-CD137 mAb in different lymphoma models would verify this hypothesis. Additionally, anti-CD137 mAb is commonly administered as an adjuvant therapy in cancer immunotherapies. The enhanced anti-tumor immune response elicited by anti-CD137 mAb could be tested in tumor models that utilize other primary therapies, such as a peptide vaccine to determine the effects of Fc-FcγR interactions on the anti-tumor function of anti-CD137 mAb. Using the adjuvant approach for anti-CD137 treatment would make the research more relevant to the clinical use of anti-CD137 mAb.

An interesting observation is the difference in the anti-tumor response elicited by the two clones of anti-CD137 mAb. Based on our blocking data, the ability of 3H3 to block CD137L interactions may potentially modulate the anti-tumor immune response. The contribution of CD137L interactions could be tested in the EL4E7 model utilizing

CD137L$^{-/-}$ mice. If CD137L is involved in modulating the 2A response then the anticipated outcome in the CD137L$^{-/-}$ mice would be similar tumor regression as seen in with 3H3 treatment.

Our study only uses one method, a tumor model in FcγRIII$^{-/-}$ mice, to identify the contribution of Fc-FcγRIII interactions that inhibit the anti-tumor immune respone elicited by anti-CD137 mAb treatment. To confirm the role of FcγRIII would be to block the receptor in WT mice, or to modify the antibody to reduce binding to FcγRIII in WT mice. The anticipated result by either approach would be an enhanced anti-CD137 mAb elicited anti-tumor immune response in the WT mice.

We have only provided a correlation between the changes in DC numbers and the enhanced anti-tumor resolution in the 2A treated FcγRIII$^{-/-}$ mice.   To confirm the role of the DC population in mediating the enhanced anti-tumor immune response we could perform two experiments: 1. deplete the DC population in the FcγRIII$^{-/-}$ mice, or 2. adoptively transfer FcγRIII$^{-/-}$ DCs into WT mice. The depletion experiment should reduce the anti-tumor response in FcγRIII$^{-/-}$ mice, whereas the adoptive transfer experiment should enhance the anti-tumor response in WT mice.

Finally, our data do not exclude a role for NK cells in modulating the anti-tumor immunity elicited by anti-CD137 mAb. NK cells regulate antigen specific CD8^{+} T cells and DCs through cytotoxicity and cytokine production, which modulates an immune response (353). We found inconsistencies between experiments in the number of NK cells in the 2A treated FcγRIII$^{-/-}$ mice. The anti-tumor response mediated by anti-CD137 mAb involves NK cells, and this confounds the results of a depletion experiment to conclusively determine the involvement of FcγRIII deficiency (192). An alternative experiment would be the adoptive transfer of WT NK cells into the FcγRIII$^{-/-}$ mice to determine how these cells influence the CD8^{+} T cells and DCs. The adoptive transfer of the WT NK cells would reduce the anti-tumor immune response and the cell populations of the 2A treated FcγRIII$^{-/-}$ mice indicating the NK cells are involved in the enhanced anti-tumor response.

This study suggests FcγRIII regulates CD137 signaling that enhances the antigen presenting function of DCs, which ultimately influences the anti-tumor immune response in the EL4E7 tumor model. This study provides supports the regulatory function of FcγRIII signaling, and provides a foundation for the further understanding of the mechanisms involved in the anti-tumor immunity elicited by anti-CD137 mAb. Finally, this study underscores the importance of understanding Fc-FcγR interactions in the function of anti-CD137 mAbs to enhance the therapeutic use of the antibody.

Appendix- Associated liver toxicity with anti-CD137 mAb therapy

Background

Antibody therapy provides the optimal surrogate stimulation of CD137 because of the ability to cross link through the Fc, which multimerizes CD137 (354). An antibody provides two key advantages over a recombinant CD137L for therapeutic use: 1. the ability to stimulate the receptor without prior multimerization for interaction, which is a requirement for CD137L interaction, and 2. an improved serum half-life reducing the dosage and frequency of dosing (355, 356). Unfortunately, anti-CD137 mAb therapy is associated with toxicities, specifically hepatitis, enlargement of lymphoid organs, and systemic inflammatory cytokine production, resulting in the early termination of clinical trials (clinical trial# NCT00351325, http://clinicaltrialsfeeds.org/clinical-trials/show/NCT00351325) (357, 358).

Four possible approaches to reducing toxicities associated with anti-CD137 mAb therapy include: 1. reducing the dose, 2. intratumoral administration of the antibody, 3. using alternative reagents, such as streptavidin linked recombinant CD137L, and 4. combining anti-CD137 mAb with other immune modulatory mAbs. The reduction in the dose of anti-CD137 mAb is currently being tested in clinical trials (clinical trial# NCT01775631, http://clinicaltrialsfeeds.org/clinical-trials/show/NCT01775631; clinical trial# NCT01775631, http://clinicaltrialsfeeds.org/clinical-trials/show/NCT01775631).

CD137 expression is highest at the tumor site so an intratumoral injection provides a means to reduce toxicity by reducing dose (203). The intratumoral injection would eliminate systemic administration, which causes the lymphocyte infiltration into the liver (203). Of course intratumoral injections are not always possible in patients.

Alternative reagents to anti-CD137 mAb for CD137 stimulation include CD137L and aptamers. The use of recombinant CD137L (4-1BBL) does not induce the same toxicities as anti-CD137 mAb while achieving similar activation of tumor specific $CD8^+$ T cells (112, 193, 357, 359). Interestingly, the use of recombinant CD137L additionally, induces CD4+ T cell mediated anti-tumor immunity (360). The downfall to recombinant CD137L is the reduced stability and required multimerization. Methods to enhance stability and multimerization of recombinant CD137L include transfecting the ligand into tumor cells, or fusing the domains with linker protein, such as streptavidin or Fc (193, 361, 362). Aptamers, high-affinity single stranded nucleic acid ligands, to CD137 are another method to induce tumor specific CD137 stimulation through linking the aptamer to biotin to multimerize the ligand and stimulate $CD8^+$ T cells (363).

A final method to improve upon associated toxicities is to combine anti-CD137 mAb therapy with other monoclonal antibodies directed against co signaling molecules. A potential combination strategy is anti-CD137 mAb with anti-CD134 (OX-40) mAb based upon the expression patterns of each receptor. CD134 and CD137 are both expressed on T cells, but CD134 is more abundant on CD4 T cells and CD137 is more abundant on CD8 T cells (334, 364). The outcome of dual stimulation of $CD8^+$ T cells through CD137 and CD134 is the induction of super effector $CD8^+$ T cells with increased expression of CD25 and IL-7Rα (365). Combined CD137 and CD134 stimulation of CD4+ T cells induces cytotoxic Th1 differentiation driven by the T-box transcription factor, Eomesodermin (366). Another potential combination is anti-CD137 mAb and anti-CD152 (CTLA-4) mAb in order to counteract the lymphocyte liver infiltration induced by CD137 stimulation, and prevent the development of autoimmunity induced by CD152 blockade (367). The addition of anti-CD137 mAb to anti-CD152 mAb therapy prevents autoimmunity by reducing anti-dsDNA autoantibody deposition, and anti-CD152 mAb to anti-CD137 mAb reduces lymphocyte infiltration into the liver (367).

Results

Using the kinetic model described in the methods chapter, we determined the infiltrating lymphocytes in the liver of tumor bearing mice either treated with 2A or Rat IgG control. We found a similar pattern of infiltration in the livers of 2A treated mice compared to the spleen (Figures 22 and 27). We did not find any reductions in the lymphocyte infiltration in the FcγRIII$^{-/-}$ mice compared to the WT mice. In fact, the same enhanced lymphocyte infiltration in the spleen of 2A treated FcγRIII$^{-/-}$ mice were found in the liver. These data suggest FcγR interactions are not involved in the toxicities associated with anti-CD137 mAb therapy.

Conclusion

The FcγRIII$^{-/-}$ mice have enhanced numbers of liver infiltrating lymphocytes after anti-CD137 mAb treatment compared to WT mice. This suggests FcγRIII engagement is minimizing liver infiltrating lymphocytes, which may reduce anti-CD137 mAb toxicity. Schabowsky *et.al.* found C1q$^{-/-}$, C3$^{-/-}$ and FcRγ$^{-/-}$ mice have similar toxicity compared to WT mice after anti-CD137 mAb stimulation, and our data further implies Fc-FcγR interactions are not involved in anti-CD137 mAb mediated liver toxicity (357).

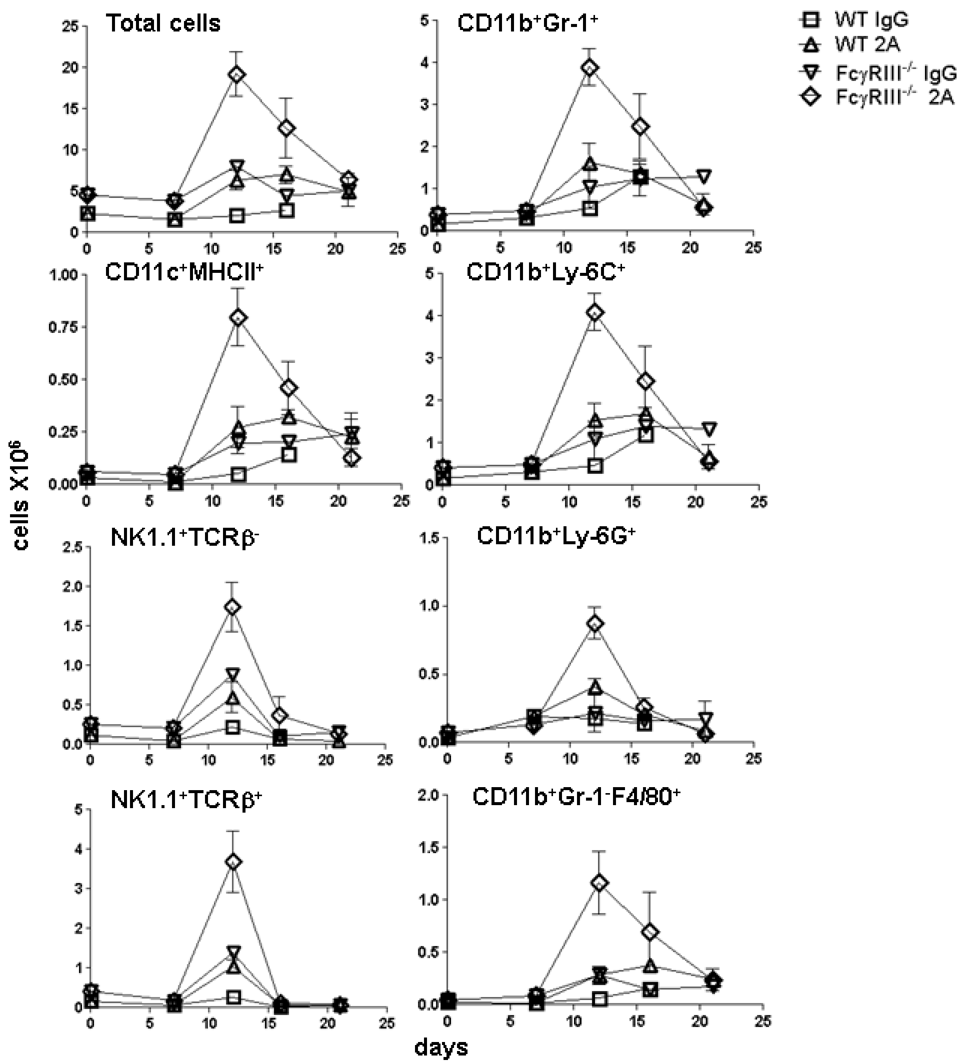


Figure 27: Leukocyte infiltration in the liver is preferentially increased in the 2A treated FcγRIII$^{-/-}$ mice

Mice inoculated with $8x10^6$ EL4E7 cells on Day 0. Mice treated with 100μg of Rat IgG or 2A on Day 7 and Day 10. Liver harvested from 2 or 3 mice per group on Days 0, 7, 12, 16, and 21. Trypan blue dye exclusion used to determine live cell population. The cell number for each organ multiplied by the percentage for a lymphocyte gate to determine total lymphocytes. Then the total lymphocyte population multiplied by the percentage for the described cell population to determine cell number per organ.

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