Understanding How the Costimulatory Molecule OX40 Engagement on T Cells Can Boost Immune Responses for Vaccine Applications and Immunotherapy.

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Understanding How the Costimulatory Molecule OX40 Engagement on T Cells Can Boost Immune Responses for Vaccine Applications and Immunotherapy

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2. OX40 is required for 4-1BB signaling to program antigen-specific CD4 T cells to express Eomes and Runx3

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Chapter 1: INTRODUCTION

Understanding how T cell biology impacts vaccine development

Vaccination

1. **History of vaccines**

Over the past century, one of the most prominent contributions to good public health both in humans and animals is vaccination. Evolution of effective methods that strengthen eradication of diseases through vaccination has been and continues to be essential for preventive medicine. A generally accepted definition of vaccination is an administration of either a live-attenuated or dead pathogen to stimulate an immune response and trigger adaptive immunity, a sub-division of immunity capable of developing a memory to specific pathogens. The weakened antigen or pathogen is presented to immune cells by the antigen presentation mechanism, the major histocompatibility complex (MHC). Memory is developed by immune cells to specific antigens thus enhancing an easier response upon subsequent infection. In addition, vaccines are usually utilized in conjunction with adjuvants, immunological agents that enhance the immune response by increasing antibody production and promoting long-lasting responses.

Both humans and agricultural subjects (farm animals) have immensely benefitted from vaccines over several decades till present. For example, one of the crowning glory of the science of vaccinology is the eradication of rinderpest for cattle [Barret et al., 2016].

Interestingly, the genesis of vaccination and other immunization methods, variolation (inoculation with live smallpox matter) has been attributed to various progenitors; yet prominent, but not the originator is Sir Edward Jenner. Edward Jenner is one of the earliest immunologists to use variolation, in that era a widely practiced immunization method to treat smallpox. He treated James Phipps suffering from smallpox with lesions from Sarah Nelms affected also with smallpox. James was administered lesions twice with a nine-day interval between treatments. Successful therapy on James concluded Edward Jenner’s unfolding of
protective immunity. Edward Jenner noted that a person inoculated with the cow-pox virus would develop a mild form of the disease. And would also attain protective immunity from impending smallpox infections leading to the discovery of the smallpox vaccine. In 1798 Jenner published his observation in his seminal work titled ‘An inquiry into the causes and effects of Variolae Vaccinae’ [Lahariya et al., 2015]. Although, Jenner’s smallpox vaccine did not confer absolute immunity and subsequent revaccination were necessary, his work was a huge immunological impact on eradicating cowpox disease.

Variolation overlapped with other previous smallpox treatments such as inoculation which had been established pre-Edward Jenner’s time by six people; Peter Plett, Benjamin Jesty Jobst Bose, and other three individuals. These individuals mostly used inoculation defined as subcutaneous instillation of smallpox virus into nonimmune persons. For example, in 1774, Benjamin Jesty’s unethical and undocumented but well-meaning experiment with cowpox matter inoculation on his wife and two children, twenty years before Edward Jenner’s time contributed to this overlap of inoculation with variolation. Likewise, scholars and physicians in Asia have been recognized to be the pioneers of inoculation. In the Common Era, primary physicians such as Thucydides in 430 BC and Rhazes (also known as Abu Bakr) an alchemist in 910 A.D were practicing inoculation [The College of Physicians of Philadelphia, www.historyofvaccines.org, 2018]. These scholars posited that people affected by smallpox were protected from future infections without treatment [Wujastyk, 2001]. Strong evidence of well-documented details also describes widely practiced inoculations in Bengal and Bombay for prevalent smallpox in the 18th century. John Zephaniah Howell one of the first surgeons to study Indian antiquities presented this proof to the Royal College of Physicians in 1767 [Coverage evaluation Survey: All India report, 2009]. Another impactful scientist was Louis Pasteur a French chemist and microbiologist, in the 19th century who discovered accidentally immunity upon affecting chicken with Pasteurella multocida which causes fowl cholera. Chicken inoculated with a month old cultured bacterium developed immunity to the fowl cholera upon re-infection. In 1870, Pasteur applied his observation as an immunization technique to diseases like anthrax and rabies.
Pasteur produced the first vaccine for rabies by growing the virus in rabbits and then weakening it by desiccating the affected nerve tissue of the rabbit [Wood et al., 2015].

The positive outcomes of vaccine development have been employed by the World Health Organization in the eradication of diseases through frequent immunization campaigns. For example, diseases such as poliomyelitis, measles, diphtheria, tetanus, cholera, smallpox, *Haemophilus influenzae* type B rubella and schistosomiasis (bilharzia) have been successfully wiped out globally. Also, a recent pandemic Ebola has been controlled considerably through successful experimental vaccination trials [Honey, K. 2004]. More presently, vaccination is being used as a therapeutic option or in conjunction with other treatments for some types of cancers and sexually transmitted infections such as Human Papillomavirus (HPV) [Stanley, M. 2008].

2. **Current vaccine development**

As history has noted, vaccinology has drifted from the era of mere implicit observations gradually over the years to a more comprehensive therapeutic paradigm supported by both basic and applied science. Parallel to this, modern-day immunologists have advanced by inculcating cutting-edge designs such as bioinformatics, genetic engineering, more purified peptides and other complex techniques to develop vaccines. Experiments postulating that plasmid DNA and RNA vaccines can induce both humoral and cellular immune responses in a variety of disease models has stimulated excitement in the vaccinology community. The basis of DNA or RNA vaccines rests on the idea of recombinant DNA or transcript transfers resulting in protein expression which can be presented by the MHC to T cells and B cells resulting in an immune response and memory. For example, hepatitis B vaccine was developed by expression of hepatitis B surface antigen in yeast [Stephene, 1990], meningococcal type B vaccine developed by reverse vaccinology [Serutto et al., 2012] and tetravalent live-attenuated dengue vaccine using reverse genetics [Guy et al., 2015]. Continuous changes in the science of vaccinology through novel ideas will spur the field
and elevate vaccine efficacy. Even so, there is a need to increase the immunogenicity of modern purified antigens and also altering antigenic epitopes.

Although vaccines have been very effective against a number of diseases, vaccines which require a productive B-cell and T-cell response are lacking against some of the major causes of mortality worldwide including malaria and human immunodeficiency virus (HIV) [Pantaleo and Koup, 2004; Hoft, 2008; Reyes-Sandoval et al., 2009]. There is an imperative need for more effective preventive vaccines against infectious diseases. Conventional vaccines comprised of attenuated antigen and adjuvant have proven to be ineffective in eliciting a robust immune response. The evidence of vaccine resistances is focused on the spread of mutants by pathogens to display different epitopes from vaccine presented epitopes, thereby escaping immune recognition [Gandon et al., 2001]. Also, vaccination against intracellular organisms such as the agents of tuberculosis, malaria, leishmaniasis, and human immunodeficiency virus infection require cell-mediated immunity that are either not available or not uniformly effective. Enforced costimulation in concomitant with antigen and an adjuvant has been successful in boosting vaccine efficacy in murine and primate experimental observations [Taraban et al., 2013]. During Cytomegalovirus (CMV) infection, presentation of a restricted set of immunodominant CMV antigens [Stephenne, 1990] requires certain costimulatory receptor-ligand pairs of which CD27–CD70 and OX40–OX40L interactions are important [Van der Burg et al., 2017]. Enforced OX40 stimulation resulted in superior mouse CMV (MCMV)-specific CD4+ as CD8+ T cell responses when applied to booster synthetic long peptides (SLP) vaccination. Furthermore, the systemic intravenous (i.v) administration of foreign proteins (ovalbumin) or derived synthetic peptides with TLR ligands and agonistic CD40 monoclonal antibodies (mAbs) can generate large numbers of CD8 T cells in mice [Celis et al., 2017]. So far, most successful vaccines rely on generating B cell memory responses via neutralizing antibodies (Abs) against a given pathogen [Pasare & Jain, 2017; Burton, 2002]. There has been a limited success in defining and inducing protective pathogen-specific T cell immunity [Pasare & Jain, 2017]. Further understanding of T cell biology and costimulatory molecules will stimulate alternative novel ways of boosting vaccine efficacy.
Basic idea of T Cell focused vaccines

The immune system is comprised of the innate and adaptive complexes made up of unique multiple cell types. The two complexes inextricably function to clear pathogens. The innate system is typically the first line of defense during an infection and the adaptive complex confers lifelong protective immunity through memory. The innate immune cells include dendritic cells, macrophages and granulocytes (neutrophils, eosinophils, basophils), mast cells and natural killer cells all arising from both the myeloid and lymphoid lineages. The adaptive immune system includes Bursa (B) and Thymus (T) cells, both arising from only the lymphoid lineage; B lymphocytes develop and mature in the bone marrow or the spleen while T lymphocytes mature in the thymus. B lymphocytes protect the humoral by secreting neutralizing or opsonizing immunoglobulins and pathogens. Opsonized extracellular pathogens are either directly detected and phagocytized by innate cells specifically macrophages. In addition, opsonized cells or pathogens can activate the complement system as a result of Fc regions of the immunoglobulin engaging an Fc receptor (FcR). T cells mediate itself or other cells’ immunity by both providing help (CD4) to innate and other adaptive cells or directly killing (CD8) intracellular pathogens.

Immune cells all express membrane bound or soluble receptors which interact with natural or artificial ligands for cellular functions. All immune cells posses’ receptors for recognition of pathogens. The innate system recognizes pathogen-associated molecular patterns (PAMP) by pathogen recognition receptors (PRR) on the cell surface, cytoplasm, and endosome for example Toll-like receptors (TLR) and Nod-like receptors (NLR). B lymphocytes are stimulated by recognition of surface immunoglobulin BCR and antigen [Cox & Coulter, 1997]. T cells recognize the tandem antigen and MHC complex through a receptor complex [T cell receptor (TCR)]. T cells are comprised of helper and cytotoxic cell subtypes marked by co-receptors CD4 and CD8 respectively. The coreceptors are inextricably involved in signaling to determine specificity to MHC molecule that presents antigen to subtype receptor complex. Upon antigenic stimulation, through MHC II naïve CD4 T cells can differentiate into a helper lineage including Th1, Th2, Th17, Th9, Tfh, and Tregs. Differentiation of T cells is dependent on several extrinsic and intrinsic factors to manipulate the
immune responses to specific pathogen invaders. CD8\(^+\) naïve T lymphocytes differentiate into a direct killing phenotype marked by secretion of granzyme and perforin after antigenic stimulation by the MHC class 1 molecule of both antigen presenting cells (APCs) or other cell types. T lymphocytes are essential for clearing intracellular invaders manifested through direct target-cell killing, the induction of apoptosis in target cells and the release of chemokines and antiviral cytokines that can clear the viral infection from target cells [Burton, 2002]. Additionally, T lymphocytes can provide help to plasma cells (matured B cells that secrete immunoglobulins) to skew isotype of antibodies secreted to neutralize extracellular pathogens.

After T cells mature in the thymus, they migrate through the lymphatic system and upon infection, are presented antigen by APCs in the nearest secondary lymphoid tissue. The dendritic cell (DC), a professional APC uptake antigen either by receptor-mediated endocytosis, or fluid-phase pinocytosis resulting in an endosome formation which fuses with a lysosome to form an endolysosome [Cox & Coulter, 1997]. The direction of dendritic cell migration to a particular regional draining lymph node or spleen is guarded by external signals in the form of chemokines and cytokines. In the dendritic cell, processed antigen encounters with MHC-II or MHC-I which have already been assembled in the endoplasmic reticulum (ER) [Cox & Coulter, 1997]. The resultant complex is then transported to the surface of the APC where the peptide is displayed in association with MHC-II [Cox & Coulter, 1997] or MHC 1 and recognized by the T cell receptor complex (TCR) together with CD3 epsilon, costimulation typically from the B7 family and CD28.

The communication between the innate and the adaptive immune system is critical for the overall protection of the host. For example, not only do APCs present antigen to T cells, APCs has also been reported to secrete Interleukin 1 (IL-1) a proinflammatory cytokine that can attract more CD4\(^+\) (adaptive immune cell) specific for the same antigen promoting clonal expansion to clear infections. T cells differentiate into effector functioning cells owing to the three-signal paradigm. The first signal provided by the innate cells is the presentation by APCs of processed antigen to the TCR complex. The second signal is provided by costimulatory molecules that are upregulated on APCs only when the antigen has associated a PAMP [Pasare & Jain, 2017]. The third signal result from innate cytokines that are produced because of PRR
activation [Pasare & Jain, 2017]. An immune response is mounted after all three signals and T lymphocytes become activated, differentiate into an effector phenotype and thus clonal expansion of antigen-specific T cells to clear the infection. Some subset of lymphocytes after antigenic stimulation differentiate into memory cells and quickly protects the host from reinfection by the same pathogen. The idea of vaccines development rests on the success of all three signals to induce a diverse, but selective T cell response and immunological memory to enhance lifelong protective immunity.

3. **Use of adjuvants in vaccine therapy**

The development of vaccines has been hampered by the poor immunogenicity of antigenic epitopes (determinant part of the antigen that the immune system recognizes), hence the requirement of potent adjuvants to elicit protective immunity. Adjuvants (from the Latin, *adjuvare* = to help) are substances that improve vaccine efficacy and have been utilized from early 1920 [Cox & Coulter, 1997]. Conventional vaccines elicit responses mostly through the adaptive immune system, hence the strategy of adding adjuvants in concomitant triggering an additive innate immune response. In 1916, Le Moignic and Pinoy described the first recorded use of mineral oil and lanolin (wool wax) as an adjuvant in a killed salmonella vaccine [Vogel, 1995]. An adjuvant is mainly grouped into particulate (salts, emulsions, liposomes, proteins, polysaccharides) and non-particulate (cytokines, polymers, toxins, and glycosides) [Cox & Coulter, 1997]. Adjuvants can boost the immune response remarkably by immunomodulation, presentation of the immunogen, induction of cytotoxicity, targeting specific immune cell types’ and molecules that control the release of vaccine contents (immunogen and adjuvant). Particulate adjuvant salts, particularly, aluminum salts and M59 (an oil-based emulsion), predominant components used in licensed human vaccines such as hepatitis A and B, human papillomavirus (HPV) and diphtheria-tetanus-pertussis (DTP) [Lindblad E. B., 2004; Didierlaurent et al. 2009] are known to be good inducers of Th2 responses and strong Immunoglobulin (IgE) responses [Cox & Coulter, 1997]. Th2 responses typically mediate parasitic (helminths) infections. Optimization of the immunogenicity of many new-generation vaccine formulations,
including combination vaccines, will require the use of adjuvants and ligands to other natural existing molecules on the cell surfaces.

4. **Combining vaccines, costimulators, adjuvants and cytokines**

The need to identify potent prophylactic and therapeutic vaccine modalities to prevent diseases such as HCMV has magnified improved therapies explore a trilogy of antigen, adjuvant and enforced costimulation [Van der Burg et al., 2017]. For example, non-particulate adjuvants and TLR ligands liposaccharide (LPS) and Polyinosinic: polycytidylic acid (Poly IC) individually or together induce Type 1 Interferon (IFN) signaling and with CD134 (OX40) costimulation, boost memory and effector response of antigen-specific CD4^+ T cells [Thompson et al., 2005; Maxwell et al. 2000]. Furthermore, they promote IFN-gamma (IFN-\(\gamma\)) secretion in antigen-specific CD4^+ T cell [Shinde Ph.D. Thesis, 2017]. Experimental results combining peptide plus MHC 1 and MHC II epitopes and OX40 activation induced polyfunctional T cell immune responses and thus suggest a new basis in the design of vaccines against persistent viral infections [Van der Burg et al., 2017].

There has been a renewed focus on optimization of prophylactic and therapeutic vaccines that employ T cell immunity against cancers (Vici et al., 2016). The allure of cancer vaccine strategies is the potential to “boost” effector and immunological memory to recognize, target, and eradicate tumor cell. The approach of such contemporary vaccines is through antigen presentation, adjuvant action, and costimulation. Combined vaccine technology-induced immune response could establish a state of immunologic memory that persists after tumor clearance indefinitely suppressing tumor regrowth and provoking rapid responses in infectious diseases [Khort et al., 2016], thus an advantage over other therapies currently in use. For example, in cancer patients, dual activation of tumor-associated antigen (TAA)–specific cytotoxic T Lymphocytes (CTLs) and helper T lymphocytes (HTLs) by DC vaccines induced superior clinical responses than the single CTL vaccine [Celis et al., 2017]. HTLs support CTLs by inhibiting activation-induced cell death and promoting T-cell memory [Celis et al., 2017], but in many instances, HTLs can
directly kill tumor cells [Celis et al., 2017]. Similar to the potential of costimulatory agonists in vaccine therapy, approved mAb antagonists to the checkpoint molecules CTLA-4 and PD-1/PD-L1 is administered to treat patients with melanoma and other advanced cancers [Brahmer et al., 2012; Hodi et al., 2010]. Hereafter regarding immune responses current vaccine experimental data suggest that dual activation of T cells results in better results overall, thus the necessity of additional signals such as enforced costimulation or even better dual costimulation to boost immunogenicity.

A. **Costimulation on T cells**

The immunological paradigm of T cell activation describes, costimulation as signal 2 after TCR engagement. Signal 2 can be a ligation of a costimulatory receptor and its ligand which typically produces an additive effect to the immune cell. Well-characterized costimulatory molecules are the T lymphocyte receptor CD28 and its ligand a transmembrane glycoprotein of the immunoglobulin gene superfamily (B7/BB1) on the APC [Shwartz, 1992, Freeman et al., 1991]. The ligation of CD28 and B7/BB1 potentiates proliferation of T lymphocytes as soon as signal 1 is engaged. Upon MHC presentation of antigen to the TCR complex, activated cytosolic tyrosine kinases p59 (Fyn), p56 (Lck), and ZAP-70 [Chan et al., 1992] downstream of the T cell phosphorylates, phospholipase C-γ1 (PC-γ1). PC-γ1 cleaves phosphatidylinositol bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (DAG). Inositol 1,4,5-trisphosphate sustains an increase intracellular calcium, and diacylglycerol activates protein kinase C. These two signaling pathways synergize to stimulate factors required for IL-2 gene transcription [Crabtree, 1989]. IL-2 (IL-2) is critical for T cell expansion. After signal 1 alone which is TCR stimulation, only minute amounts of IL-2 is produced, further signaling from the ligation of the T cell costimulatory receptor CD28 [Lindsten et al., 1989] and its natural ligand B7/BB1 is required, thus the importance of costimulatory boost. The enhancement mechanism has been shown to be achieved through stabilization of IL-2 mRNA by CD28 [Schwartz, 1992]. Costimulation has been observed to augment IL-2 function by switching from autocrine to a paracrine production (Long and Adler, 2006). Shwartz has made known that signal 1 alone via the TCR induces an anergic state and prevents subsequent T cell proliferative responses [Schwartz, 1992].
exist other costimulatory molecules such as Tumor necrosis factor superfamily (TNFSF) that may influence the duration, magnitude, and quality of T cell-mediated immune responses [Croft et al., 2010; Schreiber et al., 2012; Mittal et al., 2015]. TNFRSF members are antigen-dependent T cell costimulators and their prophylactic and therapeutic stimulation is a potential to augment vaccine efficacy.

B  Costimulation by TNFRSF

TNFSF members such as 4-1BB (CD137, TNFRSF9), OX40 (TNFRSF4) and their respective ligands, 4-1BBL, OX40L are a subset of costimulatory cell surface molecules that have been widely studied for their potential in stimulatory and inhibitory effects on T cell responses [Watts, 2005]. The costimulatory pathway begins with the ligation of TNF receptor and ligand which then recruits the adaptor proteins, TNFR associated factors (TRAFs), with their cytoplasmic tails [Watts, 2005]. TRAFs can directly or indirectly signal downstream activating transactivating factors mitogen-activating protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling cascades often ending in increased survival and proliferation of T cells [Watts, 2005]. TNFR superfamily members are commonly characterized by unique expression patterns, diverse functional activity dependent on the activation status of the specific T cell subtype. TNFRs can activate both the canonical and non-canonical NF-κB pathway [Watts, 2005]. The non-canonical pathway has been reported to occur with delayed kinetics to the classical NF-κB pathway and can sustain cytokines such as IFN-γ and TNF alpha (TNF-α) production by CD8+ effector T cells [Watts, 2005]. A significant characteristic of the TNFRSF that can trigger upon ligation is bidirectional signaling involving individual receptor and ligand (reverse signaling).

OX40 signaling contributes to CD4+ and CD8+ effector T cell activation in multiple murine tumor models. Furthermore, selective blockade and stimulation of OX40 and 4-1BB are under investigation for the inhibition of autoimmunity [Schreiber et al., 2012]. Two TNFRSF agonists; TNFRSF4 and TNFRSF25 have been shown to independently and additively costimulate vaccine-induced CD8+ T cell proliferation following both primary and secondary antigen (Ag) challenge. Although, substantial progress has been
made in elucidating the expression, function and therapeutic potential of TNRSF members, questions still remain unanswered particularly regarding the biology of OX40 and 4-1BB.

(i). **Role of 4-1BB on T cells**

4-1BB (TNFRSF9) is an immune checkpoint molecule discovered in 1989 on murine T-cell lines [Khort et al., 2016]. 4-1BB is typically expressed transiently on T cells post antigenic stimulation [Khort et al., 2016; Watts, 2005] and also expressed on natural killer (NK) cells, regulatory T cells (Tregs) and natural killer T (NKT) cells [Chester et al., 2018]. 4-1BB ligand (4-1BBL) is generally expressed by APCs including B cells, DCs, and macrophages [Chester et al., 2018]. On T cells, binding of 4-1BB by the natural ligand 4-1BBL has been described to positively regulate immunity via enhancement of T cell proliferation, Th1 cytokine production [IL-12 and IFN-γ] and secretion of pro-inflammatory cytokines. [Chester et al., 2018] When 4-1BB is engaged by its ligand the adaptor proteins, TNF-associated factors 1 and 2 (TRAF-1 and TRAF-2) are recruited. TRAFs create docking sites mediating downstream signaling scaffolds [Chester et al., 2018] and activates the master immunoregulatory transcription factor NF-κB (Fig. 1).

![Image](image_url)

**Figure 1.** 4-1BB and 4-1BBL ligation recruits the adaptors TRAF-2 and TRAF-1 to the cytoplasmic tail of 4-1BB resulting in coimmunoprecipitation and downstream signaling.

TRAF2 has constitutive polyubiquitin ligase E3 activity. Multimerization of 4-1BB upon ligand or antibody engagement brings TRAF-2 molecules into close proximity to start transubiquinating one another. These structures generate docking sites for the TAK-1-TAB1/2 kinase complex which phosphorylate downstream substrates, leading to activation of the canonical route of NF-κB via IKKβ and NEMO as well as MAP kinases via MEKK1 [Sanchez-Paulete et al., 2016].
Significantly, 4-1BB signaling prolongs survival of the T cell by contributing to upregulation of the antiapoptotic Bcl-2 family members B-cell lymphoma-extra-large (Bcl-xL), Bfl-1 and B-cell lymphoma 2 (Bcl-2), which protect against activation-induced T-cell death [Chester et al., 2018]. A unique function of 4-1BB is serving as a marker to identify tumor antigen-experienced infiltrating lymphocytes (TILs) [Chester et al., 2018]. 4-1BB agonists amplify both CD8+ T cells and NK cells secretion (Fig. 2) of perforin, granzyme that can infiltrate and lyse tumors by activating the death (Fas ligand (FasL) effector system and secreting of type 1 cytokines (IL-1 and IL-18) in the tumor microenvironment [Chester et al., 2018].

![Figure 2. The immunomodulatory effects of anti-4-1BB therapy.](image)

(A) Anti-4-1BB mAb agonist therapy induces T-cell proliferation and cytokine secretion. (B) On NK cells, 4-1BB agonism enhances Antibody-dependent cytotoxicity Antibody dependent cell-mediated cytotoxicity (ADCC). (C) Agonistic anti-4-1BB mAbs can also stimulate DCs and macrophages (Mψ) to induce antitumor immune responses. (D) Bispecific mAbs targeting both 4-1BB and tumor antigen (HER2) can lock immune effectors and lyse tumor cells in close proximity [Chester et al., 2018].

Metabolically, 4-1BB stimulation encourages oxidative phosphorylation by raising the mitochondrial membrane potential within T lymphocytes and inducing mitochondrial hypertrophy which ultimately enhances TCR signaling [Chester et al., 2018]. In addition, costimulation seems to have a global effect such that even in DCs, 4-1BB ligation accelerates maturation through upregulation of B7 co-stimulatory molecules (CD80 and CD86) and increases survival and production of IL-6 and IL-12 [Chester et al., 2018]. CD 137 single agonism trigger an immunologic memory response in tumor models such as lymphoma,
hepatocellular carcinoma, and colon cancer [Chester et al., 2018]. Currently, the two anti-4-1BB mAbs currently in the clinic are urelumab (Bristol-Myers Squibb), a fully humanized IgG4 mAb, and PF-05082566 (Pfizer), a fully humanized IgG2 mAb currently in three clinical trials, and results of the phase I monotherapy study were recently reported [Chester et al., 2018]. Interestingly, 4-1BB monotherapy has been observed to cause liver inflammation and hepatic toxicity. The adverse effect of 4-1BB monotherapy has led to the exploration of combinatorial regimens. For example, treatment of multiple tumor histology’s with anti-4-1BB plus anti-CTLA-4 (another immune checkpoint molecule) has had a huge preclinical success [Chester et al., 2018] and the potential to ameliorate the toxicities observed with 4-1BB monotherapy [Kocak et al., 2006]. In a B16 melanoma model, treatment with anti-4-1BB plus anti-CTLA-4 mAbs, synergize to induce increased CD8+ T cells and decreased Tregs infiltrating the tumor site [Chester et al., 2018]. In addition, 4-1BB mAbs combined with rituximab(anti-CD20), trastuzumab (anti-HER2), cetuximab (anti-EGFR) and elotuzumab (anti-SLAMF7) synergizes to clear tumors and prolonged survival in murine xenograft models of lymphoma, breast cancer [Chester et al., 2018] and colorectal cancers.

Mechanistically, maximal clinical efficacy will emerge with the combination of costimulation and vaccine strategies because it targets synergistic and non-redundant mechanisms to potentiate an immune response; vaccines elicit an immune response and costimulation potentiates antigen-experienced effector and memory T cell populations. Hence other costimulatory molecules are a compelling class of partnering candidates to conventional vaccines. In the HPV E6/E7-driven TC-1 cell line (murine cervical cancer model), vaccination with HPV E6/E7 peptides in conjunction with systemic 4-1BB agonistic antibody induced durable tumor regression [Chester et al., 2018]. Apart from, anti-4-1BB mAbs other TNFRSF have also proved to be superior in combinations with vaccine therapy or other costimulatory agonists, demonstrating exceptional results relative to CTLA-4 and PD-1 checkpoint inhibitors [Chester et al., 2018]. Investigating the many questions of anti-41BB combinatorial therapy is critical particularly regarding combinatorial regimens as against single agonism [Lee et al. 2004; 2007].
(ii) **Role of OX40 on T cells**

OX40 is a type 1 transmembrane glycoprotein and was originally identified in rat CD4 T cells [Croft et al., 2010]. OX40 is only constitutively expressed on Tregs, but peaks on effector T cells (Th1, Th2 and Th17) 1–2 days post Ag stimulation [Gramaglia et al., 2000; Gramaglia et al., 1998; Rogers et al., 2001; Redmond et al., 2009; Jensen et al., 2010]. The human OX40 molecule has been characterized and the receptor consists of an extracellular binding domain comprising of 4 cysteines rich domain (CRD) repeats [Compaan and Hymowitz, 2006; Bodmer et al., 2002; Naismith and Sprang, 1998, Yamniuk et al., 2016]. The natural ligand to OX40 is OX40L and is expressed on activated APC, activated endothelium and activated T cells [Croft et al., 2010]. Upon formation of a trimeric complex post ligation of OX40 and OX40L, the cytoplasmic domain creates docking sites for TRAF adaptor proteins TNFR-associated factors (TRAF-2, TRAF-3, and TRAF-5), linking receptor activation to various signaling pathways. [Watts, 2005; Arch and Thompson, 1998] TRAF-2 and TRAF-5 has been observed to activate both the canonical (NF-κB1) and non-canonical (NF-κB2) signaling pathway (Fig. 3), by interaction with the inhibitory catalytic subunit IκB kinase (IKK) and NF-κB-inducing kinase (NIK) [Kawamata et al., 1998; Song et al., 2008].

TRAF3 has been proposed to have an inhibitory effect on the NF-κB signaling pathway [Kawamata et al., 1998]. OX40 signaling through the NF-κB pathway upregulates expression of BCL-2 anti-apoptotic (inhibition of induced cell death) family proteins [Bcl-2, Bcl-xL and Bfl-1(A1)] causing suppression of apoptosis, enhanced cell survival [Rogers et al., 2001]. In addition, OX40 signaling through the NF-κB pathway amplifies optimal clonal expansion of CD4+ and CD8+ T cells in primary and secondary response.
to antigen; as well as promoting effective memory generation [Murata et al., 2000; Maxwell et al., 2000; Salek-Ardakani et al., 2003, Bansal-Pakala et al., 2004; Croft et al., 2009; Lee et al., 2006; Fujita et al., 2006]. Apart from the NF-κB pathway, other signaling pathways have been identified downstream of OX40 and OX40L interactions that account for functional consequences of T cell proliferation, survival, cytokine production and suppression of cell death [Watts, 2005; Croft et al., 2009]. For example, Ca$^{2+}$/calmodulin-dependent protein phosphatase 2B/calcineurin (CaN) regulates nuclear factor of activated T cell (NFAT) nuclear translocation inducing greater cytokine gene expression such as IL-2, (a key inducer of T cell proliferation) IL-4 (inducer of Th2 effector phenotype), IL-5 and IFN-γ. [So et al., 2006]. The prospect of OX40 signaling to influence the local cytokine environment milieu can regulate the type of T helper cell lineage that CD4$^+$ T cells differentiate into [Ito et al., 2005; Zhang et al., 2010; Xiao et al., 2016]. Also, OX40 costimulation sustains cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB) in the cytoplasm, a protein involved in the Phosphoinositide 3-kinase (PI3)/PIK pathway increasing expression of survival genes such as Aurora B kinase enhancing cell cycle progression post TCR engagement [Song et al., 2005 and 2007a].

OX40 deficient T cells have been observed to subsequently undergo extensive cell death post activation [Song et al., 2004] and poor proliferation in Leishmania, Nippostrongylus, Lymphocytic Choriomeningitis Virus (LCMV) and influenza virus infections [Croft et al., 2010]. Interestingly, OX40 ligand (OX40L) expressing APCs rescues T cell proliferation. [Croft et al., 2010] Nonetheless, notably, delineation of OX40 signaling alone is limited as with the exception of Tregs, all T cell effector phenotypes need to be activated for OX40 to be expressed. (because naive T cells do not express OX40 until they have been activated through the TCR). Like 4-1BB, OX40 has also become a target for immunotherapy using mAbs [Linch et al., 2015; Weinberg et al., 2011] which can mimic the OX40L to potentiate T cell activation, for example in immune compromised situations or blocking receptor engagement with antagonists to block hyperactive T cell pathologies [Piconese et al., 2011; Salah-Eddine et al., 2010]. Likewise, to many other TNFRSF agonists’, OX40 mAbs has numerous therapeutic benefits especially in cancers and autoimmune disorders.
In cancers, administration of OX40 mAbs has been shown to cause tumor regression, delayed tumor growth and evidence of strong memory responses dependent on expansion of CD8 and CD4 T cells mainly in immunogenic murine models [Sugamura et al., 2004; Ali et al., 2004; Gough et al., 2010; Morris et al., 2001; Song et al., 2007b]. But, studies with human OX40L suggest that higher order oligomerization is required for effective signaling to be induced and thus merely bivalent mAb stimulation of OX40 is unlikely to be optimal [Muller et al., 2008]. To date, the most impressive therapeutic benefits seen with OX40 have involved combination therapy. Hence, another important consideration will be molecules that can be combined with OX40 mAbs to improve efficacy with either in vaccine or immunotherapy.

(iii) **Dual costimulation [OX40 mAb plus 4-1BB mAb (DCO)] of T cells**

Combining immunomodulatory mAbs within the same class can augment therapeutic outcomes [Ryan et al., 2018]. It’s been firmly established that combining OX40 mAb and anti-4-1BB elicits robust CD8 CTL responses and tumor immunity [Lee et al 2004; 2007], a result also confirmed in multiple models by others in the field [Cuadros et al. 2005; Gray et al. 2008]. Simultaneous administration of agonists to the TNF costimulatory receptors OX40 and 4-1BB programs Ag-primed CD4 T cells to expand and undergo cytotoxic Th1 differentiation that enables them to control tumor burden through direct and indirect (helper) mechanisms [Mittal et al., 2015]. Also, dual costimulation programs CD4 T cells to develop cytotoxic tumor cell killing potential [Qui et al., 2011], as well as provide potent therapeutic help [Hayashi, et al. 1998]. Importantly, humanized OX40 and 4-1BB agonists have been undergoing clinical testing as monotherapies, [Cuadros et al. 2015] and a dual-costimulation clinical trial is underway (NCT02315066). Patients with cancers such as hepatocellular carcinoma, ocular melanoma, cutaneous/acral melanoma or non-small cell lung cancer will be given either OX40 agonist or Dual agonists (OX40 mAb and 4-1BB mAb).
5. **Induction of Type I IFN cytokines**

Natural IFN were initially purified, characterized and identified as an “inhibitory factor” of viral replication in the 1970s [Pestka & Langer, 1987]. The type I IFN family includes IFN-beta, IFN-delta, IFN-epsilon, IFN-kappa, IFN-tau, IFN-omega, and IFN-zeta/Limitin. IFN-alpha, IFN-beta, IFN-epsilon, IFN-kappa, and IFN-omega are all found in humans, while IFN-delta, IFN-tau, and IFN-zeta have only been described in pigs, cattle, and mice, respectively [De Maeyer and De Maeyer-Guignard, 1998]. Type I IFN can be produced by all nucleated cells and can signal through the same receptor complex [Stark et al., 1998]. The amino acid composition of cattle (IFN-aC) is very similar to that of the human and murine IFN-a’s [Pestka & Langer, 1987]. Type I IFN anti-viral effects which led to the name IFN are ascribed to the induction of IFN-stimulated genes (ISGs) that result in the production of enzymes that catalyze the formation of de novo proteins [Pestka & Langer, 1987]. Proteins function to block viral RNA translation, interfere with viral replication and degrade viral RNA [Sadler and Williams, 2008]. In addition to induction of antiviral proteins, Type I IFN receptor (IFNαR) signaling activates phagocytes, NK cells, T cells and increase in expression of some tumor-associated antigens [Pestka & Langer, 1987]. Type I IFNs can act on APCs to promote their full activation and maturation [Santini et al., 2002]. Mice lacking the IFNαR1 fail to control viral replication of vesicular stomatitis virus (VSV), semliki forest virus (SFV), vaccinia virus (VV) and lymphocytic choriomeningitis virus (LCMV) [Muller et al., 1994]. Generally, experimental results of antiviral activity of IFN alpha (IFN-α) have been employed in single or combinatorial therapy. IFNα/Ribavirin treatment of antiretroviral therapy (ART)-naïve HIV-1/hepatitis C virus (HCV) co-infected individuals was associated with a pronounced, but transient reduction in plasma HIV-1 loads and significantly enhanced expression of the antiviral proteins apolipoprotein B mRNA editing catalytic polypeptide [(APOBEC)3G, APOBEC3F, tetherin, and ISG15] in CD4+ T cells [Hotter and Kirchhoff, 2018].
Type I IFN signaling

Type I IFN family members include IFN α and IFN β. Upon ligation of either IFN α or β to the IFNα/βR1/IFNα/βR2, dimerization of the two subunits brings downstream phosphorylating enzymes Janus Kinase (Jak1) and Tyrosine Kinase 2 (Tyk2) into close proximity with each other [Sivennoinen et al. 1993] and eventually phosphorylating Tyk2. Activated Tyk2 cross phosphorylates Jak1, and the latter phosphorylates the cytoplasmic tail of IFNα/βR1 subunit, allowing docking of signal transducer and activator of transcription (STAT) proteins. STAT binding induces their phosphorylation and thus, dissociation from the receptor complex. Activated STATs proteins homo- or heterodimerize and translocate into the nucleus leading to regulation of IFN induced genes (Fig. 4) [Stark et al., 1998]. One major transcriptional complex that is formed from Type I IFN signaling is the IFN-stimulated gene factor 3 (ISGF3) complex. This complex consists of phosphorylated STAT1, STAT2, and IFN-regulatory factor 9 (IRF9) and binds to IFN-stimulated response elements (ISREs) found in the promoters of numerous IFN-stimulated genes (ISGs) [Bekisz et al. 2004]. Even though STAT1 and STAT2 are the most important mediators, IFNαR can also activate other STAT proteins with different biological effects. STAT3 homo and heterodimers in most cell types and STAT4, STAT5 and STAT6 in certain cell types [Van Boxel Dezaire et al. 2006]. IFNα/β provides pro-survival and mitogenic signals, possibly through STAT3 and STAT5, and antiproliferative signals through STAT1 [Tanabe et al., 2005]. Not only the type of STAT, but the relative abundance of these STATs may vary depending on cell type and activation state and thus, very likely to have a major impact on the overall response to type I IFN.

**Figure 4. Type I IFN signaling.**

Type I IFN binds its receptor, a heterodimer (IFNαR1 and IFNαR2). This activates two receptor-associated kinases (JAK1 and TYK2) to phosphorylate and bind STAT1 and STAT2. The STAT heterodimer binds IRF9 to form the ISGF3 complex. Phosphorylation by the kinase (PKC) activates ISGF3 and translocate to the nucleus binding IFN responsive elements (IRE) within the genome. Other enhancers and chromatin modulation complexes are recruited (not shown) to initiate interferon-induced transcriptional programs [Stark et al. 1998].
B Role of Type I IFN in vaccines

Immunostimulatory properties of adjuvants are centered on the ability to induce cytokines which in turn activate the host immune system [Singh and O’Hagan, 1999]. As an alternative to adjuvants that induce cytokine secretion by cells, cytokines can be directly used as adjuvants [Muñoz-Wolf and Lavelle, 2018]. IFN α has been implicated in numerous immunization models to cure diseases. The timing of IFN α expression after infection suggest a key role in inducing a priming state during initial immune response [Biron, 1998]. Also, IFNα has been shown to regulate the activity of other cytokines [Biron, 2001]. Most Th1 polarizing adjuvants stimulate immune responses mediated by type I IFNs (IFNα/β). For example, immunization with Poly I:C and peptides increase antigen-specific CD8 T cells, yet this phenomenon is abrogated in both effector and memory phase of IFNα/βR deficient mice [Salem et al. 2006]. Vaccination with peptides and Type IFNs as adjuvants have been shown to yield better responses. Administration of rIFNα and immunization with a Foot and mouth disease virus, induced a strong and rapid response of FMDV neutralizing antibodies, augmented lymphoproliferation and enhanced proinflammatory cytokines compared with vaccine treatment alone [Cheng et al. 2007].
II. SPECIFIC AIMS

Study 1: OX40 is not essential for antigen-specific CD4 T cells to undergo Type I IFN signaling.

Knowledge Gap 1

Our lab has previously shown that Lipopolysaccharide (LPS)-induced type I IFN is essential for effector CD4 T cells [Vella et al., 1997]. Also, lipopolysaccharide (LPS) and Poly I:C individually or together induced Type I IFN signaling with OX40 costimulation boost memory and effector response of antigen-specific CD4 T cells [Mitchell et al., 2001]. Furthermore, they promote IFN-γ secretion in antigen-specific CD4+ T cell. Type I IFN has been reported by multiple investigators to signal via STAT proteins. [Petska & Langer, 1987] How particularly OX40 links Type I IFN to induce clonal expansion of antigen-specific CD4+ T cells is not well understood. Here we address the gap by providing OX40 mAb and recombinant IFN alpha (rIFNα) or only rIFNα to TCR stimulated OX0 sufficient and deficient CD4 T cells.

Specific Aim 1: To understand how OX40 links Type I IFN to induce expansion on CD4 T cells, we hypothesized that OX40 promotes Type IFN signaling indicative by either IFNαR expression and STAT1 activation (p-STAT1).

Clinically relevant to the science of vaccinology is an opportunity to intentionally trigger OX40 either on effector, memory or both cells to induce type I IFN signaling to elicit potent immune viral responses and bacterial responses after antigenic stimulation.

Study 2: OX40 is required for 4-1BB signaling to program antigen-specific CD4 T cells to express Eomes and Runx3

Knowledge Gap 2

It has been well established that programming of cytotoxic CD4 T cells is dependent on Eomes (T-box transcription factor) and Runx3 (CD8 T cell lineage commitment transcription factor during thymic development) [Qui et al. 2011]. Furthermore, it has been shown that the mechanism is by direct repression at the DNA level by ThPOK (CD4 T cell lineage commitment transcription factor).
In our previous murine model, Eomes was induced by 4-1BB agonist mAb, but not OX40 agonist mAb administered individually [Mittal et al., 2018]. We hypothesized if OX40 signaling was important for programming cytotoxic CD4 T cells. We address this gap by in-vivo and in-vitro models. For the in-vitro model, we co-culture and individually culture OX40 deficient and sufficient CD4 T cells with Eα for several time points and observe the expansion of CD4 T cells expressing either 4-1BB or OX40. For the in-vivo model, we co-transfer or individually transfer OX40 deficient and sufficient CD4 T cells into B6 recipients and measure Eomes and Runx3 expression.

**Specific Aim 2:** To understand why exogenous OX40 mAb did not induce Eomes, we hypothesized that OX40 signaling is necessary for 4-1BB signaling to induce Eomes and Runx3.

**Study 3:** **Dual costimulation impacts TRAF1, TRAF2 but not TRAF5 in antigen-specific CD8 T cells**

**Knowledge Gap 3**

Combinatorial use of immunomodulatory mAbs especially in fighting tumor typically produces greater therapeutic efficacy [Ryan et al., 2018]. Multiple groups have stated that the efficacies of treatment of multiple cancers with individual monoclonal antibodies targeting costimulatory receptors such as the TNFRSF [Gray et al. 2008].

It is well established that dual costimulation programs tumor-unrelated CD4 and CD8 T cells to deliver therapeutic help during both the priming and effector stages of the anti-tumor response [Bos and Sherman, 2010]. In CD8 T cells, the exact intrinsic mechanisms as to how dual costimulation induces tumoricidal effector function are not well understood. Here we address this gap by providing dual costimulation to double receptor (OX40 and 4-1BB) sufficient, OX40 deficient, 4-1BB deficient and double receptor (OX40 and 4-1BB) deficient CD8 T cells post TCR stimulation and looked at differences in mRNA of (TRAFs) associated adaptor proteins.

Understanding effect of dual costimulated CD8 T cell-intrinsic mechanisms will be important in deciphering a mathematical model to predict the signaling pathway. Clinically relevant is an opportunity to
hone dual costimulation benefits. Hence knowledge can be utilized for the optimal development of vaccines against diseases such as HIV, HPV, malaria and certain forms of cancer where mAbs agonists have already been shown to be a promising adjuvant.

**Specific Aim 3:** To understand the effect of dual costimulation in antigen-specific CD8 T cell on adaptor proteins TRAF1, TRAF2 and TRAF5.
III Materials and Methods

Mice, adoptive transfers, immunizations, and costimulatory agonist treatment

Six- to 8-week-old male or female B6 mice (The Jackson Laboratory Bar Harbor, ME, USA), TEa CD4 TCR transgenic mice, TEa OX40\(^{−/−}\) and TEa WT specific to the Ea\(^{52\text{ASFEAQGALANIAVDK}A68}\) peptide and OVA (\(^{257\text{SIINFEKL}264}\))-specific OT-1 TCR Tg Rag1\(^{−/−}\) B6 mice (bred in house). All mice were maintained in the animal facility at the University of Connecticut Health Center under specific pathogen-free conditions and handled in accordance to U.S. National Institutes of Health and UConn Health Institutional Animal Care and Use Committee (IACUC) federal guidelines.

TEa TCR-Tg CD4\(^{+}\) T cells\(1−3 \times 10^{6}\) specific to an I-Ab–restricted I-Ed–derived epitope (E\(a^{52\text{ASFEAQGALANIAVDK}A68}\)) WT CD4\(^{+}\) T cells (Thy1.1\(^{+}\)) and WT, lacking OX40 (purchased from The Jackson Laboratory and backcrossed onto TEa) prepared from spleens and transferred either separately or combined into congenic (Thy1.2\(^{+}\)) B6 recipients. B6 mice were immunized intraperitoneal (i.p.) with 250 mg Ea peptide along with rat IgG (75 mg; Sigma-Aldrich), or OX40 (OX86 mAb, 50 mg; Bio X Cell), or 4-1BB (3H3 mAb, 25 mg; Bio X Cell) agonists administered individually or in combination 1d following adoptive transfer. Spleens were analyzed 5 d post transfer.

Tissue processing

Tissue processing was consistent throughout all three studies. Isolated splenocytes and lymphocytes from mice were treated with 150 mM ammonium chloride (NH\(_4\)Cl) to lyse Red blood cells (RBC). Lymphocytes from all of the tissues were suspended in BSS (supplemented with HEPES, L-glutamine, penicillin, streptomycin, and gentamicin sulfate) and strained twice to remove unwanted debris.

In-vitro stimulation assays

B6 or TEa WT and TEa OX40\(^{−/−}\) CD4\(^{+}\) T cells (0.1 - 1\(\times\) 10\(^{6}\)) cells were suspended in 200\(\mu\)l CTM (MEM, supplemented with 10% FBS, dextrose, salts, amino acids, and antibiotics). 96 well flat bottom plates were
coated with anti-CD3 (5μg/ml) and anti CD28 (5μg/ml) overnight. CD4+ T cells were stimulated with Eα (1μg/mL), OX40 mAb (5μg/ml) or Isotype IgG control antibody (5μg/ml) and rIFNα (2μl) for the 24h respectively in round-bottom 96-well plates.

T£a TCR-Tg splenocytes 2–3 × 10^5 that were WT or OX40− (separately or cocultured at a 1:1 ratio in 200uL CTM) in round-bottom 96-well plates with 1 mg/mL Eα peptide for 24 and 48h.

OT-1 TCR Tg Rag1−/− B6 mice CD8 T cells (1 × 10^6) cells were stimulated for the 12 and 24h stimulated with 257 SIINFEKL peptide (NE BioLabs) and unlinked costimulators OX40 (OX86) 4-1BB (IAH2) or control polyclonal rat IgG in 200ul CTM.

Purification

Using Dynabeads (Thermo Fisher Scientific) according to manufacturer’s protocol, CD4+ T cells were negatively selected and purified from a total of 130 × 10^6 splenocytes from B6 mice.

Using Stem Cell EasySep Mouse CD8+ T Cell Isolation Kit, OT-1 Tg CD8 T cells were purified according to the manufacturer’s protocol and prepared for RNA isolation.

Flow cytometry

For surface staining, 0.05–1 × 10^6 cells were suspended in a staining buffer (BSS, 3% FBS, and 0.1% sodium azide), and nonspecific antibody binding was mitigated with Fc block [mouse serum (Sigma-Aldrich)]. Cells were stained on ice for 30 mins in the dark and then washed and suspended in the staining buffer or fixed with 2% paraformaldehyde in staining buffer for analysis the next day. For intracellular cytokine detection, the cells were washed with staining buffer and stained for CD4, CD8 Vα2, and Vβ6. Cells were then fixed with 2% paraformaldehyde. For phosphorylated STAT1 (p-STAT1) intracellular staining, splenocytes were stimulated with recombinant type I IFN alpha (rIFNα) for 20 mins. After stimulation, cells were fixed immediately with 1.5% paraformaldehyde and permeabilized with ice-cold methanol for 25 mins (after this, cells were stored at 20°C and stained at a later time point). For surface and
intracellular staining, cells were first washed with 200μl cold PBS twice to remove the methanol and stained with CD4, Vα2, Vβ6, and anti-p-STAT1 antibodies in PBS containing 0.5% BSA and 0.01% sodium azide (NaN₃) (phosphate buffer) at room temperature for 30 mins. Samples were run on a Becton Dickinson (BD) LSR II (BD Biosciences, San Jose, CA, USA), and data were analyzed using FlowJo 10.2 software (Tree Star, Ashland, OR, USA). The mAb were purchased from BD PharMingen (San Diego, CA, USA; Vβ6-allophycocyanin), eBioscience (San Diego, CA, USA; Vα2-PE-Cy7, Armenian hamster IgG-allophycocyanin), BD Biosciences (CD4-V450, p-STAT1-AF647, CD90.1-FITC, CD90.2-PE), Mouse IgGκ, Rat IgGκ, IFNαR1-PE, OX40-PECy7, CD25-PerCp-Cy5.5, Mouse IgG2a-AF647, Rat IgG2b-V450, Thermo Fisher Scientific (LIVE/DEAD InfraRed780), or Tonbo Biosciences (San Diego, CA, USA; Ghost Dye Red 780).

TCR-Tg (specific) CD4⁺ T cells (CD4⁺Thy1.1⁺) recovered from B6 spleen, and from 24 or 48h in vitro culture, were analyzed for either surface or intracellular expression. The surface staining mAbs used were CD4-BV711, CD8a-BV650, CD90.1-APC, CD90.2-FITC, CD44-AF700, Rat IgG2b-AF700, Rat IgG2a-PerCP-ef710/FITC, eBioscience [4-1BB 17B5 mAbs], OX40 (OX86 mAbs), CD25 (PC61.5). The intracellular mAbs were Mouse IgG1-PE intracellular Granzyme B-PE, Eomesodermin-PerCP-ef710, and Runx3-PE directly ex vivo.

Statistics

Graphs were generated and analysis performed using GraphPad Prism (GraphPad Software, Inc.) in all studies with statistical analysis only performed on Study 2.

Real-time PCR

RNA was prepared from four pulled wells CD8⁺ T cells (0.2 × 10⁵) and purified (Fig. 7-2) or not (Fig. 7-1). mRNA was isolated using an RNeasy Mini Kit (QIAGEN Valencia, CA, USA) according to the manufacturer’s protocol, which was then reverse transcribed using an iScript Reverse Transcription Supermix for qRT-PCR using SSOAdvanced Universal SYBR Green Supermix on a CFX96 Real-Time
System C1000 Touch Thermal Cycler (all from Bio-Rad). Data were normalized to β-actin. The following primers were generated by inputting the National Center for Biotechnology Information accession number for each gene in the primer bank website [PrimerBank, pga.mgh.harvard.edu/primerbank 2006] to get a validated forward and reverse primer sequence that was then purchased from Molecular core Integrated DNA Technologies (Coralville, IA, USA; Supplemental: TRAF1 forward, 5’-AGGTTGTGGAATTACAGCAA-3’ and reverse, 5’-GCAGTGTAGAAAGCTGGAGAG-3’ (primer bank); TRAF2 forward, 5’-AGAGAGTAGTTCGGCTTTCC-3’ and reverse, 5’-GTGCATCCATCATGGGACAG-3’ (primer bank); TRAF5 forward, 5’-TTTGAGCCCGACACCGAGTA-3’ and reverse, 5’-AGAGACCGGATGCACTGCT-3’ (primer bank); BIRC5 (Survivin) forward, 5’-AGAGCTGCTTCTCACCAGTCC-3’ and reverse, 5’-AGAGACCGGATGCACTGCT-3’ (primer bank); GLUT1 forward, 5’-TCTCGGCTTAGGGCATGGAT-3’ and reverse, 5’-TCTATGACGCGTCTTGCTC-3’; GLUT1 forward, 5’-TCTCGGCTTAGGGCATGGAT-3’ and reverse, 5’-TCTATGACGCGTCTTGCTC-3’; mKi67, forward 5’-ACCCTGGAGTCTTTATCTGGG-3’ and reverse, 5’-TGTTTTCCAGTGCTCCAGTTCTTCT-3’; and β-actin forward, 5’-AAGGCAAACCGTGAAAAGAT-3’ and reverse, 5’-GTGGTACGACCAGGGCACAT-3’. [PrimerBank, pga.mgh.harvard.edu/primerbank 2006]
IV RESULTS

Study 1: **OX40 is not essential for antigen-specific CD4 T cells to undergo Type I IFN signaling.**

Induction of Type I IFN signaling by multiple adjuvants and TLR agonists plus OX40 agonism is established [Uematsu and Akira, 2007]. Yet, the role of OX40 is not clearly defined. To uncover the presence of a link between, OX40 and Type I IFN signaling, we processed splenocytes from a B6 mouse and stained for IFNαR, IFNγR and CD25 (IL-2R) (Fig. 5–1A). Gating on only CD4⁺ T cells (Fig. 5–1B), we observed an average of 9.59% CD25, 1.61% of IFNαR1 and 0.867% of IFNγR1 positive CD4⁺ T cells (Fig.5-1C). The data shows basal expression of CD25 in some subset of CD4⁺ T cells but no IFNαR and IFNγR expression.

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**Figure 5-1. OX40 sufficient CD4 T cells express CD25 at basal level.**

B6 splenocytes (1 × 10⁸) were isolated and stained. (A). Gating scheme of lymphocytes to identify CD4⁺ and CD8⁺ T cells (B). CD4⁺ T cells were analyzed for the percent of CD25, IFNγR1, IFNαR1 by flow cytometry (C). The data is representative of 1 study and presented as percent CD4⁺ T cells expressing CD25, IFNγR1 and IFNαR from all mice.
To assess the role of OX40 signaling in IFNαR, IFNγR, and CD25 expression. CD4+ T cells were purified by negative selection and confirmed by flow (Fig. 5-2B) from whole splenocytes. CD4+ T cells were stimulated in vitro overnight with plate-bound anti-CD3 and anti-CD28. The next day CD4+ T cells were stimulated with OX40 mAb mAb or Rat IgG mAb (Fig. 5-2A). We observed 57.49%, 0.31% and 61.42% of CD25, IFNγR, IFNαR1 expression on OX40 treated CD4 T cells respectively (Fig. 5-2C). Compared to 51.18%, 0.51% and 54.77% of CD25, IFNγR, IFNαR1 expression on IgG treated CD4 T cells respectively (Fig. 5-2C). We also looked at 48h timepoint under similar conditions and the trend observed was no different from 24h but elevated numbers (The data is not shown). The data suggest that CD25 and IFNαR expression by some subset of CD4+ T cells is independent of OX40 signaling. Also, post-TCR activation, CD4+ T cells do not express IFNγR.

Figure 5-2. OX40 sufficient CD4 T cells express IFNαR and CD25 post-TCR stimulation independent of extrinsic stimulus.

Isolated splenocytes from B6 mice were depleted of CD8+ T cells and stimulated with anti-CD3 plus anti-CD28 and either OX40 mAb or IgG control antibody for 24h (A). Flow cytometry analysis showing pre and post CD8+ negative selection (B). CD4+ T cells were stained and analyzed for OX40 CD25, IFNαR1, IFNγR1 (C). The data is representative of 1 study, presented as percent CD4+ T cells from all mice.
To assess the role of OX40 on the Type I IFN low-affinity receptor (IFNαR) expression on antigen-specific CD4 T cells, we utilized the transgenic mice which TCR is specific for the Eα peptide. We performed two in-vitro assays whereby we stimulated splenocytes with two different concentrations of Eα (1 and 10ug/ml), OX40 mAb (OX40 mAb) or both and after 24h treated with rIFNα (1ul) for 20 mins (Fig. 5-3A). The first assay included CD4 T cells from TEa WT (Thy 1-1+) and TEa OX40-/- (Thy 1-2+) mice stimulated individually in different wells. The second assay included a mixed ratio of 1:1 of CD4 T cells from both TEa WT (Thy 1-1+) plus TEa OX40-/- (Thy 1-2+) mice stimulated in the same well. The gating scheme to determine specific phenotype prior to in vitro stimulation is shown as TEa WT (Thy 1-1+) and TEa WT (Thy 1-2+) (Fig. 5-3B). The gating scheme to confirm the equal mixture of TEa WT (Thy 1-1+) plus TEa WT (Thy 1-2+) prior to in vitro stimulation is shown in Fig. 5–3D.

We saw that OX40 deficient CD4 T cells showed a higher expression of IFNαR in all treatment conditions compared with OX40 sufficient CD4 T cells at both peptide concentrations. IFNαR expression was amplified upon augmented TCR stimulation with 10ug/ml peptide stimulation. CD4 T cells treated with 1ug/ml peptide showed an average value of ~20% IFNαR expression and CD4 T cells treated with 10ug/ml peptide showed an average value of ~38% IFNαR expression. The trend of higher IFNαR expression in OX40 deficient compared with OX40 sufficient CD4 T cells was observed in both individually stimulated cells (Fig. 5-3D-G) and co-cultured cells (Fig. 5-3I, J) with (Fig.5-3D, E) or without rIFNα (Fig. F, G).

An average difference of 13% in IFNαR expression between TEa WT and TEa OX40-/- was observed when cells were stimulated in separate wells (Fig. 5-3D-G). Compared with 6.5 % when cells were equally mixed and cultured in the same well (Fig.5-3I). We also performed experiments for 48h and 72h time points (The data is not shown). IFNαR expression was similar between OX40 sufficient and deficient mice at 48 and 72h time points. The data suggest that OX40 is not essential for IFNαR expression at the early time points.
CD4 splenocytes $1 \times 10^6$ isolated from TEa Thy 1.1 WT and TEa Thy 1.2 OX40$^{-/-}$ mice were separately or mixed (ratio of 1:1) and stimulated with Eα, OX40 mAb or both. After 24 h, separate or mixed cultures were stimulated with or without rIFNα for 20 mins (A). Gating scheme of lymphocytes in identifying TEa WT and TEa OX40$^{-/-}$ CD4$^+$ cells before separate in-vitro stimulation (B). Bar graphs showing percent TEa CD4$^+$ T cells expressing IFNαR1 when treated with rIFNα (D, E) or without rIFNα. (F, G). Gating scheme of lymphocytes confirming mixture of TEa WT and TEa OX40$^{-/-}$ CD4$^+$ cells before in-vitro stimulation (H). Bar graphs showing percent TEa CD4$^+$ T cells expressing IFNαR1 when treated with rIFNα (I) or without rIFNα. (J). The data representative of independent duplicate wells (D-G) in a single study (D-G, I and J) and is presented as percent CD4 T cells expressing IFNαR from all mice.
To assess the role of OX40 on Type I IFN signaling in antigen-specific CD4 T cells, we utilized the transgenic mice which has a TCR specific for the Eα peptide. We performed an *in-vitro* assay of mixed cells TEa WT (Thy 1-1+), TEa OX40+/− (Thy 1-2+). in a ratio of 1:1. The cells were stimulated with Eα, OX40 mAb (OX40 mAb) or both and after 24h treated with rIFNα for 20 mins. (Fig 5-4A) The gating scheme to determine specific cell type prior to *in vitro* stimulation are shown as TEa WT (Thy 1-1+) plus TEa WT (Thy 1-2+) (Fig. 5-4B). We saw a higher expression of pSTAT1 in OX40 deficient CD4 T cells measured as mean fluorescence intensity (~3500) in all treatment conditions with (Fig. 5-4C) or without rIFNα (Fig. 5-4D) at 24h. Compared with OX40 sufficient CD4 T cells measured as mean fluorescence intensity (~2000) at 24h. A similar trend was observed at an earlier time point of 12 h and later time points of 48h and 72h stimulation (The data is not shown). The data suggest that OX40 is not essential for Type I IFN signaling.

**Figure 5-4. OX40 deficient antigen-specific CD4 T cells express phosphorylated STAT1.**

CD4 T cells isolated from Thy 1-1+ TEa WT mice and Thy 1-2+ TEa OX40+/− mice were mixed at approximately a 1:1 ratio to generate an equal percentage of Thy 1-1+ (WT) and Thy1-2+ (OX40−/-) before culturing. Mixed CD4+ TEa cells were treated with either (Eα) or OX40 mAb and both. After 24 h time point mixed cells were stimulated with or without rIFNα for 20 mins and stained for pSTAT1 (A). Gating scheme of lymphocytes confirming mixture of TEa WT and TEa OX40+/− CD4+ cells before *in-vitro* stimulation (B). Bar graphs showing percent TEa CD4+ T cells expressing pSTAT1 when treated with rIFNα (C) or without rIFNα. (D). The data representative of a single study and is presented as percent CD4 T cells expressing IFNαR from all mice.
To reveal the role of exogenous rIFNα on IFNαR signaling in antigen-specific CD4 T cells, we utilized the transgenic mice which TCR is specific for the Eα peptide. We performed an *in-vitro* assay whereby either splenocytes from TEa WT (Thy 1-1⁺) and TEa OX40⁻/⁻ (Thy 1-2⁺) mice were stimulated in separate cultures. The cells were stimulated with or without Eα for 24h and treated with (Fig. 5-5B) or without (Fig. 5-5C) rIFNα for 20 mins. (Fig. 5-5A) We observed that OX40 deficient CD4 T cells showed augmented pSTAT1 expression in all treatment conditions compared to OX40 sufficient CD4 T cells (Fig. 5-5B). The data showed that rIFNα is necessary for Type I IFN signaling independent of TCR stimulation.

**Figure 5-5. Exogenous rIFNα is required for CD4 T cells to express phosphorylated STAT1**

CD4 T cells isolated from Thy 1-1⁺ TEa WT mice and Thy 1-2⁺ TEa OX40⁻/⁻ mice were separately treated with or without Eα and after 24 h stimulated with or without rIFNα *in vitro* (A). Bar graphs showing mean fluorescence intensity TEa CD4⁺ T cells expressing pSTAT1 (B). The data is representative of 1 study and presented as mean fluorescence intensity WT (Thy 1-1⁺) and OX40⁻/⁻ (Thy1-2⁺) T cells from all mice.

**Study 2: OX40 is required for 4-1BB signaling to program antigen-specific CD4 T cells to express Eomes and Runx3**

Administration of 4-1BB agonists alone induces expression of Eomes and Runx3 in WT CD4 T cells but not in OX40⁻/⁻ CD4 T cells post TCR stimulation.

To reveal the role of OX40 in the induction of cytotoxic CD4 T cells, we performed an *in vivo* and *in vitro* assay. The *in vivo* model involved i.v transfer of individual or combined TEa WT Thy 1-1⁺ or TEA OX40⁻/⁻ and Thy 1-2⁺ CD4 T cells into B6 recipients. On day 1 the mice were immunized with Eα peptide along with anti-4-1BB agonist mAbs and cells were analyzed on day 5 (Fig. 6-1A). We saw an induction of Eomes (~30 %) and Runx3 (20%) in OX40 sufficient cells compared with Eomes (10 %) and Runx3 (5%) in
OX40⁻ CD4 T cell (Fig.6-1B and C). The data shows that OX40 together with 4-1BB signaling is required for induction of Eomes and Runx3 in antigen-specific CD4 T cells.

Next, to discover the role of OX40 in 4-1BB expression, we performed two in vitro assays. TEa WT (Thy 1-1⁺) and TEa OX40⁻ (Thy 1-2⁺) CD4 T cells were stimulated individually in separate wells. An equal mix of both TEa WT (Thy 1-1⁺) plus TEa OX40⁻ (Thy 1-2⁺) was stimulated in the same wells. The cells were stimulated with Ea for 24 and 48h. (Fig 6-2A). We observed an induction of 4-1BB⁺ antigen-specific CD4

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**Figure 6-1** OX40 and 4-1BB signaling are both required for induction of Eomesodermin and Runx3 in antigen specific CD4 T cells

TEa WT (Thy1.1⁺) or OX40⁻ (Thy1.2⁺) CD4 T cells 1 × 10⁶ were transferred or co-transferred into B6 recipients and were immunized with Ea peptide along with rat IgG or OX40 or 4-1BB agonists administered individually or in combination. Spleens were analyzed 5 d post transfer. Scatter plots show the percentage of specific TEa CD4 T cells that express Eomes (B) and Runx3 (C) and the total number of specific CD4 T cells (D) n = 4 per group in all three experiments. *p > 0.05, **p > 0.01, ***p > 0.001
T cells of ~25% at 24h and ~40% at 48h in both separate and co-cultures respectively. (Fig. 6-2B-E) The data shows that OX40 is not required for 4-1BB induction on antigen-specific CD4 T cells.

Isolated splenocytes were depleted of CD8\(^+\) T cells. TEa WT (Thy1-1\(^+\)) and OX40\(^{-}\) (Thy1-2\(^+\)) CD4 T cells 1 \times 10^5 cultured separately (upper panels) or together at a 1:1 ratio (lower panels) in the presence of 1 mg/ml Ea peptide. Percentage of WT and OX40\(^{-}\) TEa CD4 T cells expressing 4-1BB (B), OX40 (C), and CD25 (D) that had been cultured for 0, 24, and 48h. Percentage of TEa WT and OX40\(^{-}\) CD4 T cells expressing 4-1BB in a second independent trial that used a similar experimental setup. In both experiments, the 24h and 48h values represent the mean \pm SEM calculated from triplicate or quadruplicate wells.

**Figure 6-2. OX40 is not required for 4-1BB expression on antigen-specific CD4 T cells**
Study 3: **Dual costimulation impacts TRAF1, TRAF2 but not TRAF5 in antigen-specific CD8 T cells**

Dual costimulation on CD8 T cells has been shown to induce an output of potent expansion markers CD25 and inflammatory cytokine secretion such as IFN-γ. [Ryan Ph.D. Thesis, 2018] To uncover the potential of CD8 T cell-intrinsic mechanisms after engagement of Dual costimulatory receptors with artificial ligands, we perform an *in vitro* assay with OT-1 CD8 T cells. Splenocytes were stimulated with concentrations of (5, 10, 15pg/ml) SIINFEKL peptide and dual costimulation (DCO) 0.2ug/ml (Fig. C, E, G, I) or not (Fig. B, D, F, H). After 24h time point, four wells were pulled together and mRNA was isolated. We performed qRT-PCR with the genes of interest TRAF1 (Fig. 7-1 B, C), TRAF2 (Fig. 7-1D, E), TRAF5 (Fig. 7-1F, G) and BIRC5 (Fig. 7-1 H, I) that have been reported to be recruited downstream of costimulatory signaling upon ligation of OX40 or 4-1BB with ligand OX40L and 4-1BBL respectively [Kohrt et al., 2016; Cragg et al., 2071].

TRAF1, TRAF2, and TRAF5 were downregulated whereas BIRC5 was upregulated in both media (Fig. 7-1B, D, F, H) and DCO (Fig. 7-1C, E, G, I). treated CD8 T cells in a peptide-dependent manner. The data shows that dual costimulation does not impact TRAF1, TRAF2, TRAF5 in splenocytes since down-regulation was almost similar in both DCO treated and non-treated cells.
Figure 7-1. Dual costimulation does not impact TRAF1, TRAF2, and TRAF5 in splenocytes.

OT-1 Rag\(^{-}\) WT, OT-1 Rag\(^{-}\)OX40\(^{-}\} OT-1 Rag\(^{-}\) 4-1BB\(^{-}\} mice cells were stimulated with antigen (SIINFEKL) 5 pg/ml, 10 pg/ml or 15pg/ml and 0.2 µg/ml DCO or media for 48 h (A). RNA was isolated for qRT-PCR. Bar graphs are showing delta CT values normalized to \(\beta\)-actin from either DCO (right) or media (left) treated OT-1 Rag\(^{-}\) CD8\(^{+}\) T cells respectively from all mice. n=2. The genes of interest are; TRAF1 (B, C), TRAF2 (D, E), TRAF5 (F, G), BIRC5 (H, I). The data is representative of 1 study.
Because of the contribution of mRNA from other cells in the spleen, for example, DCs, we performed another experiment to identify direct effect of dual costimulation on CD8 T cells. We performed a similar in vitro assay whereby we stimulated splenocytes from OT-1 Rag\(^{-}\) WT, OT-1 Rag\(^{-}\) OX40\(^{-}\), OT-1 Rag\(^{-}\) 4-1BB\(^{-}\) and OT-1 Rag\(^{-}\) OX40\(^{-}\) and 4-1BB\(^{-}\) with 10 and 20pg/ml SIINFEKL peptide. After 0, 12 and 24h cells pulled from multiple wells were purified to obtain only CD8\(^{+}\) T cells which were confirmed by Flow cytometry (The data is not shown). qRT-PCR was performed on the mRNA isolated from the CD8 T cells (Fig. 7-2A) for the following genes of interest: TRAF1 (Fig. 7-2B, C), TRAF2 (Fig. 7-2D, E), TRAF5 (Fig. 7-2F, G) and BIRC5 (Fig. 7-2H, I), GLUT1 (Fig. 7-2J, K), MKI67 (Fig. 7-2L, M). We observed a downregulation of TRAF1 and 2 in OX40 sufficient CD8 T cells. In the OX40 and 4-1BB sufficient CD8 T cells TRAFs 1 and 2 were subtly expressed when compared to single receptor knockouts pre-TCR stimulation and post-TCR stimulation except in the double receptor knockouts. 4-1BB sufficient CD8 T cells for TRAF1 we observed 0.018 expression at 10pg/ml dose of peptide and 0.017 at 20pg/ml pre-TCR stimulation. TRAF1 expression increased to 0.025 and 0.018 over 12 h upon peptide 10 and 20pg/ml stimulation respectively. At 24h all CD8 T cells downregulated TRAF1 expression. Double receptor knockouts expressed 0.01 and 0.006 TRAF1 prior TCR stimulation. Upon TCR stimulation there was an immediate upregulation of TRAF1 in both peptide treatments which was lost at 24h.

For TRAF2, in 4-1BB sufficient CD8 T cells we saw 0.004 delta CT values at both peptide concentrations. TRAF2 expression decreased to 0.0038 and 0.003 over 12 h upon peptide 10 and 20pg/ml stimulation respectively. At 24h TRAF2 was downregulated to 0.002 and 0.0015 at 10 and 20pg/ml peptide stimulation respectively. Double receptor knockouts expressed 0.02 TRAF2 prior to TCR stimulation. Upon TCR stimulation there was an immediate upregulation to 0.0025 of TRAF2 in both peptide treatments which was lost at 24h to 0.0018.

For TRAF5, in 4-1BB and OX40 sufficient CD8 T cells, we saw 0.003 delta CT values at both peptide concentrations pre TCR stimulation. TRAF5 expression decreased to 0.001 over 12h and 24h upon 10 and 20pg/ml peptide stimulation respectively. In both single knockouts we saw 0.004 TRAF5 expression which
was downregulated at 12 and 24h timepoint upon TCR stimulation. Double receptor knockouts expressed 0.01 and 0.006 TRAF1 prior TCR stimulation. Upon TCR stimulation there was an immediate upregulation of TRAF1 in both peptide treatments which was lost at 24h.

BIRC5, GLUT1 and MKI67 were upregulated in a peptide dependent manner. (Figure 7-2B-M). The data suggest that DCO does impact TRAF1, TRAF2, BIRC5, GLUT1 and MKI67 but not TRAF5 in antigen-specific CD8 T cells.

Figure 7-2. Dual costimulation impacts TRAF1 and TRAF2 but not TRAF5 in antigen-specific CD8 T cells

OT-1 Rag\(^{-}\) WT, OT-1 Rag\(^{-}\) OX40\(^{-}\), OT-1 Rag\(^{-}\) 4-1BB\(^{-}\) and OT-1 Rag\(^{-}\) OX40\(^{-}\}4-1BB\(^{-}\) splenocytes were stimulated with antigen (SIINFEKL) 10 pg/ml or 20 pg/ml and 0.2 ug/ml DCO (A). After 12 and 24 h in-vitro stimulation, CD8\(^{+}\) T cells were negatively selected and mRNA was isolated for qRT-PCR. Bar graphs are showing delta CT values normalized to β-actin from 10 pg/ml and 20 pg/ml SIINFEKL treated OT-1 Rag\(^{-}\) CD8\(^{+}\) T cells respectively from all mice. n=2. The genes of interest are; TRAF1 (B, C), TRAF2 (D, E), TRAF5 (F, G), BIRC5 (H, I), GLUT1 (J, K), MKI67 (L, M). The data is representative of 1 study.
V DISCUSSION

Study 1: **OX40 is not essential for antigen-specific CD4 T cells to undergo Type I IFN signaling.**

Previously, our lab has demonstrated that combining Poly I:C with OX40 agonist promotes enhanced CD4 T cell expansion and Th1 differentiation that is dependent on type I IFN signaling [Shinde Ph.D. Thesis, 2017]. Also, optimal CD4 T cell priming has been observed after LPS-based adjuvanticity with OX40 costimulation. This enhanced CD4 T cell response was dependent on induction of OX40L by CD8+ DC’s [Thompson et al., 2005; McAleer et al., 2007]. Also, it has been well established that Poly I:C induces type I IFN [Field et al., 2010].

In our *in vitro* model we tested this question of the link between OX40 and Type I IFN signaling by stimulating CD4 T cells with artificial ligand and recombinant IFNα in OX40 sufficient and deficient mice with or without TCR stimulation.

Our *in vitro* data suggest that OX40 alone is not essential for Type I IFN signaling. This is consistent with previous literature which has shown minimal induction of IFN-β (Type I IFN) in OX40 sufficient CD4 T cells in the absence of TCR stimulation. *In vivo* models of immunization with poly I:C, induced ~180-200pg/ml of type I IFN (IFN-β) in serum, during both Eα plus Poly I:C or Eα plus Poly I:C and plus OX40 mAb immunization, whereas administration of Eα plus OX40 mAb induced minimal levels of type I IFN (~25-30pg/mL) [Shinde Ph.D. Thesis, 2017]

We reason that upon TCR stimulation, OX40 is induced and ligated by artificial OX40 mAbs signaling to induce Type I IFN receptor expression. Particularly, the upregulation of the low-affinity receptor chain IFNαR1 expression dependent upon TCR stimulation and OX40 induction at 24h is consistent with what has been seen by other groups. [Kurche et al., 2012] OX40 is not constitutively expressed but found on T cells 1–2 days after activation. [Croft et al., 009; Croft et al.,2010] The kinetics of receptor expression are determined by a number of factors including; T cell subset, persistence of antigen as well as immunogenicity, inflammatory environment i.e. cytokine milieu, as well as the presence of other co-
stimulatory molecules or innate cells. [Jensen et al., 2010]. Kurche et al has also reported that OX40L induction dependent on Type I IFN signaling in DCs and optimal CD4 T cell responses upon administration with OX40 mAbs. Our data is not consistent with this model. In our model OX40 was not essential for CD4 T cells to undergo Type I IFN signaling (Fig 5-4C, D and Fig. 5-5) and was dependent on CD4 T cell IFNαR expression and exogenous IFN alpha (Fig. 5-2C and Fig. 5-3D-G, I and J). TCR stimulated and purified CD4 T cells expressed IFNαR and CD25 independent of OX40.

When OX40 sufficient and deficient CD4 T cells were mixed in a 1:1 ration before culture (Figure 5-4) IFNαR1 expression was rescued. Our study provides evidence of OX40 playing an inhibitory role in IFNαR1 expression and pSTAT1 signaling. Hence it seems that the lack of OX40 causes T cells to be more activated to make up for the lack of extra costimulation. Although, DCs are a source of IFNα, yet IFNαR expression was observed in purified CD4 T cells. Thus, suggesting that the mechanism of IFNαR1 expression is independent of DC secretion of IFNα. But as to what induces transcription and translation of IFNαR1 we are not certain and further work needs to be done to elucidate the exact signaling mechanism.

However, potential questions on OX40 signaling still remain unanswered in the field regarding the structural interactions with mAbs and natural ligand. if OX40L binding is a prerequisite for signaling or indeed if mAb binding alone in some circumstances/at certain epitopes is sufficient for full signaling. [Cragg et al., 2017] Also how do Fcγ receptors (FcγR) modulate antibody induced OX40 signaling? These extrinsic mechanisms can be possible in modulating effects of OX40 costimulation on CD4 T cells

Understanding the biology of OX40 and the link to IFN secretion holds the promise to better answers for anti-viral immunity.
Study 2: **OX40 is required for 4-1BB signaling to program antigen-specific CD4 T cells to express Eomes and Runx3**

Dual costimulation by TNFR costimulatory receptors OX40 and 4-1BB programs Ag-primed has been shown to expand CD4 T cells to undergo cytotoxic Th1 differentiation that enables them to control tumor burden through direct and indirect (helper) mechanisms [Bos and Sherman 2010]. Previously our lab has shown cytotoxicity in dual-costimulated CD4 T cells depends on the T-box transcription factor eomesodermin (Eomes) and Runx3 [Mittal et al., 2018].

The *in vivo* and *in vitro* data confirms that although that OX40 is not necessary for induction of 4-1BB expression after antigenic stimulation, however either its presence or endogenous signaling is required for Eomes and Runx3 expression. This study confirms a previous report that 4-1BB agonist, but not OX40 agonist, administered individually is sufficient to induce Eomes in CD4 T cells [Mittal et al., 2018].

Further work to investigate the CD4 T cell intrinsic mechanisms will be clinically beneficial in tumor pathologies whereby TCR antigenic stimulation is limited and dual costimulation can be administered to elicit cytotoxic CD4 and CD8 T cells responses.

**Study 3:** **Dual costimulation impacts TRAF1 and TRAF2 but not TRAF5 in antigen-specific CD8 T cells**

OX40 and 4-1BB dual costimulation on resting effector CD8 T cells express high levels of granzyme B (cytotoxic marker) and CD25 (activation marker), migrate into peripheral tissue and elicit robust tumoricidal killing function [Glimcher et al., 2004; Lee et al. 2004 and 2007]. 4-1BB associates with the adaptors TRAF-2 and TRAF-1 in its cytoplasmic tail, resulting in coimmunoprecipitation leading to ultimate activation of NFκB pathway.

Although the best read out is western blot to investigate signaling of proteins in dual costimulated CD8 T cells, we employed RNA measure due to the capabilities of our lab.
Our study investigates the intrinsic mechanisms specifically looking at adaptor proteins genes (TRAF1, TRAF2, TRAF5), survival marker gene (BIRC5) glucose transporter gene (GLUT1), and proliferation genes (MKI67). TRAF1, TRAF2 and TRAF5 genes were downregulated upon increasing TCR stimulation when either 4-1BB or OX40 signaling was absent. Contributions from TCR signaling is prominent since expression of OX40 is Ag dependent and 4-1BB stimulation increases signaling through the T-cell receptor (TCR) and amplifies the cytotoxicity of CD8+ T cells. [Khort et al., 2016] The data suggest an inter reliance of both receptors to regulate downstream TRAF1, TRAF2 and TRAF5 post Ag stimulation. 4-1BB associates with (TRAF1 and TRAF2) and OX40 motif in the cytoplasmic domain is able to recruit TRAF2, 3 and 5. [Sanchez-Paulete et al., 2016; Arch & Thompson, 1998] However, data from the first experiment cannot be used to infer any conclusions as the total mRNA isolated from cells included whole splenocytes.

The second experiment whereby CD8 T cells were purified, we observed a redundant effect of the TRAF1, 2 and 5 when both OX40 and 4-1BB were present. TRAF2 and 5 is reported to have overlapping binding motifs. [Xie, 2013] Hence a possibility of close proximity of transcription sequence in the genome. It is important to note these are mere scientific speculations since data although of good fidelity, we were unable to predict what was really going on mechanistically.

The data is reliable because GLUT1 serves as a positive control encoding glucose transporter. Our data is consistent with the reported literature on induction of transcription factors such as Myc and HIF1, and the glucose transporter (Glut1) pivotal for the increase of glucose uptake after T cell activation and costimulation. [Patsoukis et al., 2017] Furthermore another internal control showing that the data has good fidelity is the upregulation of MKI67 at a later timepoint of 24h which is consistent with the reported literature of expression peak during G2 phase of cell cycle (24h timepoint). [Sobecki et al., 2017] Glut1 upregulation post TCR stimulation tracks with activation of PI3K, Akt, and mTOR, MAPK downstream of dual stimulation which triggers the switch to anabolic metabolism and is mandatory for cell cycle progression and cytokine production. [Patsoukis et al., 2017]
Understanding CD8 T cell specific intrinsic mechanisms can play a key role in advancing the capabilities of cancer combination therapy especially with other TNFRSF such as GITR which typically engage similar adaptor proteins downstream. Importantly, mounting evidence implicates TRAFs in the pathogenesis of human diseases such as cancers and autoimmune diseases.
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