THE ROLE OF IL-27 IN SUBUNIT VACCINE ELICITED CELLULAR IMMUNITY

by

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ABSTRACT

Vaccination represents one of the most impactful medical interventions in the history of human health staving off death and infirmity in the lives of millions over the past 250 years of formal practice. To date, clinical vaccination success has been predicated upon the successful employment of B cells to generate antigen-specific high affinity antibody. This vaccine approach however has had limited impact on disease outcomes related to chronic viral infection and cancer. Successful outcomes of these afflictions instead correlate with the magnitude and functionality of antigen specific T cells. In apposition to intact pathogen inoculations, subunit immunizations represent a modality that is safer and easier to manufacture and distribute worldwide. Unfortunately, subunit vaccines in current clinical practice fail to evoke robust cytotoxic T cell responses, highlighting a critical objective for vaccine research.

Previous work has reported on a dual agonist (TLR/CD40) subunit vaccine platform that uniquely generates robust T cell responses comparable to those achieved by pathogen inoculation. Here we report on the elucidation of one of the key dependencies for this robust T cell response, the generation and perception of IL-27. Through the use of mixed bone marrow chimeric mice, the IL-27 dependency of a variety of subunit immunization is demonstrated to have a T cell intrinsic component not observed in immune responses to pathogens. Deficits in IL-27 receptor (IL-27R) engagement on T cells during subunit vaccination results in an impaired ability for T cells to control a secondary challenge by an infectious bacterium consequent of IL-27RαKO T cells being compromised in their ability to achieve large numbers and produce the effector cytokine IFNγ. Utilizing the OT-1 transgenic system we documented deficits in...
the up-regulation of the transcription factor Eomesodermin as well as the cell surface receptor CD122. Additional experiments implicate a role for IL-27 in DC priming of T cell responses to the TLR2/1 agonist, Pam3Cys. Collectively we observe a well-orchestrated dynamic between IL-27 stimulation of T cells and DCs that subsequently enhances the recognition of IL-15 by T cells. Ultimately this is highlighted by an analogous and obligate dependency on IL-15 for subunit immunization efficacy.

The form and content of this abstract are approved. I recommend its publication.

Approved: Ross M. Kedl
DEDICATION

This dissertation is dedicated to all of those who have lost loved ones to medically confounding illness. There is some hope and solace in understanding the afflictions that will take us from this life. We will never have the cure for this mortal body, but hopefully we can understand why it must end when our time is near.

This work would have been impossible without my family. Dad you gave me the belief that there is always an answer and encouraged me to go work hard and find it. For the most part I am still looking. I will never forget what you told me before you left us about what a blessing this grad school experience was for my family and I. As usual you were right, and this was the right place at the right time. I tried hard not to take this opportunity or my family for granted. I credit you for the blessing and curse that is my scientific curiosity. Mom, you have always given me a safe place to stand in life. I know you are always behind me and always for me. That’s not to say that I am never wrong in your eyes and I am grateful for your willingness to point out the error of my ways. Thank you for showing me that most of the time people are more than important that products or progress. Both of you have shown me how to work hard and care much for people.

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Without a doubt the most profound influence and direction of this thesis came from the Kedl lab at the University of Colorado. Catherine Haluszczak was an invaluable professional and personal confidant and support. We had so many great conversations where we both walked away convinced that we were both almost normal or at least similarly crazy and therefore not alone. Dr. Matt Burchill, Dr. Jon Kurche and Dr. Beth Tamburini were genuinely intellectually involved in all aspects of the lab and this was greatly appreciated and an important motivation for choosing to pursue my thesis work in this lab. Conversations and disagreements with Beth in the lab about kids and science generated a wonderful blend of science and family inside the lab. Eric Cross and Jason White kept the lab light and vibrant and yet were willing to carefully and insightfully converse about science at the drop of a hat, providing much needed balance. Elizabeth Cheney was my sister in sarcasm and Alisha Chitrakar had the daring to try to understand my random ramblings while providing global insight to the less well traveled and cultured, namely myself. This lab was a phenomenal and enjoyable place to spend a short 5 years.
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CHAPTER I

INTRODUCTION

1.1 A short history of vaccination

A simple definition of vaccination could be the controlled introduction of all or a piece of pathogen for the purpose of generating enhanced immunity to subsequent pathogen encounter. Under this definition, the first accounts of the process of vaccination started more than 800 years ago in India in a process known as variolation or inoculation\(^1\). In order to control small pox, an infected individual’s pox would be removed by blade and introduced into a small incision made into a limb of a healthy subject. This subcutaneous introduction of pathogen was not without complications carrying with it an estimated mortality rate of 2-3% to the vaccine itself. Importantly this risk was substantially smaller than the chance of surviving a natural encounter with small pox and importantly conferred lifelong protection against the pathogen. This is the first recorded recognition that the human body carried the capacity for specific enhanced protection upon purposeful prior pathogen education.

As international and intercontinental trade increased, home grown and adapted pathogens were ferried alongside of commerce. These old pathogens in new lands found ideal reservoirs in the altered customs and inexperienced immune systems of people around the world. The increasing rapidity and frequency of travel meant that people in early stages of illness could easily and unknowingly rapidly spread disease to the corners of the earth. The magnitude of pandemic illnesses now abrogated by vaccines is difficult to grasp. However, over the past 150 years alone the worldwide pandemic of small pox has claimed hundreds of millions of lives\(^2\).
Along with the spread of illness also came the spread of knowledge. Starting in the 11th and 12th century regular trade was established between Britain and India first by land and then by sea. Desperate to protect their communities, knowledge of the practice of variolation began to circulate widely. Although not universally practiced, stories of variolation efficacy increased the use of this duplicitously life-giving yet dangerous practice in both England and in the Americas. It is against this backdrop of human history that the term vaccination would enter the lexicon.

As the story is told, an English doctor and Royal Society member named Edward Jenner made an important correlation between occupation and resistance to small pox. Jenner observed that milk maids rarely contracted a severe case of small pox and wondered if like the practice of variolation, the milk maids’ prior introduction to a similar yet less fatal pathogen endemic to cows, cow pox, had conferred resistance to small pox. Importantly, and yet by today’s standards unethically, Jenner set out to prove this hypothesis by purposefully introducing cow pox, through inoculation, to a group of people and tracked their susceptibility to subsequent small pox. After filing a report on the success achieved with a number of patients in 1798, Jenner moved forward with what would be a lifelong dedication to the recruitment and treatment of ever more individuals. He additionally worked on the culturing of cow pox in efforts to improve, what would be termed his “vaccine.” The term vaccine itself comes from the Latin root “vacca” meaning “cow”, and the vaccinia virus being the pathogenic source of cow pox.

With his efforts, Jenner formalized a practice of culturing pathogens for the right balance of immunogenicity and safety (attenuation), coined the term vaccination/vaccinology and additionally formally tested the principle of
heterologous immunity, protection conferred against one pathogen by education to a similar yet distinctly different pathogen\textsuperscript{2}. Additionally the benefits of his relatively benign treatment for small pox (compared to variolation with small pox itself) help standardize the idea that immunizations were a safe and effective means of thwarting disease. So much so such that by 1840 the practice of variolation was banned in the United Kingdom and vaccination for small pox (by use of cow pox) was offered freely to the public.

A few decades later the bone surgeon William B Coley from Memorial Hospital in New York City was reviewing patient cases in an attempt to understand why some bone cancer patients achieved better outcomes than others. He drew a positive correlation between post-operative infections and regression of cancer and hypothesized that something about the active infection spurred the immune system to fight off cancer\textsuperscript{5}. In efforts to test his theory, Coley injected at first live and subsequently killed bacteria (toxins) and achieved varying degrees of success. During his time “Coley’s toxins” were never truly accepted as a successful medical intervention, however this practice achieved a certain level of fame in medical lore. In so doing he is credited with the idea that disparate pathogens, or in this case even killed pathogens, could stimulate the immune system to be employed towards unrelated targets. This very concept to many is the beginning of the understanding of the term adjuvant. A biologically disparate substance used to boost the immune response to a secondary target.

In 1926 Alexander Glenny\textsuperscript{6}, building upon early observations of Gaston Ramon\textsuperscript{7}, showed that diphtheria toxin absorbed onto aluminium salts generated robust immune responses in guinea pigs conferring protection to diphtheria. Thus the prototypical subunit vaccine was born in which the metal salt aluminium was the adjuvant, conferring an effective, non-infectious, versatile, stable and
relatively cheap vaccine platform\textsuperscript{8,9}. Various pieces of multiple pathogens could be combined with “Alum” in order to confer protection against a variety of diseases, leading to the widespread use it maintains today.

Over the past 90 years, shockingly little has changed. Aluminium salt subunit vaccines make up a substantial portion of immunizations administered. Live attenuated and killed pathogen vaccines make up the remaining fraction and collectively account for more than 25 injections covering greater than 10 pathogens currently recommended during the first 2 years of life by the American Board of Pediatrics. Vaccines have forever changed the face of global health, perhaps highlighted most by the effectual eradication of small pox and the control of polio across the planet.

1.2 Humoral vs cellular immunity

The key to the birth of vaccinology was the ability to quantitatively study “protection” as an output from successful vaccines. In Jenner’s time this was simply done by observing rates of onset or severity of disease course after vaccination and subsequent exposure to the pathogen. In between the reports of Jenner and Glenny, Paul Ehrlich proposed his side chain theory of immunity\textsuperscript{10} essentially describing the role of antibodies as both cell bound and serum present agents of protection. Ehrlich’s push for the quantifiable established comparative titrated limits of protection for chemotherapeutics and serums (antibody titer). This new gold standard for quantifiable quality control paved the path to evaluating vaccine efficacy. It was against this backdrop that Glenny was able to demonstrate the value of his Aluminum salt adjuvants. Consequently by default, preeminent immunologist at the time related the quality of vaccine induced immunity to production levels of antibodies. This study of the cell-free
transferable protection component of serum became known as humoral immunity.

Opposed to this cell-free immunity, cell-associated immunity was advocated as the source of immunization mediated immune protection by Ilyich Mechnikov. Mechnikov illustrated that as opposed to the transfer of the humors alone, bacteria co-cultured with stimulated cells (macrophages) would be consumed (phagocytosed) and cleared\(^\text{11}\). For their work on these two “arms” of immunity (humoral and cellular) Ehrlich and Mechnikov shared the 1908 Nobel Prize in Medicine.

Since this time, other mechanisms of cell-associated, or “cellular” immunity have been documented. Cellular immunity now often refers to the killing of infected cells by activated NK cells and T cells. Opposed to macrophage consumption, NK cells and T cells utilizing contact-dependent proteins on their surface focus effector molecules towards a compromised/or infected cell and induce apoptosis or lysis of that cell\(^\text{12}\). For T cells, cell death can be triggered through FAS ligand (FASL) expression on T cells. FASL on T cells crosslinks the death inducing Fas receptor expressed on the surface of stressed cells, resulting in apoptosis\(^\text{13}\). Alternatively, perforin and granzymes molecules (proteases) in the T cell are coordinated by engagement of the T cell receptor with the peptide-MHC (pMHC) molecules on the surface of the target cell. The pMHC interacting with the TCR results in the directional release of digestive enzymes into the cytosol of targets cells. Similarly, engagement of NKG2D on the surface of T cells and NK cells with the MHC like ligands MICA, MICB, Rae1, or ULBP on targets cells also direct perforin and granzyme towards target cells\(^\text{14,15}\). In a tip of the hat to the original concept of cell-mediated immunity, cytotoxic T cells and NK cells also produce large quantities of effector cytokines (i.e. IFN\(\gamma\)) that modify the
inflammatory environment including the stimulation of macrophages, enhancing their phagocytic (cytotoxic) and antigen presentation properties (Figure 1.2.1)[16].

**Figure 1.2.1 Overview of humoral and cellular immunity.** In the humors (body fluid, lymph and blood (tan)) antibodies generated by antigen-specific B cells (green) are circulating, along with complement (C1q). Antibody binds to pathogens (light green) allowing for clearance of pathogen through macrophage (red) uptake mediated by Fc receptor (FcR). Complement (C1q) binds to antibody targeted pathogens for direct attack by the complement cascade. Cellular immunity (white) requires cell to cell contact. The principle cell types in cellular immunity are macrophages, NK cells (orange) and cytotoxic T cells (blue). NK cells and cytotoxic T cells target infected cells by recognition of MHC I by TCR, and MICA/RaeI by NKG2D. Interactions with these receptor and ligands direct NK cell and T cells release of lytic granules perforin and granzyme (gray cloud with red and yellow stars). T cells can also instigate cell death by FAS-L expression engaging the receptor FAS (purple) on target cells inducing apoptosis. NK and T cell activation results in the release of IFNγ (triangles) that binds to receptors on macrophages enhancing phagocytic functional characteristics.

Since these early days of immunological exploration, what is now clear is that a delicate orchestration of innate cells (macrophages and dendritic cells) and both arms of the adaptive response (T cells and B cells) are required to generate robust, high affinity class switched antibody responses to both vaccination and natural pathogens. As the adjuvant of choice for generating antibody, Alum by inference must succeed to some degree on all these fronts. While the
mechanism of Alum induced immunity is still under debate and investigation\textsuperscript{17,18}, what we have learned is that Alum does indeed induce cytokine secreting antigen-specific T cells. The magnitude and location of this stimulation is clearly sufficient to generate the required levels of T cell cytokines necessary for the generation of robust class switched antibodies. Despite all that it does well in supporting humoral immunity, Alum is a poor inducer of cytotoxic CD8 T cell immunity\textsuperscript{19}. In short what is good enough for humoral immunity is not sufficient for robust clinically relevant cellular immunity.

As stated previously, historically, the efficacy of vaccines was correlated to the generation of high affinity antibody and of course ultimately revealed by the protection they conferred the host. Live attenuated and dead pathogen vaccines as well as subunit immunization had successfully protected hosts from a wide range of viruses and bacteria. However traditional humoral immune vaccine approaches have fallen short repeatedly in generating vaccines against persistent viral infections such as HIV and Hepatitis C as well as to a broad range of cancers, must notably solid tumors. In 2010, Silverstein et al\textsuperscript{20} documented that vaccine efficacy with regard to viral and tumor clearance, regardless of type of immunization, was mathematically related to the density of the CD8+ T cell response in relationship to the established pathological burden. While heavy on math and short on mechanism, this paper confirmed what every T cell vaccinologist already knew, size matters.

The goal has been come clear for therapeutic vaccines, the generation of robust antigen-specific CD8 T cell numbers is prerequisite for favorable outcomes. This concept has only been further reinforced with the inspirationally successful outcomes of artificially generated antigen-specific T cells transferred to a host through the use of chimeric antigen receptor, and recombinant antigen
receptor T cell technology\textsuperscript{21}. Efforts to optimize these platforms has furthered basic science research into answering the question of what is required to generate large and durable expansions of fully functional cytotoxic T cells.

1.3 T cell programming - The Signal Three Hypothesis

The hallmark of the adaptive immune response is antigen specificity. For T cells this specificity comes from the randomly and uniquely generated surface protein, the T-cell receptor (TCR). Random rearrangements of gene segments in the TCR alpha and beta locus generate a dual chain surface receptor selected to recognize peptides in the unique context of MHC. This tri-molecular (peptide-MHC-TCR) interaction guides an immature T cell progenitor cell through the thymus and insures a sufficient yet not too robust interaction with self MHC and self-peptide\textsuperscript{22–25}. These selection processes generate a vast repertoire of potentially pathogen recognizing “naïve” but mature T cells. These mature “thymocytes” then exit the thymus and populate the periphery.

In the periphery there is a limited economy of circulating necessary survival factors for T cells. These thymic processes of positive and negative selection ensure that these limited resources are consumed only by T cells that have undergone useful (MHC recognizing) gene rearrangements and also put into place a mechanism to protect against autoimmunity by the death of overly self-reactive T cells (central tolerance)\textsuperscript{26,27}.

Also in the periphery, professional antigen presenting cells (APCs) consume and breakdown pathogens or portions of pathogens. These pathogen proteins are degraded and its peptides “presented” on the surface of the APC associated with either MHC I or MHC II. T cells of varying TCRs will scan the surface of these APCs, looking for a “cognate” signal between TCR and peptide bound MHC (pMHC). If there is a biochemical compatibility the engagement of
the TCR with the pMHC will trigger an initial activation cascade. This antigen specific signal has become known as the signal 1 for T cell activation. While requisite there is great breadth to the strength of signals (affinity and avidity) that will be sufficient for cellular activation.

Peptide-MHC interactions alone however are not sufficient for inducing robust immunity. Strong signal one activation will induce proliferation as a means of increasing the cell number of potentially relevant pathogen/antigen-specific T cells. However, in the absence of additional cellular inputs, the T cell will either become anergized (unresponsive) or undergo cell death (deletion)\textsuperscript{28}. This is useful as yet another means of preventing detrimental aberrant self-recognition in the periphery. This safeguard is overcome however with the introduction of aforementioned adjuvant.

Classically, adjuvants do not work on the T cell directly but rather instigate an inflammatory milieu that drive the activation of antigen presenting cells. Once a portion of a pathogen or an adjuvant engages with a pattern recognition receptor on a professional antigen presenting cell, that receptor signals an activation cascade that results in the upregulation of a number of surface proteins on the APC. Allison and colleagues\textsuperscript{28,29} demonstrated that agonism of CD28 through the upregulation of the surface molecules B7-1 and B7-2\textsuperscript{30,31} on antigen presenting cells was critical to enhancing T cell expansion and survival. These molecules engage with the surface receptor CD28 on the T cell and provide what has been termed signal 2 of T cell activation. Intracellular motifs of CD28 recruit and enhance downstream scaffolding proteins and protein kinases to the region of TCR activation\textsuperscript{32}. Consequently once engaged by B7-1 and B7-2, CD28 upregulates IL-2 production and consequently upregulation of the anti-apoptotic molecules Bcl2 and Bcl-x\textsubscript{l}\textsuperscript{28,33}. In a feedback loop, IL-2 signals
through the functional high affinity IL-2 receptor (CD25/CD122/CD132) to reinforce PI3K34 signaling and mTOR activation. Ultimately this results in T cells that not only divide but survive during the primary response.

To be useful, T cells need to do more than just divide and survive. Their utility in the adaptive immune response is derived from their ability to execute effector functions and to persist long term in order to respond quickly to secondary infections. Two broad classes of effector functions of T cells are cytokine production and cytotoxicity (granzyme and perforin production-discussed earlier).

The requirement of additional outside signals beyond signal 1 and 2 to program effector functions and memory formation, necessitated the adoption of a more complicated model35. Now termed signal 3, several groups have documented the capacity of various cytokine receptors on T cells to program specific CD4 T helper phenotypes capable of executing specialized cytokine secretion profiles36–38. The importance of cytokines in cytotoxic CD8 T cell programming is also appreciated and a canonical orchestration of type I IFN and IL-12, along with their receptors have been demonstrated to be instrumental in programming the robust generation of IFNγ, perforin and granzymes, mediated through the transcription factor T-bet37–39 (Figure 1.3.1).
**Figure 1.3.1 Signal Three Hypothesis.** Dendritic cells, DC, (green) engaged by a pathogen (red) or piece of pathogen bind to pattern recognition receptors. Upon activation the dendritic cell secretes IL-12 and IFNα/β. These type I IFNs then signal back on the DC to upregulate costimulatory molecules (signal 2) necessary for T cell (blue) activation. Pathogen that enters the cell is digested into small protein pieces that are presented to T cells in the context of peptide MHC complexes (pMHC), serving as the antigen specific signal 1. IFN made by the DC engages type I IFN receptor (IFNAR) on the surface of the T cell, signaling through STAT1 (and STAT2) IFN upregulates gene expression of IL-12 receptor (IL-12R) which then engages with DC generated IL-12. IL-12 binding to IL-12R initiate STAT4 signaling that ultimately upregulates expression of the canonical T cell transcription factor T-bet (signal 3).

Generally speaking cytokine receptors carry out this transcriptional modulation through the following generalized JAK-STAT signaling pathway. Cytokine (ligand) binding to cytokine receptors stabilizes functional dimerization and induces conformational changes of these receptors. These conformational changes induces activation of the constitutively associated kinase molecules Janus associated kinase (JAK). JAKs propagate the signal ultimately by phosphorylation of STAT molecules. STATS then homo or heterodimerize and associate with other accessory molecules to form active transcription factor complexes. These complexes then bind to specific sequences of DNA in the various promoters of relevant genes, either promoting or inhibiting transcription.
and subsequently translation. More specific and detailed examples of this process will be discussed throughout this work.

Observations by several groups have called into question the completeness of even the signal 3 hypothesis. Aside from cytokines other surface receptors, specifically TNF receptor family members, may be capable of fulfilling at least some of the roles of signal 2 or signal 3\textsuperscript{40–42}. One of the principle observations in our lab demonstrate that a fulminate primary and intact memory response can be generated in response to the robust subunit immunization delivered by the dual TLR/CD40 agonist vaccine platform independent of any contribution by IFNAR or IL-12R\textsuperscript{43}. Consistent with this observation we have observed no deleterious effects to T cell effector function or memory generation in T cells deficient in either STAT1 (IFNR associated STAT) or STAT4 (IL-12R related STAT). Conversely this immunization was critically dependent upon the TNFR family member CD27 and to a lesser extent OX40 for both primary and memory responses\textsuperscript{44}. Others have also observed this dependency on CD27 in response to pathogen infection specifically for the survival of low affinity T cells\textsuperscript{45}. Several groups have reported the contribution of TNFR family member to the survival of T cells as well as other cell types through NF\kappa B mediated upregulation of pro-survival molecules Bcl2 and Bcl-xL\textsuperscript{46,47}. In this way TNFR family members augment and support signal 2 functions of cell survival. The reported effects on effector differentiation and memory formation would also support a designation as signal 3 mediator.
1.4 Effector – memory balance: The roles of STAT1, STAT4 and STAT3 in cell fate programming

While the role of cytokines can vary from one immunological insult to another, a general paradigm has been amassed to explain the roles that cytokines and in particular individual STAT signaling pathways play, in the programming of either short lived or long lived T cells. First a T cell that starts out as naïve and omni-capable, will undergo some degree of differentiation with regards to their effector “personality.” Classically speaking most of this differentiation is programmed in large part by cytokines. The particular effector cytokines a T cell will produce are driven by the transcription factors induced. T-bet drives the Th1 phenotypic cytokine IFNγ, while GATA3 leads to IL-4 production and RORγT is responsible for the generation of IL-17. In cytotoxic T cells an additional transcription factor, Eomesodermin (Eomes) both enhances and modifies T-bet like functions and can support the differentiation of cells that produce IFNγ, perforin and granzyme.

Another cellular decision that cytokines influence is the long term role of the differentiated T cell. If T-bet and another transcription factor Blimp-1 are preferentially over expressed a T cell will trend to being a robust effector cell specializing in assisting the clearance of the acute challenge. In extreme cases these T cells will clonally expand greatly and secrete large quantities of cytokines, after which the cell and its progeny will completely die out (terminal effector differentiation). Conversely if a T cell receives programming that suppresses these transcription factors and instead greatly favors the upregulation of the transcription factors Bcl6 and Eomes, that T cell and its genetic clones will persist in the host for a long period of time to generate
immunological memory\textsuperscript{55,56}. In excess these transcription factors will cause these memory-biased T cells to proliferate minimally and secrete very little cytokine, a phenotype I have termed "trivial" immunologic memory.

For vaccination the goal is achieve a balance between these extremes. The optimal balance will allow for rapid clearance of the acute response yet preserving functional memory for future re-expansion upon secondary exposure. The model that has been established to integrate cytokine signaling and STAT regulation of transcription factor expression for determination of T cell fate, states that the role of type I IFN in the primary response is to signal and activate STAT1 mediated transcription of IL-12R expression\textsuperscript{38}. In turn IL-12R expression, in response to milieu IL-12, drives STAT4 translocation to the nucleus, where it transcriptionally upregulates T-bet and Blimp-1\textsuperscript{57}. In opposition to these signals, STAT3 mediated cytokines (i.e. IL-6, IL-10 and IL-21) result in transcriptional upregulation of Bcl6 and Eomesodermin. Additionally STAT3 signaling upregulates the negative regulator of cytokine signaling Suppressor of Cytokine Signaling (SOCS) 3. SOCS3 reinforces the Eomes, Bcl6 driven memory bias by directly engaging and deactivating the cytokine receptor-associated JAK Kinases. This impairs STAT4 phosphorylation signaling downstream of the IL-12R\textsuperscript{58}. This reductionist model of cytokine signaling emphasizes the importance of overall balance between STAT1 and STAT3 cytokines signals. Applied more universally this model would assert that all cells that demonstrate responses to these cytokines (other immune cells, tumors, etc.) can be predicted to have a cell fate dictated upon this overall balance, for which there is great body of experimental evidence to support.
Figure 1.4.1 Effector/memory programming by STAT1, STAT3 and STAT4.
Type I IFN signaling through the IFNAR results in the phosphorylation (green circles) of STATS 1 and 2. These STATs heterodimerize and form a complex with IRF9 (not pictured) and translocate to the nucleus to bind ISRE in the promoter of the IL-12R. Upregulation of IL-12R and subsequent engagement by IL-12, phosphorylates STAT4, which in turn dimerize and binds promoter regions of BLIMP-1 and T-bet driving their elevated transcription. IL-6 and IL-21 binding to their respective receptors result in phosphorylation and dimerization of STAT3. STAT3 binds to the promoter of Bcl6, Eomes and SOCS3 elevating their expression. SOCS-3 biases the differentiation towards memory programming by directly binding IL-12R associated JAKs impairing IL-12 signaling. This impairs the transcriptional support of T-bet and Blimp-1 expression.

1.5 Subunit immunization vs live attenuated pathogens

As described previously, the earliest vaccines/inoculations were by necessity and convenience pathogens themselves. Nature had provided the danger signals (natural adjuvant) in the form of foreign proteins, sugar and lipids on the pathogen that were recognized by pathogen recognition receptors of the host. For those that survived the infection, host/pathogen adaptation had selected for an immune milieu sufficient for eradication of the primary infection balanced with the generation of lifelong immunity. The variability of this outcome among the world population however illustrates exactly the complication of utilizing pathogens themselves as vaccines.
Our interest are particularly focused on the generation of cellular immunity. In general fewer vaccine platforms are capable of generating cellular immunity than humoral immunity. There are several model pathogens however that have been explored as vaccine vectors that readily generate large antigen specific T cell responses (*Listeria monocytogenes*, Vaccinia Virus, Vesicular stomatitis virus, LCMV etc.)\(^ {59-62}\). As with all live pathogenic vaccines there are several limitations to their application. Live pathogens must be “attenuated” or restricted in their virulence so that the average healthy host does not succumb to the vaccine pathogen. Complicating this end point is the reality that chronically infected and cancer-laden subjects, targeted populations for cellular immune support, are not the picture of health. Their immune system is inevitably altered, and consequently they maybe more susceptible to reversion to virulence or have a lower threshold for pathogen tolerance. This can result in the patient succumbing to the pathogen vaccine itself or unable to mount an antigen-specific response to the targeted molecule.

In addition to the health concerns revolving around live vaccines\(^ 2\), the generation of live vaccine is a costly, time consuming and resource depleting. All pathogens take time to grow, and often must be stored in cold conditions and have a limited shelf life. These factors restrict their worldwide distribution. Furthermore, adding additional target antigens to the vector require time consuming reengineering of the host pathogen. Pathogens that serve as the vaccines against themselves are sometimes difficult to grown in culture or lose significant immunogenicity having gone through the attenuation process. Similarly, while the use of dead pathogens removes the fear of reversion or lethality to susceptible populations it also weakens the effect of the vaccine for secondary protection\(^ {63-65}\).
In principle, all of these concerns could be addressed by a simple adjuvant containing subunit vaccine. Subunit immunizations incorporate only a portion of the pathogen and therefore harbor no concern of succumbing to the immunization. Additionally most subunit vaccines are composed of simple adjuvants that can be readily and rapidly mixed with any antigenic target. Furthermore, many adjuvants do not require continuous refrigeration. The safety, flexibility, manufacturing and storage advantages to subunit vaccines in principle make subunit immunization the goal of modern vaccinology. Subunit vaccines have indeed worked very well for a multitude of immunizations, providing detectable humoral immunity. Unfortunately, while both CD4 and CD8 T cells are activated with subunit adjuvants such as Poly I:C and Alum, the magnitude of their expansion provides no real clinical benefit with regard to cellular immunity19.

1.6 Dual agonist (TLR/CD40) subunit vaccine platform – synergy mystery

With all the above factors taken into consideration one of the “holy grails” for vaccine research has been the establishment of a subunit vaccine platform that elicits robust and long lived cytotoxic T cell responses. Such a vaccine could then be employed to address the health concerns of an established viral infection and cancer. Mechanistic explorations of such an immunological unicorn would also further inform future vaccine design and likely broaden the scope of clinically useful subunit adjuvants.

In 2004 our lab published the discovery of profound increases in the generation of antigen-specific cytotoxic CD8 T cells from the co-administration of two soluble agonists66. In this report, it was demonstrated when one of any number of TLR agonist was combined in solution with an agonistic CD40 antibody and an antigenic target, the result was a synergistic increase in the
number of antigen-specific CD8+ T cells in mice (Figure 1.6.1). The resulting response was on par with idealized cytotoxic T cell generating pathogen mediated vaccines in both magnitude and quality of T cells generated. The focus over the past 11 years in the Kedl lab has been to understand how this combined subunit adjuvant platform achieves T cell responses that single adjuvants do not.

**Figure 1.6.1 Schematic of dual agonist vaccine platform.** For the work recorded here we utilize the model antigen chicken ovalbumin, ova (depicted as an egg). Combining 100-150ug of antigen, 50ug agonistic anti-CD40 antibody (clone FGK-45, \( \alpha \)CD40) and any number of innate immune receptor agonistic (represented by TLR, i.e. Pam3Cys-Pam, Poly I:C ) results in a Day 7 peak of primary splenic antigen-specific CD8+ T cell response 10-100 x greater than single adjuvants alone. We determine antigen-specific cells by gating on B220-, CD3+, CD8+, CD44 high, tetramer+ cells. Ovalbumin contains the immunodominant CD8+ T cell epitope SIINFEKL. Accordingly antigen-specific T cell responses are determined by use of \( K^b \)-SIINFEKL tetramer.

Following the established paradigms of T cell biology discussed above, the Kedl lab has been interrogating which signals on the T cell are required for the synergistic increase observed in response to this dual agonist immunization. Signal 1 concerns antigen specificity and given the relative increases of antigen specific T cells within the CD8+ T cell compartment, the dependence on signal 1 is implicit. Likewise mice immunized with TLR/CD40 require classic signal 2. This
has been demonstrated through immunization of WT mice or mice deficient for the CD28 stimulatory ligands B7-1/B7-2 (Figure 1.6.2). Without CD28 stimulation, no antigen-specific T cell response is observed at the peak of the response.

**Figure 1.6.2 Dual agonist vaccine requires signal 2, B7-1/B7-2.** WT C57BL/6 (B6) mice and B6.B7-1/B7-2 double KO mice were immunized with 50ug Poly I:C, 50ug αCD40 and 100ug whole ova. 7 days post immunization spleens and blood were harvested and processed to single suspension for tetramer staining and flow analysis. Shown above are representative tetramer plots from representing two independent experiments. Averages and SEM were accumulated for n = 6, WT and n = 7 for B7-1/2KO.

As eluded to in the signal 3 subsection, a cloud of mystery settled over the TLR/CD40 vaccine platform when it was thoroughly demonstrated that unlike classical T cell responses to pathogens, robust antigen-specific cytotoxic T cells were evoked in T cells lacking the canonical signal 3 cytokine receptors IFNAR and IL-12R. IFNAR/IL-12R receptor-deficient T cells generated fully functional effector and memory cells that persisted in the host and provided protection against antigen-specific pathogen challenge. Further investigations revealed that production of these cytotoxic antigen-specific cells was critically dependent upon the TNFR-L interactions of CD27-CD70 and OX40-OX40L. Based upon not
only the impaired primary response but also the augmented memory response it was speculated that TNFR-L interactions served as an alternative signal 3.

Given the established paradigms of STAT-mediated upregulation of the fore-mentioned transcription factors necessary for shaping the character and memory fate of antigen activated T cells, it was not immediately clear how TRAF-mediated TNFR signaling could likewise supplement the transcriptional profile induced by these cytokines. For this reason we speculated cytokines outside of the canonical Type I IFNs and IL-12 utilizing different receptors, could achieve the same T cell outcomes. In the cytokine family, one unique member had been documented to play semi-redundant roles for both type I IFN^{69} and IL-12 signaling^{70}. This cytokine, IL-27, through its capacity to efficiently utilize both STAT1^{71} and STAT3^{72}, became the focus of my thesis investigations.
CHAPTER II
IL-27 IS REQUIRED FOR SHAPING THE MAGNITUDE, AFFINITY DISTRIBUTION AND MEMORY OF T CELLS RESPONDING TO SUBUNIT IMMUNIZATION

2.1 Introduction

The efficacy of vaccination exploits the highly specific adaptive arm of the immune response. To date, the objective of most clinical-use vaccines has been the generation of high titers of antigen-specific neutralizing antibodies. Initially antibody production was achieved through direct exposure to attenuated pathogens. However, a host of issues (manufacturing, stability, toxicity, and virulence) limit the use of these types of vaccines.

An alternative strategy constructs vaccines using only strategic portions of pathogens combined with innate immune agonists. These subunit vaccines are more stable, versatile, and safe relative to traditional attenuated pathogen vaccines. Combined, these platforms have saved countless lives in a little over 200 years of practiced vaccinology. Despite this success, vaccination has been unable to consistently achieve medically meaningful responses against most solid tumors and several persistent viral infections (i.e., HIV and hepatitis C). Interestingly, the major correlate for sterilizing immunity to both viral and tumor challenge is not antigen-specific antibody titer but rather the number of antigen specific T cells generated, known as cellular immunity. Unfortunately, T-cell responses to subunit immunization typically require multiple boosts to achieve even detectable antigen-specific T-cell numbers, which often have little clinical impact. As such, identifying the factors that dictate the magnitude of antigen-

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1 Adapted from Proc Natl Acad Sci U S A. 2014 Nov 18;111(46):16472-7
specific T cells in response to immunization is of paramount importance.

Classically, robust CD4+ and CD8+ antigen-specific T-cell responses are dependent upon multiple inputs derived from various kinds of receptors on the T-cell surface. Particular cytokine receptors, such as the type I interferon receptor and IL-12R, execute targeted up-regulation of key transcription factors necessary for supporting T-cell expansion and the initiation of both T-cell effector and memory-fate programs. Encounters that produce longstanding cellular immunity induce a balanced cytokine milieu, using both stimulatory (STAT1) and suppressive (STAT3) signaling pathways. IL-27 is a member of the IL-12 family of cytokines and, via its’ signaling through both STAT1 and STAT3, contributes to a spectrum of T-cell functions and phenotypes.

Although in vitro studies demonstrate a role for IL-27 in CD4 Th1 differentiation, IL-27 deficiency in vivo also leads to severe inflammatory immunopathology in parasite/pathogen infection models as well as in vaccination-induced autoimmunity. Additionally, IL-27 displays different effects on CD4+ and CD8+ T cell responses, enhancing tumor-specific CD8+ T-cell responses while also inducing IL-10 producing CD4+ T cells and Tregs.

We report here an unexpected and central requirement for T cell-intrinsic IL-27 signaling in the generation of maximal T-cell responses to subunit vaccination. Besides dictating the overall magnitude of the T-cell response, IL-27 was also required for the survival of high-affinity antigen-specific cells. In the absence of IL-27, the pool of memory T cells was of lower affinity, was of reduced effector function, and was less protective on a per-cell basis against infectious challenge. Importantly, these observations are unique to subunit immunization because the T-cell responses to infectious challenge remain intact.
in an IL-27Rα–deficient environment. Furthermore, the influence of IL-27 on CD8+ T-cell expansion, affinity, function, and memory programming was mediated via a STAT1/3-dependent mechanism. Collectively, these observations point to a unique and previously unappreciated role for IL-27R signaling on T cells in response to subunit vaccination.

2.2 Results

2.2.1 Vaccine Adjuvant-Elicited Cellular Immunity Is Dependent on IL-27Rα Signaling in T Cells

Previously we reported on large, durable antigen-specific CD4+ and CD8+ T-cell responses generated through the combined use of Toll-like receptor (TLR) and CD40 agonists (combined TLR/CD40 vaccination)\textsuperscript{44,66}. In contrast to other forms of vaccination/immunization, T-cell intrinsic stimulation from classical Signal 3 cytokines such as type I IFN or IL-12 were not required for T-cell expansion, polarization, or memory generation\textsuperscript{68,90}. Literature searches for other cytokines capable of dramatically influencing T-cell polarization/differentiation drew attention to the IL-12 family member IL-27\textsuperscript{79,91,92}. To investigate the potential role of IL-27 signaling in response to our subunit vaccination (TLR/CD40), IL-27RαKO and wild-type (WT) mice were immunized with antigen in the context of either Poly I:C/αCD40 or Pam3cys/αCD40, and the magnitude of the antigen-specific T-cell responses was monitored by tetramer staining at the peak of the response (day 7). Surprisingly, we found a significant role for IL-27 in mediating the maximal T-cell response to this vaccination. The CD8+ T-cell response in the IL-27RαKO mice was substantially reduced (∼5- to 10-fold) in both the peripheral blood (Fig. 2.2.1.1A) and spleen (Fig. 2.2.1.1B) to either vaccination, compared with WT hosts. This reduction was apparent based on the assessment of the percentage and total numbers of antigen-specific CD8
+ T cells (Fig. 2.2.1.1C). This IL-27 dependency was not solely a feature of the CD8+ response, being also observed in the CD4+ T-cell response to the 2W1S peptide antigen (Fig. 2.2.1.1D). Thus, both CD8+ and CD4+ T-cell responses to combined TLR/CD40 vaccination are dependent upon IL-27.

These data were in apparent contrast to previous reports where IL-27 deficiency in vivo more often led to an elevation in the magnitude of T-cell response. However, these published data monitored T-cell responses during infectious challenge, raising the possibility that the IL-27 dependence might be unique to the cellular response elicited by subunit vaccination. We therefore next examined the CD8+ T-cell responses in WT and IL-27RαKO mice to primary challenge with either *Listeria monocytogenes* or vaccinia virus. Consistent with previous reports, there was no defect in the CD8+ T-cell response to either of these pathogens in IL-27RαKO mice (Fig. 2.2.1.1E). These data therefore demonstrate a significant, but selective, IL-27 dependency of the CD4+ and CD8+ T-cell responses elicited after combined TLR/CD40 vaccination but not infectious challenge.

Modern subunit vaccines may be composed of one or multiple agonists for innate receptor pathways. As the response to both Pam3/αCD40 and Poly I:C/αCD40 were IL-27–dependent, we questioned whether this dependency was applicable only to combined adjuvants using αCD40 or whether a broad range of innate receptor agonists shared this trait. Therefore, WT and IL-27RαKO mice were immunized with antigen in the context of single adjuvants, and the T-cell responses were analyzed by tetramer staining of cells in the blood and spleen as before.
Figure 2.2.1.1 Combined agonist TLR/CD40 vaccination-induced T-cell responses are IL-27Rα–dependent.
Figure 2.2.1.1 Combined agonist TLR/CD40 vaccination-induced T-cell responses are IL-27Rα–dependent. IL-27RαKO and WT mice were immunized with Pam3Cys/αCD40 (25ug/50ug) or Poly I:C/αCD40 (50ug/50ug), and 100ug ova. Seven days post-immunization (dpi), T-cell responses in the peripheral blood lymphocytes (PBL) (A) and spleens (B) were determined by tetramer stain as described in detail in Materials and Methods. All data are gated on B220−CD3+CD8+ cells. (C) Absolute number of splenic antigen-specific T cells. Data shown are representative of at least five independent experiments. (D) IL-27RαKO and WT mice immunized as in (A) except inclusion of 150ug of the I-Ab binding CD4+ T-cell epitope peptide 2W1S and stained with I-Ab/2W1S tetramer on CD3+CD4+B220− gated events. Data shown are representative of two independent experiments. (E) Numbers of CD8+ B8R (Vaccinia-virus CD8 T cell epitope) specific splenic T cells in WT B6 and IL-27RαKO mice challenged with Listeria monocytogenes- genetically engineered to express B8R, Lm-B8R(2000 c.f.u/mouse), or with vaccinia virus (Vv) (1x10⁷pfu/mouse). Mice were harvested at peak of T cell response, Day 8. Blood and spleens were collected, processed to single cell suspension and stained with Kβ-B8R tetramer (B220−, CD3+CD8+). Data shown are representative of three independent experiments.
Surprisingly, all single adjuvant-treated IL-27RαKO mice showed a reduced percentage of antigen-specific T cells (Fig. 2.2.1.2A and 2.2.1.2B). IL-27 dependency applied to more robust single adjuvants, able to produce 1–3% antigen-specific T cells in the WT host (αCD40, αGalCer), as well as to adjuvants that produce overall weaker (<1%) cellular responses (Poly I:C, Pam3Cys). These data therefore demonstrate a previously unappreciated central and broad requirement for IL-27 in multiple vaccine adjuvant-elicited cellular immune responses.

Potent immune modulation by IL-27 has been observed within both T cells and dendritic cells (DCs)\textsuperscript{95}. It was therefore feasible that our observed IL-27 dependency of the T-cell response to vaccine adjuvants could be due to a requirement for IL-27 stimulation of T cells, DCs, or a combination of different cell subtypes\textsuperscript{96}. To understand which cell type required IL-27R signaling for the cellular response to vaccination, we generated IL-27RαKO:WT mixed bone marrow chimeras (BMCs). These hosts have both WT and IL-27RαKO DCs and T cells, allowing the assessment of the T-cell dependency of IL-27 signaling in an environment that has competent, WT antigen-presenting cells. Vaccination of these chimeric mice with either dual agonist vaccine (Fig. 2.2.1.2C) or single adjuvants (Fig. 2.2.1.2D) recapitulated the impairments in the CD8+ T-cell compartment observed in the IL-27RαKO mice regardless of the innate receptor agonist used. Although these data do not eliminate the possibility that IL-27 signaling may also be important for some aspects of DC activation/maturation, the disparity between WT and KO CD8+ T cell responses within the same host demonstrates that IL-27 dependency of the vaccine-elicited T cell response is T cell intrinsic.
Figure 2.2.1.2 IL-27Rα dependency of subunit vaccine-elicited T cell responses is T cell intrinsic.
Figure 2.2.1.2 IL-27Ra dependency of subunit vaccine-elicited T cell responses is T cell intrinsic. (A and B) WT and IL-27RaKO mice immunized with ova in conjunction with indicated innate receptor stimulus. For the indicated mouse the following doses of adjuvant were used 25ug flagellin, 25ug Pam3Cys, 50ug Poly I:C, 50ug αCD40 and 2ug αGalCer. Seven dpi, spleens were harvested and stained with Kb-SIINFEKL tetramer as in Fig.2.2.1.1. Data shown are representative of three independent experiments for each adjuvant. (B) B6-Ly5.2 (CD45.1) were irradiated with 2 x 500 rads and reconstituted with equal fractions of mature lymphocyte depleted (B220, CD3, NK1.1, F4-80, CD11b, CD4, CD8) bone marrow from IL-27RaKO (CD45.2) and B6 (CD45.1) donors. Twelve weeks after reconstitution, mice were immunized with Pam3cys/αCD40/ova (25ug/50ug/100ug) or Poly I:C/αCD40/ova (50ug/50ug/100ug) (C), or with the indicated single adjuvants in the presence of 100ug ova (D). Seven dpi, spleens were harvested and stained with Kb-SIINFEKL tetramer and antibody for congenic CD45.1. Magnitude of antigen-specific T cell response is measured as % antigen specific of the individual congenic CD8 T cell pool (C) Representative of four independent experiments. (D) Representative of two independent experiments. Black line denotes limit of detection for tetramer.
2.2.2 IL-27 Signaling Shapes the Affinity Distribution of T cells

In addition to the overall numbers of tetramer-positive cells being dramatically reduced, we observed that the residual pool of tetramer staining T cells in the IL-27Rα-deficient host had a lower level of tetramer fluorescence intensity (FI) compared with the WT cells (Fig. 2.2.2.2A). However, because tetramer staining varies directly as a function of TCR levels, this lower tetramer staining could be the result of lower TCR expression in the IL-27RαKO host. We therefore adopted two methodologies for normalizing our data for relative quantities of surface TCR complex. A commonly used method restricts analysis of tetramer fluorescence to narrow ranges of CD3 expression (Fig. 2.2.2.1A). After controlling for TCR levels in this fashion, statistically significant differences between WT and IL-27RαKO tetramer mean fluorescence intensity (MFI) remained (Fig. 2.2.2.1B) indicating a broad difference in affinity between WT and IL-27Rα-deficient cells.

This form of analysis is limited however, taking into account only the events within each CD3 gate. We therefore confirmed our results by correcting for CD3 levels on a per-cell basis. We defined an output parameter that divided the tetramer FI for each event by the CD3 FI for that event (tet FI/CD3 FI). This parameter allowed for the inclusion of a data point for every detectable antigen-specific T cell. Plotting these ratios for each tetramer-positive cell for each mouse confirmed a paucity of high-affinity (high tetramer/CD3 ratio) tetramer-positive cells in both the CD4+ and CD8+ T-cell pools from IL-27RαKO mice in response to combined TLR/CD40 immunization (Fig. 2.2.2.2B). To concisely represent and statistically validate these observations, the average Tet FI/CD3 FI for each mouse was determined. These average ratio values were then compared between cohorts by t test.
Figure 2.2.2.1 Relative tetramer staining compensated by TCR expression.

(A) Thin CD3 Slices. Splenocytes from WT and IL-27RαKO mice immunized with Poly I:C/αCD40/ova (50ug/50ug/100ug) were analyzed by flow cytometry for antigen-specific tetramer positive cells. Populations of tetramer-positive cells were sub-gated based upon differing expression of surface CD3 (y axis) as indicated by arrows. (B) Tetramer gMFI of thin CD3 slices. Geometric Fluorescence Intensity (gMFI) for each of the sub-gates from (A) was then determined by flow analysis and plotted individually for each mouse and separated and grouped by strain. Student’s t-test determined statistical significance. (A & B) are representative graphs from greater than 3 experiments, each containing greater than 3 subjects per strain.

This unbiased method of analysis confirmed significant differences in the affinity distribution of the responding CD8+ and CD4+ T cells in WT and IL-27RαKO hosts (p value < 0.01) (Fig. 2.2.2.2 C-D). This conclusion was further supported through results obtained by more traditional tetramer disassociation assays (Fig. 2.2.2.3 A-D). The reduced affinity of responding T cells in the IL-27Rα deficient host was not due to an altered naive repertoire because Lm-ova challenged IL-27RαKO mice produced T cells with a broad distribution of tetramer/CD3 ratios (Fig. 2.2.2.2E). The overall average affinities of IL-27RαKO and WT T cells in Lm-ova–challenged mice were statistically indistinguishable from one another and from WT mice immunized with Poly I:C/αCD40 (Fig. 2.2.2.2F). Thus, IL-27 signaling influences the generation of high-affinity antigen-specific CD4+ and CD8+ T cells, but only in response to subunit vaccination.
Figure 2.2.2.2 High affinity T cells responding to vaccination are dependent on IL-27. Indicated mice were immunized and T-cell responses were analyzed as described in Fig. 2.2.1. (A) Tetramer fluorescence intensity (FI) histogram for tetramer-positive cells by gating on B220−CD3+CD8+CD44Hi cells positive for K b-SIINFEKL tetramer for either WT (gray filled) or IL-27RαKO (white open) mice. (B) Output scatter plot of tetramer FI/CD3FI for each tetramer-positive cell, K b-SIINFEKL for CD8 or I-A β-2W1S + for CD4+. Columns represent tetramer events within a mouse. (C) Display of the mean tetramer FI/CD3 FI for antigen-specific CD8+ T cells for each mouse. (D) Tet FI/CD3 FI (relative affinity) for antigen specific CD4 T cells from (B). Statistical significance determine by t test. Data shown for A–F are representative of three independent experiments. (E and F) WT or IL-27RαKO immunized with Poly I:C/αCD40/ova (50ug/50ug/100ug) or 3,000 cfu of Lm-ova and subjected to same relative affinity metric and display.
Figure 2.2.2.3 Tetramer dissociation assay compared to relative affinity.

Vβ5 mice have an elevated precursor frequency of ova-specific (SIINFEKL recognizing) antigen-specific T cells, providing an elevated number of cells at the peak of primary response to increase sensitivity and accuracy of statistical calculations performed on the IL-27RαKO background. WT Vβ5 and IL-27RαKO Vβ5 cohorts were immunized with Poly I:C/αCD40/ova (50ug/50ug/100ug). At day 7, the peak of primary response, splenic suspensions were stained first with Kb-SIINFEKL-PE tetramer and then with antibodies for immune subsets. Unbound tetramer and antibody were washed free from samples, and aliquots for a 0 time point were then removed and fixed with 1% PCHO-3% sucrose for 10 minutes. Then, 25D1 antibody against Kb-SIINFEKL was added at a final concentration of 20 μg/mL to the remaining bulk of tetramer-stained cells. Aliquots were removed and fixed at 0.5 min, 2 min, 4 min, 6 min, 8 min, 10 min, and 20 min. (A) Geometric MFI (gMFI) of tetramer-positive cells was obtained by flow cytometry and plotted for each mouse. (B) Data was fit to a second-order polynomial resulting in the displayed R^2 line fit values. (C) From the equations of the line, the time for each time 0 sample to have half of the tetramer competed off by 25D1 was determined and plotted, reflecting the average TCR affinity for ova for each sample. (D) The same samples were treated to the Tet FI/CD3 FI analysis described in Fig.2.2.2.2 and plotted.
2.2.3 IL-27 Alters Programming and Effector Function of T Cells in Response to Subunit Immunization

The larger goal of vaccination is the generation of long-lasting, protective immune memory. Our data thus far have characterized defects in the primary T-cell numbers in response to subunit vaccination in the absence of IL-27 signaling. We therefore examined immune memory formation and function in the IL-27RαKO/WT mixed BMCs. IL-27RαKO/WT BMCs were given a primary immunization with Poly I:C/αCD40 (IL-27–dependent) or Lm-ova (IL-27–independent). Fifty days later, the mice were boosted with either Vv-ova or Poly I:C/αCD40, and the secondary T-cell response was observed 5 d later. Two observations are noteworthy. First, despite having similar resting memory populations (Fig. 2.2.3.1A), IL-27Rα–deficient T cells from Poly I:C/αCD40-primed mice showed defective secondary expansion to Vv-ova challenge, compared with their WT counterparts (Fig. 2.2.3.1C and 2.2.3.2A). Thus, in the absence of IL-27 signaling, primary subunit vaccination results in a deficit in memory programming. Second, the secondary response of IL-27Rα–deficient T cells was also reduced in mice primed with Lm-ova and boosted with Poly I:C/αCD40 (Fig. 2.2.3.1C and Fig. 2.2.3.2A). These data indicate that even pathogen-elicited memory T cells also have an acute requirement for IL-27 signaling in response to subunit vaccination. Importantly, IL-27RαKO T cells in these chimeras respond comparable to WT cells if primed with Lm-ova (Figure 2.2.3.1B and boosted with VV-ova (Fig. 2.2.3.1C), once again reinforcing the unique role of IL-27 in subunit vaccination compared with infectious challenge.
Figure 2.2.3.1 WT and IL-27RαKO mBMC response to secondary challenge.

WT/IL-27RαKO mixed bone marrow chimeras (mBMCs) were generated as described in Fig. 2.2.1.2. Mice were the immunized with Poly I:C/αCD40/ova (50ug/50ug/100ug) or Lm-ova (2000 c.f.u./mouse). Chimeric mice were then assessed for their primary response to Lm-ova (B) and (A) their resting memory as a percentage of their congenic CD8 compartment 50 d.p.i.. Mice were then challenged with either Vv-ova (1x10^7 p.f.u./mouse) or Poly I:C/αCD40/ova (50ug/50ug/100ug). (C) The KO percentage of WT fold change was calculated for each mouse by calculating the fold change from rest for WT and IL-27RαKO T cells after secondary challenge. This was determined by dividing the fold change in antigen-specific IL-27RαKO T cells by the fold change of WT T cells, and is expressed as a percentage. # - denotes no statistical difference, p= 0.2554. For differences between WT and KO T cell populations paired Student’s t-test was used for determining statistical significance. For difference between treatment groups, group values were compared base upon Student’s t-test. Data are displayed and analyzed in aggregated from two separate experiments.
To demonstrate the impact of all of these factors in aggregate, we compared the protective capacity of antigen-specific WT and IL-27RαKO T cells in response to an infectious challenge. Equal numbers of either WT or IL-27RαKO antigen-specific memory T cells (generated by Poly I:C/αCD40 vaccination) were transferred into congenically disparate naive WT B6 mice and subsequently challenged with a lethal dose of Lm-ova. Five days later, mice were killed, and the bacterial load in the spleens was determined as a measure of the protective capacity of the transferred cells (Fig. 2.2.3.2B). Mice receiving IL-27RαKO T cells showed elevated bacterial load over a broad range of transferred T cells, indicating that IL-27Rα-deficient T cells are less protective on a per-cell basis relative to WT cells. Collectively, we conclude from these data that vaccine-induced protective T-cell memory is compromised in the absence of IL-27.

IL-27 signaling after subunit vaccination could conceivably be influencing T-cell proliferation, survival, differentiation, or some combination of all of the above. To detect which of these factors was influencing the abundance of antigen-specific T cells, we transferred equal numbers of congenically marked WT (CD45.1/2) and IL-27RαKO (CD45.1/1) ova-specific TCR-transgenic T cells (OT-1) into B6 recipients (CD45.2/2) and subsequently immunized them with Poly I:C/αCD40/ova. The response of the transferred T cells was then monitored each day to determine the functional consequences of IL-27 deficiency on early events in the T-cell response.

By transferring carboxyfluorescein succinimidyl ester (CFSE)-labeled OT-1s, we were able to observe that IL-27RαKO T cells had no deficit in antigen recognition or cellular division because they display similar numbers of divisions as WT OT-1s throughout the first 2–3 d after immunization (Fig. 2.2.3.2C). However, by monitoring the ratio of WT/IL-27RαKO OT-1s, we observed a
striking increase in the ratio of WT to IL-27RαKO OT-1s between the peak of the OT-1 response at day 5 and growing through day 7 (Fig. 2.2.3.2D). Thus, either the survival of IL-27RαKO T cells was compromised past day 3 or their division was reduced compared with WT. Intracellular staining for the cell cycle-regulated protein Ki67 showed no differences at any time between WT and IL-27RαKO OT-1s in the proportion of cells undergoing division (Fig. 2.2.3.2E). We therefore concluded that it was likely that survival of IL-27Rα-deficient T cells is compromised at late times after subunit vaccination.

We next examined the role of IL-27 in shaping the effector functions and transcriptional profile in response to the subunit immunization. Intracellular cytokine staining of the co-transferred WT/IL-27RαKO OT-1 T cells 7 d after vaccination revealed a deficiency in IFNγ production by IL-27RαKO T cells (Fig. 2.2.3.2F). No deficits in granzyme A or granzyme B were noted, and all of these observations were consistent with previous reports of IL-27Rα deficiency in other systems\textsuperscript{97,98}. Although alterations in expression levels of a variety of transcription factors; IRF4, T-bet, Gata3, c-MAF, Blimp-1, Bcl6, and eomesodermin (Eomes) were easily observed between naive and vaccine-experienced T cells, the only transcription factor that showed a difference between WT and IL-27RαKO OT-1s in expression profile was Eomes. IL-27RαKO OT-1 T cells had an early and persistent decrease in Eomes expression (Fig. 2.2.3.2G), consistent with previous reports demonstrating the ability of IL-27 to amplify Eomes expression via STAT3\textsuperscript{72,99}. We confirmed the functional significance of this reduced Eomes expression by observing a subsequent reduction in the expression of CD122 (Fig. 2.2.3.2H), a protein known to be regulated by Eomes and to support the proliferation and cell survival of T cells via IL-2/15 signaling\textsuperscript{100,101}. 

Figure 2.2.3.2 IL-27 shapes T-cell memory, function, and survival.
Figure 2.2.3.2 IL-27 shapes T-cell memory, function, and survival. (A) Memory fold expansion assay of WT and IL-27RαKO mixed BMCs as described in Figure 2.2.3.1. Briefly, fifty d.p.i. mice immunized with either Poly I:C/αCD40/ova (50ug/50ug/150ug) or Lm-ova (2000 c.f.u./mouse), mice were boosted with either Vv-ova (1x10^7 p.f.u./mouse) or Poly I:C/αCD40/ova. Fold expansion was calculated as fold increase of the percentage of tetramer positive cells from rest to peak of secondary response (day 5) within a congenic group. (B) Lm-ova protection assay. WT Vβ5 and IL-27RαKO Vβ5 mice were immunized with Poly I:C/αCD40/ova (50ug, 50ug, 100ug) and allowed to rest more than 35 days. Mice were sacrificed and CD8+ T cells purified by antibody-mediated magnetic negative selection. The numbers of antigen-specific T cells were determined by tetramer stain and equal numbers (as indicated in graph) of antigen-specific cells were transferred into congenically disparate WT mice. The following day mice were given a lethal challenge of Lm-ova (2.5 x10^6 c.f.u./mouse). Mice were sacrificed 5 days later and spleens harvested and plated on BHI agar plates containing 5ug/mL erythromycin for 36 hour incubation at 37°C to count Lm-ova burden. (C–H) WT (CD45.1/2) and IL-27RαKO (CD45.1) OT-1s were co-transferred into WT B6 (CD45.2) hosts and immunized with Poly I:C/αCD40/ova. Mice were killed at the time points indicated, and OT-1s were compared for (C) CFSE dilution days 1–3, (D) ratio between WT/IL-27RαKO OT-1s, (E) proportion of cycling cells by % Ki67 intracellular stain, (F) IFNγ production by intracellular cytokine staining 7d.p.i. after 4 hr in vitro incubation with SIINFEKL peptide and brefeldin A, (G) eomesodermin by intracellular transcription factor antibody staining, and (H) CD122 expression by surface stain and flow cytometry. A–H are representative data from single experiments repeated at least twice with an n greater than or equal to 3 for each group. For experiments employing separate hosts for WT and KO T cells (B) Student’s t-test was used for statistical significance. For A, C–H, single time point significance was determined using paired student’s t test whereas time course significance was determined by two-way ANOVA.
The functional IL-27R, composed of the unique IL-27Rα and shared gp130 subunits, allows signaling through both STAT1- and STAT3-dependent signaling pathways. To address whether either of these signaling pathways was responsible for the unique defects we have observed in IL-27RαKO mice, we immunized mice with T cells deficient in STAT1 or STAT3. Due to the known effects of STAT1 deficiency within dendritic cells on antigen presentation and T-cell costimulation\textsuperscript{102,103}, we generated STAT1KO/WT mixed bone marrow chimeras and examined the magnitude and phenotype of STAT1KO T cells compared with WT T cells.

In the chimera, we observed no differences in the magnitude (Fig. 2.2.3.3A) or in the affinity distribution between STAT1KO and WT T cells in response to either Pam3/αCD40 or Poly I:C/αCD40 immunization. Similarly, we found in experiments using conditional deletion of STAT3 within the T-cell compartment (STAT3 fl × CD4 cre)\textsuperscript{104}, the magnitude of the T-cell response to subunit vaccination was largely unaffected (Fig. 2.2.3.3B). Interestingly, however, the responding CD8+ T-cell pool did show a skewed affinity distribution similar to that observed in IL-27RαKO T cells (Fig. 2.2.3.3C and D), indicating the importance of STAT3 in mediating IL-27–mediated expansion/survival of high-affinity T cells.

To address the possibility that STAT1 and STAT3 share redundant functions downstream of IL-27R signaling, we generated STAT1 fl × STAT3 fl × CD4 CRE (+/−) mice. Due to concerns with a more general STAT1 defect in the STAT1 fl mice\textsuperscript{105}, we again generated congenic mixed BMCs using WT bone marrow mixed with either Cre-sufficient or Cre-deficient STAT1 fl × STAT3 fl bone marrow. As before, mBMCs were immunized with Poly I:C/αCD40, and the
magnitude and affinity distribution of the responding CD8+ T cells were analyzed.

In contrast to the loss of either STAT1 or -3 alone, T cells deficient in both STAT1 and -3 showed a reduction in the magnitude of the T-cell response similar to that observed in the IL-27RαKO T cells (Fig. 2.2.3.3E). This reduction was not observed for cre deficient STAT1/3 fl/fl T cells (Fig. 2.2.3.3E), indicating once again that STAT1/3 dependency was T cell-intrinsic.

Figure 2.2.3.3 STAT1 and STAT3 deficiency in T cells recapitulates IL-27RαKO deficiency. (A) STAT1KO:WT mBMCs were immunized with either Poly I:C/αCD40/ova (50ug/50ug/100ug) or Pam3/αCD40/ova (25ug/50ug/100ug) as indicated, and the percentage of tetramer positive cells within each congenic T cell group determine 7 d.p.i. from the spleen. (B–D) STAT3 fl mice ± CD4-Cre were immunized with Poly I:C/αCD40/ova (50ug/50ug/100ug) and 7 d.p.i were analyzed for (B) number of tetramer positive cells and (C) the tetramer staining histograms of tetramer of positive cells. STAT3 fl + Cre (white) and without cre mice (filled) are overlaid. (D) Ratio of tetramer FI/CD3 FI was determined as described in Fig. 2.2.2.2 (E and F) STAT1fl × STAT3 fl ± CD4 Cre:WT BMCs were immunized with Poly I:C/αCD40/ova (50ug/50ug/100ug) and examined 7 dpi in blood for (E) the percentage of tetramer+ CD8+ T cells and (F) Tet FI/CD3 FI. Data displayed are from single experiments (A–F). A–D are representative of experiments repeated two or more times with n greater than 3 for each experiment. E and F were powered to determine n required for determination of statistical significance. Significance was determined by unpaired (B–D) and paired (A,E and F) Student’s t-tests.
Similar to both IL-27RαKO and STAT3 KO T cells, the affinity distribution of the CD8 T-cell response in the STAT1/3 double deficient T cells showed a loss of high-affinity responders (Fig. 2.2.3.3F). Taken together, our data reveal both unique and redundant roles for STAT1 and STAT3 in IL-27–mediated survival and affinity distribution for T cells responding to subunit vaccination.

2.3 Discussion

Our data identify a previously unobserved, highly selective, non-redundant role for IL-27 in instigating the survival of subunit vaccine-elicited cellular immune responses, contrasting with the role for IL-27 in pathogen-elicited cellular immunity. Consistent with previously published literature\textsuperscript{97,98,100}, IL-27 has effects on T-cell effector function (IFNγ) and transcriptional profile through the augmentation of expression of eomesodermin. In light of the highly pleiotropic nature of IL-27 signaling in both inflammation and immune regulation, we uncovered a surprisingly consistent and dramatic effect of IL-27 on the magnitude of T-cell responses across a spectrum of vaccine adjuvant-elicited cellular responses. Our data join only a few existing publications\textsuperscript{78,87,106} directly demonstrating in vivo a direct positive role of IL-27R signaling in non-regulatory T-cell phenotypes. Many examples exist in the literature with indirect evidence of a positive role for IL-27 in enhancing CD8 T-cell responses, particularly in cancer therapy\textsuperscript{85,86}, but rarely is the direct effect of IL-27 on T cells examined.

More recently, another report\textsuperscript{78} also demonstrated an in vivo, T cell-intrinsic requirement for IL-27R signaling in the preservation of activated effector CD4 T-cell numbers undergoing homeostatic expansion. In this report, IL-27 mediated the survival of activated T cells through up-regulation of cFLIP, which counteracted Fas-mediated caspase 8 activation. Thus far, we have observed that none of the classical instigators (Fas; caspase-1, -3, or -8; and Bim) or
inhibitors (Bcl2 and BclxL) of caspase-mediated cell death appear to be involved in IL-27–mediated T-cell survival after subunit vaccination. In fact, observed decreases in active caspase-8, -3, and -1 in IL-27RαKO OT-1 T cells suggested less cell death by extrinsic apoptosis, intrinsic apoptosis, and pyroptotic pathways, respectively.

Although these observations leave open the question as to how IL-27 mediates T-cell survival in the setting of subunit vaccination, our observation of decreased Eomes/CD122 in IL-27RαKO T cells may be consistent with a role for these molecules in survival and metabolic regulation of proliferating T cells.

During rapid growth, such as that experienced by T cells after antigenic stimulation, T cells switch their energy metabolism from favoring oxidative phosphorylation (TCA cycle) to aerobic glycolysis, a process that can be enhanced by IL-15 signaling through CD122\(^{107}\). Cells that are stuck favoring the TCA cycle and unable to meet increased energy demands are more likely to die from necroptosis\(^{108}\). Necroptosis is another form of programmed cell death often mediated by the protein kinases RIPK1 and RIPK3\(^{109,110}\) and enhanced by the presence of reactive oxygen species. Importantly, necroptotic pathways have been shown to be in direct opposition to apoptotic and pyroptotic cell death. This pathway is consistent with our preliminary caspase observations in IL-27Rα–deficient T cells responding to subunit vaccination.

Whichever cell death pathway is involved, our data indicate that its prevention requires STAT1/3 signaling. Our use of both IL27RαKO and STAT1/3 KOs in mixed bone marrow chimeras not only confirms the T-cell intrinsic dependency of IL-27 and STAT1/3 but also demonstrates the exclusive dependency of the vaccine-elicited response on IL-27. A host of STAT1/3-signaling cytokines are induced during the course of vaccination, some of which
(IL-21, IL-10) are downstream of IL-27\sup{81,88,111,112}. In the absence of the mixed BMC data, it could be argued that IL-27 is simply the initial cytokine in a cascade of STAT1/3-dependent cytokines. Indeed, Braciale and co-workers demonstrated that, in the response to influenza, IL-27 acts directly on CD4 T cells to induce their production of IL-10, which ultimately affects the magnitude of the primary response and the differentiated state of the memory cells\sup{89}. However, because WT-derived T cells in the mixed chimeras did not provide any rescuing effect to IL-27Rα-deficient T cells, this argues against any causal role for paracrine cytokine factors. Consistent with this, abrogation of IL-6\sup{113} or IL-10 (Fig. 2.3.1), both STAT3 cytokines, had no impact on the vaccine-elicited T-cell response. Thus, not only is the dependency of IL-27 subunit-elicited cellular immunity inverse from what is observed in primary T-cell responses to pathogen, but the pathways downstream of IL-27 are not conserved between pathogen exposure and subunit immunization.

Perhaps even more surprising is the central importance of IL-27 signaling via STAT3 for the response of high-affinity T cells. STAT3 signaling often participates in resolving inflammation and suppressing immune responses and was recently shown to be critical for generation of memory CD8 T cells, ultimately through induction of SOCS3 and suppression of T-bet\sup{114}. These and other studies indicate that unabated pro-inflammatory signals lead to terminal-effector differentiation and immune exhaustion. Seen in this context, STAT3 signals might facilitate the survival/transition of high-affinity T cells into long-lived memory T cells, as has been demonstrated in both mouse and human\sup{84,87,114–116}. In our system, IL-27, signaling through STAT3, would serve as the immunological restraint, critically dampening the higher perceived signal within the higher-affinity T-cell clones.
Figure 2.3.1 IL-10R blockade does not alter T-cell expansion or WT/IL-27RαKO antigen-specific T-cell ratio. Three thousand WT and IL-27RαKO OT-1 T cells of different congenic background were purified by CD8 PE-mixture negative selection to greater than 90% purity and then transferred 1:1 into congenically distinct WT host. The following day, mice were immunized with Poly I:C/αCD40/ova (50μg/50μg /150μg). To prevent interference with early innate IL-10 signaling, we chose to block IL-10R starting 72 h after immunization immediately before T cells should begin to produce effector cytokines. Then, 72 h and 96 h post immunization, half of the mice were given 250 μg per mouse anti-IL-10R antibody. Mice were killed at peak of OT-1 primary response (day 5), and (A) total numbers of OT-1 T cells were enumerated, and (B) the ratio of WT/IL-27RαKO OT-1 T cells was determined. Student’s paired t-test determined the statistical significance between WT and IL-27RαKO responses for a given treatment whereas an unpaired Student’s t-test was used for comparing between treatment groups.

Finally, it is interesting to note that, in the absence of IL-27Rα, responses to the combined TLR/CD40 adjuvant platform more closely resemble the magnitude of responses of WT mice to single adjuvants. Consistent with this, use of recombinant IL-27 increases antigen-specific T-cell responses above use of αCD40 alone (Fig.2.3.2). However, these responses are well short of the 50- to 100-fold increases reported previously from combined TLR/CD40. This failure to recapitulate the magnitude of the T-cell response to Poly I:C/αCD40 indicates that IL-27 cannot completely replicate the complex inflammatory environment instigated by the TLR agonist.
Recombinant IL-27 enhances T-cell responses to αCD40 immunization. Recombinant IL-27 (IL-27p28/EBI3) was produced and purified as Fc-fusion proteins as described in detail Materials and Methods. Three thousand purified WT OT-1s were adoptively transferred into congenically disparate host. Mice were then immunized i.v. with αCD40/Ova (50μg/150μg per mouse). Half of the recIL-27 was papain digested to liberate functional IL-27 from the Fc portion and then repurified by dialysis to separate from papain digestion reagents. When mice received recIL-27, they were immunized with 250 μg of rec IL-27 per treatment (125μg of papain digested and 125μg of Fc-conjugated recIL-27). One cohort of mice was immunized (1x) with rec IL-27, 2 h after αCD40 immunization, and another cohort received an additional 250μg of recIL-27 injections on day 3 and day 4 (∗3). At peak of OT-1 response (day 5), mice were killed, and the numbers of OT-1s were enumerated. Statistical differences were determined by Student’s t-test between cohorts.

We and others previously published on an important role for CD70–CD27 interactions in the T-cell response to combined TLR/CD40 immunization as well as to infectious challenge/vaccination. Since IL-27 and CD27 separately are required for maximal T-cell responses after subunit vaccination, neither alone is therefore sufficient for maximal T-cell expansion. All available data suggest that it is the synchronized delivery of signals through these two signaling pathways that is at the heart of the combined TLR/CD40 subunit vaccine adjuvant potency. Thus, we propose that future efforts at novel vaccine adjuvant discovery would do well to monitor the kinetics and magnitude of DC expression of IL-27, as well as the ligand for CD27, CD70.
2.4 Model of T cell dependency on IL-27

In conclusion we propose the following molecular model (Figure 2.4) for how IL-27 enhances T cell number, survivability and function. Upon cytokine binding to the functional IL-27R, a unique milieu of STAT1, STAT3 or STAT1/3 dimers translocates to the nucleus driving gene transcription and therefore translation of Eomesodermin. Eomes itself then binds to DNA elements responsible for the upregulation of CD122 and production of IFNγ. Deficits in IFNγ are most certainly responsible for the observed impairments of T cell protection in the Lm challenged system. CD122/IL-15 on the other hand is known to play a role in the maintenance of memory CD8 T cells and has been shown to enhance T cell number and survival in response to simple adjuvants, and strong TCR signals. In the next chapter we test this model formally by investigating the role of IL-15/CD122 in the dual agonist vaccine platform.
IL-27 is a heterodimer of the unique subunit IL-27p28 (dark green, white writing) and the EBI3 subunit (light green, black writing) shared with IL-35. Correspondingly, IL-27p28 subunit associates with the unique IL-27Rα chain while EBI3 utilizes the more promiscuous cytokine receptor gp130. Functional cytokine engagement results in JAK transphosphorylation of the cytokine receptor cytoplasmic tails. Phosphorylation sites act as binding sites for SH2 domains of STAT molecules. STAT1 associates with IL-27Rα while STAT3 associates with gp130. While bound to the cytoplasmic tails the STATs themselves are acted upon by the JAKs. Once phosphorylated they dimerize and translocate to the nucleus to bind to specific DNA motifs to enhanced transcription and translation of Eomesodermin. As transcription factor itself Eomesodermin binds to DNA and enhances expression of CD122 and IFNγ.
CHAPTER III
IL-15 DEPENDENCY OF SUBUNIT ADJUVANTS

3.1 IL-27RαKO T cells, like CD122KO T cells display a “trivial” memory phenotype

The model of T cell IL-27R dependency for subunit vaccination described above ultimately (Fig. 2.4) relies heavily on the observed decreased expression of CD122 to account for reduced cell number and functionality of responding antigen-specific T cells. We have shown above that the numbers of antigen specific IL-27RαKO T cells are drastically decreased in response to subunit immunization, however a resting population of memory T cells are easily observable (Fig 2.3.1A). Furthermore the remaining antigen-specific T cells do expand but to a very limited extent, produce less IFNγ and offer poor protection against subsequent pathogen challenge (Figure 2.3.2). These initial observations fit the description of an extreme central memory T cell phenotype which I refer to as “trivial” memory. Supporting this claim, we have observed that IL-27RαKO antigen-specific T cells at the peak of response have reduced KLRG1 expression, as well as decreased downregulation of CD27 and an impaired ability to upregulate the active isoform of CD43 (Figure 3.1). This is demonstrated here by employing the adoptive transfer OT-1 system utilized previously and interrogating the T cells at the peak of the primary response. We have examined these features on endogenous T cell populations from WT and IL-27RαKO mice and noted the phenotype is conserved between the two systems. We choose to continue to employ the OT-1 system when possible to avoid phenotypic variations subsequent of altered affinity repertoire and to be able to ascribe alterations to intrinsic T cell defects.
Figure 3.1 Antigen-specific IL-27RαKO T cells have trivial memory phenotype. 3000 of each congenically disparate WT OT-1 and IL-27RαKO OT-1s were adoptively co-transferred into a congenic WT host. Mice were immunized with Poly I:CαCD40/ova (50ug/50ug/100ug) (A&B) or by Pam3αCD40/ova (25ug/50ug/100ug) (C) by intravenous (i.v.) injection. 5 days later at the peak of the OT-1 response splenic OT-1s were phenotyped by flow cytometry for surface expression of the activation isoform of CD43 (A), CD27 (B) or KLRG1 (C). Gates were set for positive expression based upon unstained, isotype and FMO controls. For (A) light gray bar represents naïve endogenous T cells while dark histogram is WT OT-1s at day 5 post immunization. For histogram (B) Light gray plot represent FMO control and dark gray WT-1 OT-1s at peak of the primary response. Data displayed and quantified are from one experiment for each agonist and chosen to be representative of a body of data repeated greater than 3 times within each the endogenous and OT-1 adoptive transfer systems. Statistical significance was determined by paired Student’s t-test.

Interestingly a recent report\textsuperscript{121} demonstrated the modification of CD43 glycoforms was correlated to IL-15-mediated upregulation of intracellular glycosylases. Along these lines another report investigating the effect of altered CD122 signaling observed the importance of varied CD122 signal strength in dictating the outcomes of CD8 T cell activation\textsuperscript{122}. In this report they illustrated that high CD122 was necessary for the development of both terminal effector and effector memory cells while central memory phenotype cells could survive with very little CD122 signals. The surface phenotype of these residual memory T
cells was that of central memory and correspondingly they were located in the lymph node. However these antigen-specific T cells of low CD122 where unlike conventional central memory cells in that they afforded little protection against pathogen challenge consequent of a reduced ability to expand and produce cytokine. Additionally, like the IL-27RαKO T cells these CD122 low T cells exhibited altered CD43, KLRG1 and CD27 expression patterns, and in so doing recapitulate the observed phenotype for IL-27RαKO T cells of “trivial” memory. This report reinforces the possibility that lowered CD122 could result in the observed IL-27RαKO antigen-specific phenotype.

3.2 CD122 signaling by IL-2 and IL-15

CD122 is the shared beta subunit of the cytokine receptor complexes for both IL-2 and IL-15. CD122 is constitutively expressed at varying levels on multiple subtypes. Within the T cells subtypes conventional CD4 cells express lower levels of CD122 than CD8 T cells. After activation CD122 is upregulated on all T cells and remains elevated on memory T cells. Both cytokines also utilize the common gamma chain (CD132) cytokine receptor subunit shared by the appropriately named common gamma family of cytokines (IL-2, 4, 7, 9, 15 and 21). Unique signaling is conferred between IL-2 and IL-15 based upon the presence of the unique alpha subunit for each receptor complex. For IL-2 the unique alpha receptor is CD25 and it is also known as the high affinity IL-2 receptor subunit for its ability to generate IL-2 specific responses in combination with CD122 and CD132. CD25 is most notoriously constitutively expressed on T regulatory cells (Treg) tracking well with the functionally IL-2-addicted personality of Tregs. CD25 expression is induced to varying degrees on non-Treg CD4
and CD8 T cells based upon cellular activation conditions. CD25 operates in cis, with CD122 and CD132 to coordinate IL-2 signaling.

As for the IL-15Rα expression, it can be induced to be expressed on multiple cell subtypes, however during the steady state it is only appreciably expressed on dendritic cells. A unique group of experiments utilizing bone marrow chimeras, illustrated that unlike IL-2 which requires expression of CD25, CD122 and CD132 on the same cell, IL-15Rα can signal in trans\textsuperscript{124,125}. This paradigm has gone on to reinforce the importance of the magnitude and location of IL-15 and IL-15Rα production in relationship to its observed biological function. In vivo, most activities of IL-15 are accounted for by trans-presentation. In vitro however, several people have clearly demonstrated cis activation\textsuperscript{122}. Lacking a signaling domain within the intracellular tail, IL-15Rα binding of IL-15 seems to function to stabilize, orient and localize IL-15 signals to nearby cells expressing the signaling subunits CD122 and CD132.

CD122 and CD132 being the primary molecules responsible for signal transduction of IL-2 and IL-15, their proximal intracellular signaling pathways have been well explored. Like many canonical cytokine receptors, CD122 and CD132 are constitutively associated with Janus Kinases, JAK1 and JAK3, respectively. Upon ligand binding the receptor undergoes a conformational change activating the JAKs. Traditional JAK-STAT cascades show activated JAKs phosphorylating tyrosine residues in the intracellular tails of the cytokine receptors. The spacing and phosphorylation status of key residues differentially recruits various scaffolding and adaptor proteins to the membrane bound receptors. For canonical IL-2 JAK-STAT signaling, SH-2 domains on STAT5 bind to phosphorylated tyrosines of the cytokine receptors intracellular tails.
Transiently docking in these locations places the STAT5 molecule within proximity to be acted upon directly by the JAKs. Once they are phosphorylated STAT5 molecules release from the cytokine receptor tails, dimerize and translocate to the nucleus to bind to DNA and alter gene transcription\(^{126}\).

**Figure 3.2 Overview of CD122/IL15-IL-2 signaling cascades.** CD122/CD132 are responsible for the IL-15 and IL-2 intracellular signaling cascade. The unique cytokine receptor alpha subunits are differentially expressed with IL-2 being on the same cell as CD122 and CD132 (cis) while IL-15R\(\alpha\)-IL15 complexes are presented effectively by a neighboring dendritic cell (green) (trans). Three pathways are known to be downstream of CD122/CD132 signaling in response to either IL-2 or IL-15. Canonical JAK-STAT signaling leads to STAT5 dimerization and translocation to the nucleus altering gene transcription. SHC1 binding to CD122 recruits RAS to the membrane resulting in activation of the MAPK cascade. Lastly direct activation of JAKs on PI3K can activate Akt and subsequently suppress Foxo1 while activating mTOR pathways.

Alternatively several groups have shown the scaffolding and adaptor proteins Shc and subsequently Grb2, binding to the phosphorylated regions of the intracellular cytokine tail and subsequently recruiting the small GTPase RAS\(^{127}\). Positioning RAS closer to the membrane leads to downstream activation of the MAPK/ERK pathway. A third pathway exists where JAK1 and JAK3 in concert or independently of the above scaffolding directly activate PI3K.
activates AKT which in turn negatively regulates FOXO1 and activates mTOR\textsuperscript{128}.

Curiously while IL-2, IL-2R\textsubscript{α}, IL-15 and IL-15R\textsubscript{α} knockouts all have distinctly different phenotypes in various T cell priming conditions, no completely unique intracellular signaling pathways are present between IL-2 and IL-15. This relegates the differences observed between IL-2 and IL-15 pathways largely attributable to the differences in location, timing and magnitude of alpha chain and cytokine expression\textsuperscript{122,129,130}.

With this in mind, we return to reconsider the previously referenced paper\textsuperscript{122} that established variable dependency on CD122 for modulating memory T cell outcome. When this group repeated their observations in IL-15KO mice they saw alteration to the effector memory, central memory balance as observed before with CD122 mutants. This relegated these early memory differences to the effect of IL-2 rather than IL-15.

3.3 IL-15KO T cells recapitulate the phenotype of IL-27R\textsubscript{α}KO in response to subunit immunization

Ultimately the validity of the model proposed in 2.4 rests on whether or not in our system alterations in CD122-transmitted signals have any consequence to the primary response. As discussed previously the delineation as to whether the CD122 signals are being propagated by IL-2 or IL-15 can be deduced by the timing and magnitude of cytokine production and/or expression of the unique cytokine alpha subunit (CD25 for IL-2, CD215 for IL-15). To determine if the differences seen in CD122 would be related to alterations in IL-15 or IL-2 relayed signaling we examined CD25 expression in the OT-1 adoptive transfer system.
At the peak of the primary response, day 5, we observed very few CD25+ cells in either WT or IL-27RαKO T cells (Fig. 3.3.1). Furthermore there was no difference in the relative number of T cells expressing CD25 between WT and IL-27RαKO OT-1. This is consistent with the current literature which supports very little CD25 upregulation on CD8+ T cells that are primed in vivo, while more robust CD25 expression results from in vitro priming. Based upon this finding we hypothesized IL-2 has little impact on the dual agonist system and therefore deprivation of CD122/IL-15 signaling is ultimately what results in the generation of fewer antigen-specific cells down stream of IL-27Rα deficiency.

![Figure 3.3.1 CD25 expression of WT and IL-27RαKO OT-1.](image)

**Figure 3.3.1 CD25 expression of WT and IL-27RαKO OT-1.** 3000 of each congenically disparate WT OT-1 and IL-27RαKO OT-1 T cells were adoptively co-transferred into WT B6 host, then immunized with Poly I:C/αCD40/ova (50ug/50ug/100ug). Day 5 post immunization, spleens were harvested and cells stained for expression of congenic markers and CD25 expression. (A) CD25+ gates were set based upon FMO and isotype controls. As a reference for these gates, displayed are endogenous CD8+ T cells from naïve B6 (left panel) and a representative plot of transferred WT OT-1s, day 5 post immunization with Poly I:C/αCD40/ova (right panel). (B) % CD25+ expression of co-transferred WT and IL-27RαKO OT-1s collected from one experiment. Experiment was repeated greater than 2 times with at least 4 mice per group.

Consistent with this hypothesis IL-15KO antigen-specific T cells have routinely demonstrated lower levels of IFNγ production, similar to our IL-27RαKO phenotype. Furthermore, several groups have shown that addition of IL-15 and IL-15/IL-15Rα complexes to simple adjuvants results in enhanced effector CD8+...
T cell numbers\textsuperscript{131,132}. However no literature illustrates an obligate role for IL-15 in subunit immunization.

Problematic to our hypothesis that IL-15 has any role in the IL-27R\textsubscript{α}KO phenotype is a large body of evidence that illustrates that IL-15 deficiency has no dramatic impact on the magnitude of the antigen-specific T cell primary response to multiple pathogenic challenges\textsuperscript{133–135}. What is firmly established is the requirement for IL-15 to maintain the memory pool of CD8+ T cells during the contraction and stable memory phase of the T cell immune response\textsuperscript{136–138}. The results from out IL-27R\textsubscript{α}KO studies have illustrated that cytokine dependencies for T cell priming are not conserved between pathogen and subunit immunizations. Therefore it is difficult to predict T cell outcomes based upon literature from different immune challenges.

To address our hypothesis and build upon the literature of IL-15 in the context of subunit immunization, we immunized IL-15KO and WT B6 mice with Poly I:C/\textsubscript{α}CD40 or Pam3cys/\textsubscript{α}CD40 subunit adjuvant, plus antigen, i.v.. For antigen, mice received whole ovalbumin, containing the immune-dominant CD8+ T cell epitope SIINFEKL in conjunction with the CD4+ T cell MHCII epitope 2W1S peptide following the same protocol described earlier. We examined the magnitude and character of splenic antigen-specific CD4+ and CD8+ T cells at the peak of the primary response, 7 days post immunization. Like the IL-27R\textsubscript{α}KO mouse we observed significant deficits in both the relative number (Fig.3.3.2B & E) and absolute magnitude (Fig. 3.3.2C & F) of antigen-specific T cells in the CD8+ (Fig. 3.3.2 A-C) and CD4+ (Fig. 3.3.2 D-F) T cell compartments.
Figure 3.3.2 Primary T cell responses in IL-15KO mice to dual agonist vaccine.
Figure 3.3.2 Primary T cell responses in IL-15KO mice to dual agonist vaccine. WT B6 and IL-15KO mice were immunized with either Poly I:C/αCD40/ova (50ug/50ug/100ug) or Pam3/αCD40/ova (50ug/50ug/100ug). 7 days post immunization at the peak of the primary response spleens were harvested and stained with tetramer for enumeration of antigen-specific T cells. CD8+ T cells were gated as B220-, CD3+ CD8+ (A) a representative profile of CD44 and Kb-SIINFEKL tetramer staining in WT and IL-15KO mice for both immunization conditions (B) % tetramer positive cells within the CD8+ T cell compartment (C) the absolute number of CD8+ antigen-specific T cells in the spleen. CD4+ T cells were gated as B220-, CD3+ CD4+ (D) a representative profile of CD44 and I-A^b tetramer staining in WT and IL-15KO mice for both immunization conditions (E) % tetramer positive cells within the CD8+ T cell compartment (F) the absolute number of CD4+ antigen-specific T cells in the spleen. Statistical significance was determined by Student’s t-test between WT and IL-15KO cohorts. Data displayed are from one experiment, experiment was repeated twice with n greater than or equal to three.
It is worth highlighting the surprising result that CD4 T cells are also significantly impaired in the absence of IL-15. CD4s are known to readily upregulate CD25 after 48 hours of stimulation both in vitro and in vivo in response to a broad range of activation conditions. With an understanding of the redundancy for IL-2 and IL-15 signals, the dependence upon IL-15 is surprising as it illustrates that the perception of IL-15 signals must occur in a time critical for CD4 T cells that cannot be supplemented by IL-2 signaling. Speculating on this further it is worth noting that the primary source of IL-2 production is activated CD4 T cells. Under the conditions of this experiments, with completely naïve animals, both CD25 upregulation and IL-2 production would be present at substantial levels all time points with the exception of the first 24-36 hours. The idea that day 7 responses are impaired suggests the importance of an enhanced (inflammatory vs homeostatic) IL-15 response during the early innate response. To more appropriately discern the importance of IL-15 to CD4+ T cells, studies with transgenic antigen-specific TCR T cells should be used in an adoptive transfer model under conditions of the dual agonist system. Similar to what was done for the CD8+ T cell compartment, this would allow for greater sensitivity in determining the kinetics of CD25 upregulation and IL-2 production. This kinetic importance is elaborated on in subsequent sections.

Returning to the day 7 T cell response, we further investigated the characteristics of the remaining T cells in the IL-15KO mice. Utilizing the previously established Tet/CD3 relative affinity metric, we observed the same trend in the IL-15KO mice that was observed in the IL-27RαKO mice. The few antigen-specific CD8+ and CD4+ T cells are of lower relative affinity (Fig 3.3.3A-B). This observation highlights the importance of bioavailable IL-15 for the generation of robust T cell responses during the primary response to subunit
immunization. Like IL-27, IL-15 is critical for all T cells, but disproportionately important for those of high affinity.

Figure 3.3.3 Phenotype of antigen-specific T cells in the IL-15KO. WT and IL-15KO mice were immunized with Poly I:C/αCD40/ova/2W1S (50μg/50μg/150μg/150μg) or Pam3/αCD40/ova/2W1S (25μg/50μg/150μg/150μg). Day 7 post immunization at the peak of primary response, spleens were harvested and processed for flow cytometric staining. (A) B220-CD3+CD8+ Kb-SIINFEKL tetramer+ cells were subjected to Tet FI/CD3 FI relative affinity measurement as described in Figure 2.2.2.2. (B) B220-CD3+CD4+ I-Ab-2W1S tetramer + cells were also analyzed by TetFI/CD3FI measurement. (C) % KLRG1 surface staining + cells of B220-CD3+CD8+ Kb-SIINFEKL tetramer+ cells from mice immunized with Pam3/αCD40/ova. Data displayed are from a single experiment representative of total of 2 experiments. Significance was determined based upon statistical analysis by Student’s t-test.

Interrogating the antigen-specific cells even further we observed yet another similarity between the IL-15KO and IL-27RαKO mice. KLRG1, a marker of effector differentiation, is also lower in the remaining IL-15KO antigen-specific CD8+ T cells. This is most easily observable by comparing differences consequent of immunization with Pam3/αCD40 (Fig 3.3.3C). This is somewhat surprising. While the expression of KLRG1 positively correlates with effector T cell differentiation, it is thought that the fraction of KLRG1 expressing cells correlates with the relative abundance of IL-12 in the inflammatory milieu. This assumption is based upon the model of STAT1/STAT3 balance presented earlier and further supported in our data by the greater proportion of KLRG1 expressing cells in the Pam3/αCD40 immunization compared to Poly I:C/αCD40. Given that
IL-27R has been well documented to facilitate the upregulation of IL-12R, it was not all that surprising to observe the earlier described decreases in KLRG1 + cells in the IL-27RαKO mice. With the IL-27R intact in the IL-15KO mice we would have not have predicted the IL-15KO T cells to have shared this phenotype. These data suggest while IL-27R may be enhancing T effector differentiation through IL-12 receptor, perception of IL-15 signals, which are impaired in both IL-27RαKO (lower CD122) and IL-15KO, must be of greater importance for effector T cell expansion and/or survival.

3.4 Discussion

In this chapter we have shown that apart from the functional impairments demonstrated earlier, IL-27RαKO antigen-specific T cells have phenotypic characterizations consistent with those previously reported on CD122 low antigen-specific T cells. These cells are KLRG1 low, CD43 active isoform low, and CD27 high and behave like “trivial” or useless memory. Trivial memory cells are unable to expand extensively nor do they produce levels of cytokine secreting cells expected of a functional memory response. In determining which pathway, IL-2 or IL-15, to pursue for validation of the importance of CD122 levels in antigen-specific T cell response to the dual agonist vaccine, we report that CD25 levels are essentially non-existent for CD8+ T cells in both IL-27RαKO and WT OT-1s. Based upon these data we acquired and tested T cell responses in IL-15KO mice. In contrast to the existing pathogen response literature, responses in IL-15KO mice were dramatically blunted to the dual agonist vaccine. Furthermore, like the IL-27RαKO mice, the remaining antigen-specific T cells were of low affinity and KLRG1 expression. These observations serve to reinforce the hypothesis that one critical feature of IL-27R expression on antigen
specific T cells is to facilitate the recognition of milieu IL-15 through upregulation of the CD122 receptor.

While the resulting T cell responses in the IL-15KO mice look strikingly similar to those demonstrated to be consequent of T cell intrinsic deficits of IL-27R, we have not yet formally demonstrated that IL-15 specifically impacts the T cells directly during the primary response. NK cells and DCs have both been shown to be modulated by interactions with IL-15. Data too preliminary to present here support the idea that these pathways (IL-27 and IL-15) are in series to one another. Preliminary investigations with WT OT-1 and IL-27RαKO OT-1 cotransfers into WT and IL-15KO hosts show that IL-27RαKO OT-1 responses are equal in the WT and IL-15KO hosts. Furthermore, WT and IL-27RαKO OT-1 responses are equal within the IL-15KO host. This suggests that the environments for these T cells are not redundant to one another and therefore likely in series. Unfortunately limitations in the number of available IL-15KO mice and IL-27RαKO OT-1s have not allowed for a repeat of these data. If this observation holds true it would greatly reinforce the connection between IL-27R and IL-15. To further demonstrate the importance of the T cell intrinsic nature of IL-15 dependency we have also generated CD122KO OT-1s. Again unfortunately at the time this dissertation is being prepared we have been unable to undertake any preliminary experiments.

In the future as mice become available it would be good to quantify the response of IL-27RαKO and CD122KO OT-1s in both the WT and IL-15KO environments. As we have demonstrated, both IL-15 and IL-27 have different effects on T cell responses than would be predicted by pathogen challenge. For that reason it would be intriguing to extend our studies on subunit adjuvants to
examine single adjuvants and common clinical use adjuvant (Alum, monophosphoryl Lipid A (MPL) and Addavax (squalene emulsion)) with IL-27RαKO OT-1s, CD122 KO OT-1s and in IL-15KO mice. Unfortunately time and reagent limitations prohibited these studies from being included in time for this dissertation.
CHAPTER IV
THE INNATE ROLE OF IL-27 IN FACILITATING IL-15-MEDIATED T CELL RESPONSES

4.1 Introduction

The data presented so far point to the importance for T cell cognition of IL-27 and IL-15 cytokines. Highlighting the importance of the availability of IL-27 to boost adjuvant vaccine efficacy we previously reported through the adoptive transfer OT-1 system, 2-3 fold enhancement of T cell responses from a mediocre adjuvant (αCD40) with IL-27 supplementation (Fig 2.3.2). In this experiment we utilized the co-adoptive transfer of congenically disparate WT and IL-27RαKO OT-1s. Upon closer examination we realized that a population that we had intended as control (IL-27RαKO OT-1) also experience modest yet non-dose-dependent, not stat increases in T cell number (Fig 4.1).

Having demonstrated different vaccines platforms (pathogen vs subunit) generate unique milieus that vary in their IL-27R dependency, one interpretation of this response is an introduction of a contaminant along with the recombinant IL-27 augmented the IL-27 dependency of the OT-1 and allowed for more of the IL-27RαKO T cells to grow apart from any dependency on IL-27. If this were the case we would expect a shrinking of the ratio between WT and IL-27RαKO OT-1s as seen earlier with pathogen priming. This was not observed to a significant degree. This means that instead both WT and IL-27RαKO T cells both experienced a new form of cellular support in response to IL-27 that helped them equally but does not overcome the relative deficit imposed upon the T cell by IL-27R deficiency. We observed additional increases in WT OT-1 T cell responses with additional temporally separated IL-27 administrations occurring 24 hours.
apart from one another. However for the IL-27RαKO OT-1 T cells, a single administrations 2 hours after αCD40 administration seemed as beneficial as multiple injections. This sharpened our focus to the effects IL-27 might be having on cells during the early innate immune response.

**Figure 4.1 Recombinant IL-27 enhances IL-27RαKO T-cell responses to αCD40 immunization.** Three thousand each of purified WT and IL-27RαKO OT-1s were adoptively co-transferred into congenically disparate host. Mice were then immunized i.v. with αCD40/Ova (50μg/150μg per mouse). Half of the recIL-27 was papain digested to liberate functional IL-27 from the Fc portion and then repurified by dialysis to separate from papain digestion reagents. When mice received recIL-27, they were immunized with 250μg of rec IL-27 per treatment (125μg of papain digested and 125μg of Fc-conjugated recIL-27). One cohort of mice was immunized (1x) with rec IL-27, 2 h after αCD40 immunization, and another cohort received an additional 250μg of recIL-27 injections on day 3 and day 4 (×3). At peak of OT-1 response (day 5), mice were killed, and the numbers of IL-27RαKO OT-1s were enumerated and are depicted in the figure. Statistical differences were determined by Student’s t-test between cohorts.

We have established the importance of IL-15 and IL-27 on the primary immune response to both of the combined dual agonist subunit vaccines. Both of these cytokines are known to be generated in response to type I IFN generating TLR agonists, like Poly I:C as well as to αCD40 engagement141,142. These activities have been most potently recorded to be executed by dendritic cells and within the first 48 hours of innate engagement. While its obvious that IL-27 and IL-15 would be elicited from Poly I:C/αCD40 immunization we did not
understand how the combined Pam3/αCD40 adjuvant influenced the dynamics of cytokine production compared to single adjuvants alone to result in a synergistic increase in T cells. In our model (Fig 2.4), ultimately IL-15 is the downstream determinate for T cell numbers. Therefore we first sought to determine how individual components of the synergy system collaborated to generate bioavailable IL-15.

4.2 Both agonists are required for coordinated upregulation of IL-15 and IL-15Rα

In exploring IL-15 generation we took note that cognition of available IL-15 is predicated upon the successful presentation of this protein by IL-15Rα. As detailed earlier, this is predominantly done in vivo through trans-presentation of IL-15 in complex with IL-15Rα which is expressed highly on the surface of dendritic cells. We first wished to address the dynamics of IL-15 and IL-15Rα expression in response to individual components of the synergy system as well as combined in the dual agonist platform. To do this we immunized mice i.v. with either Poly I:C, Pam3 or αCD40 in isolation or the previously described dual agonist combination. Subsequently mice were sacrificed and spleens removed and processed to single cell suspensions by collagenase and DNase treatment. Reporter mice for IL-15 (known as TE20s) were used to determine the successful generation of translated IL-15 message mice as determined by GFP expression while both WT and TE20 mice were used for determination of IL-15Rα expression based upon flow cytometric staining. Preliminary time course studies determined that 18 hours represented the optimum peak of co-expression of GFP and IL-15Rα.
It is well appreciated that a diversity of dendritic cells exists. In secondary lymphoid organs dendritic cells are principally divided up into two distinct subset based upon their surface expression of CD11b and CD8 (Fig 4.2A). These subsets are distinct in the PAMP receptor expression and lineage derivation. It has been documented that amongst these two subsets, CD8+ DCs of the spleen are predisposed to a greater efficiency of the specialized process of cross-presentation\textsuperscript{143}. Additionally it has been documented that CD8+ DCs constitutively express elevated levels of IL-15 and express higher levels of basal IL-15R\textsubscript{α}\textsuperscript{144,145}.

When we qualitatively examine our results from the in vivo dendritic cell priming assays we recapitulate these previously reported steady state dynamics of IL-15 and IL-15R\textsubscript{α} (Fig. 4.2B & C). When TLR agonist or αCD40 are introduced to the system, depending upon the agonist, both cell types are able to express IL-15 to some degree. Despite the abundance of reports illustrating IL-15 upregulation in response to type I IFN and αCD40 we noted that the coordinated maximal expression of IL-15 and IL-15R\textsubscript{α} occurs from the dual agonist system and more so on the CD8\textsubscript{α}+ DC subset (Fig. 4.2D&E). Furthermore note that the Pam3/αCD40 profiles is most similar to that of Poly I:C/αCD40. Administration of only Pam3 upregulates IL-15 minimally overall and specifically on CD11b+ DCs and without the coordinated upregulation of the IL-15R\textsubscript{α} (Fig. 4.2D&E). The activity observed by αCD40 alone cannot account for the IL-15/IL-15R\textsubscript{α} dynamic observed from the Pam3/αCD40.
Figure 4.2 Vaccine elicited IL-15/IL-15Rα expression on dendritic cells. WT or TE20 mice were immunized with Pam3/ova (25ug/100ug), Poly I:C/ova (50ug/100ug), αCD40(50ug/100ug), Poly I:C/αCD40/ova (50ug/50ug/100ug) or Pam3/αCD40/ova (25ug/50ug/100ug). 18 hours post immunization spleens were harvested, collagenase and DNase digested, prepared into a single cell suspension. (A) Flow cytometric gating strategy of dendritic cell populations as follows; size exclusion, doublet exclusion, B220-NK1.1-CD3-CD11c+MHCIIHi cells were considered a pure dendritic cell population that could then be divided into CD11b+ and CD8α+ subsets for further characterization. (B) representative CD8α+ DC subset IL-15GFP by IL-15Rα contour plots from TE20 mice (C) representative CD11b+ IL-15GFP by IL-15Rα contour plots from TE20 mice. CD8α+ DC (D) and CD11b+ DC (E) surface expression by gMFI of IL-15Rα across adjuvants (TE20 and WT mice included in data).

Overall in the diagrams displayed it is IL-15Rα expression that is more conservatively expressed, and done so only with a concomitant increase of IL-15. It is worth noting that without IL-15Rα expression, IL-15 cannot signal. Therefore
the bioavailable form and therefore biologically relevant form of IL-15 is actually IL-15/IL-15Rα of which IL-15Rα seems to be the limiting agent. For these reasons, as well as concerns regarding the transgenic nature of TE-20 mice themselves, we have focused on the quantifiable expression of IL-15Rα (Fig. 4.2D&E) moving forward.

4.3 Non T cell STAT1 expression is necessary for T cell responses

In previous work the Kedl lab has shown variable dependence for the type I IFN receptor (IFNAR) on dendritic cells for evoking a synergistic primary immune response\textsuperscript{117}. The variability of this dependence perfectly reflects what has been known about the unique milieus generated by the individual PAMP receptor agonists. Specifically, agonism of TLR3, TLR7, TLR8, TLR9 and MDA5 generate robust type I IFN response and are dependent upon the perception of that IFN by dendritic cells for maximal T cell responses. A lingering question is what acts in the place of type I IFN transmitted signals to generate robust T cell responses in response to Pam3/αCD40 administration. Along these lines we have now observed that neither Pam3 nor αCD40 alone can recapitulate bioactive IL-15 production profile leaving us to speculate what signals are amplified or unique among the combined Pam3/αCD40 adjuvant that could drive IL-15 production in the place of type IFN. Since type I IFN acting upon dendritic cells has been proven to be sufficient to achieve maximal T cell responses in the past, and it has been shown that Poly I:C is both a robust inducer of type I IFN and also capable of generating a coordinated IL-15 responses via STAT1, we hypothesized STAT1 to play a central role for the induction of all T cell responses, even those that are IFNAR independent.
To test this hypothesis we immunized STAT1KO mice with either Pam3/αCD40/ova or Poly I:C/αCD40/ova and monitored the resulting magnitude of T cell response. We have shown previously (Fig. 2.2.3.3A) that STAT1 is dispensable within the T cells for maximal primary and memory T cells responses to synergy. Therefore any augmentation in T cell response must be consequent of a role for STAT1 in a non-T cell population. Confirming our hypothesis both the Poly I:C/αCD40 and Pam3/αCD40 immunizations were dependent upon the presence of STAT1 for the generation of an antigen-specific primary response (Fig. 4.3A & B).

**Figure 4.3** Dual agonist immunizations are non-T cell STAT1 dependent. WT B6 or STAT1KO mice were immunized with either Pam3/αCD40/ova (25ug/50ug/100ug) or Poly I:C/αCD40/ova (50ug/50ug/100ug). 7 days post injection, at the peak of the primary response, spleens were harvested and stained for B220-CD3+CD8+ T cells that were antigen-specific by Kβ-SIINFEKL tetramer binding. (A) representative contour dot plots of WT and STAT1KO CD8+ T cells (B) % tetramer + of CD8+ T cells by treatment group and mouse cohort. Data shown are cumulative over 2 experiments where n is greater than or equal to 3 for each experiment. Statistical significance was determined by Student’s t-test.
4.4 IL-27Rα expression is necessary for maximal IL-15Rα response

Several deficits have been noted in other systems among STAT1KOs, including deficits in B7-2 upregulation, MHCI upregulation, cross-presentation and enhanced dendritic cell death\textsuperscript{102,146,147}. Type I IFNs signaling through STAT1/STAT2 heterodimers in complex with IRF9, are known to participate in the regulation of all of these functions. As mentioned previously, type I IFN is well documented to promote IL-15 production as well. All of these factors considered, it is understandable how type I IFN through STAT1 signaling would enhance T cells responses to Poly I:C/αCD40. The question for PAM3/αCD40 vaccination is which non-type I IFN STAT1 cytokine plays an analogous role for maximizing IL-15 expression and enhancing T cell output.

In contemplation of this question we returned to the earlier recorded observations that 1.) IL-27 can signal through both STAT1 and STAT3 2.) IL-27 administration enhanced the T cell responses of IL-27-indifferent OT-1s. Collectively this lead us to hypothesize that IL-27 working on dendritic cells can participate in STAT1-mediated signaling. One would predict the presence of IL-27 would be of more importance for adjuvants providing sub-optimal IFN responses like Pam3/αCD40.

Knowing that IL-15 is of critical importance for the dual agonist response we examined what effect the presence of IL-27Rα on dendritic cells might have on maximal IL-15 presentation. We were particularly interested in interrogating the role of IL-27Rα in response to the Pam3/αCD40 immunization since previous work has demonstrated the critical nature of IFNAR on dendritic cells in Poly I:C/αCD40 responses. From the previous experiments we observed that it is the upregulation of IL-15Rα that seems to be the more tightly regulated component
in generating bioactive IL-15. There are multiple sources of soluble IL-15, however its presence is only relevant if IL-15Rα is also present to allow for efficient presentation. With this in mind we examined the upregulation of IL-15Rα in the WT and IL-27RαKO dendritic cells 18 hours post Pam3/αCD40 immunization. Supporting our hypothesis we observed that maximal IL-15Rα expression in response to Pam3/αCD40 immunization was blunted in the absence of the functional IL-27R (Fig. 4.4)

**Figure 4.4 IL-27Rα is necessary for maximal IL-15Rα expression on DCs in response to Pam3/αCD40.** WT and IL-27RαKO mice were immunized with Pam3/αCD40/ova (25ug/50ug/100ug)i.v.. 18 hours later spleens were harvested, collagenase and DNase digested and processed into single cell suspensions. Cells were then stained based upon the strategy display in Fig 4.2A along with antibody against IL-15Rα. (A) Representative histograms of CD8α+ (left panel) and CD11b+ (right panel) DCs in WT animals untreated (Untx, clear) and WT (gray) or IL-27RαKO (blue) mice immunized with Pam3/αCD40. (B) enumeration of geometric mean fluorescence intensity (gMFI) of DCs across experimental groups. Data displayed is from one experiment, representative of two experiments with greater than 3 mice per group. Statistical significance was determined by Student’s t-test.

### 4.5 IL-27Rα rescues Pam3 instigated cell death of CD8α+ DCs

While examining the DC populations 18 hours after various immunizations we observed an increased ratio of CD11b+ to CD8a+ DCs (Fig 4.5A). This occurred in WT animals treated with Pam3 alone or in IL-27RαKO animals who received Pam3 or Pam3/αCD40 (Fig. 4.5B). We further examined the absolute numbers of DCs to delineate whether this alteration in ratio was
consequent of migration and reproportioning or selective cell death. We consistently observed lower numbers of CD8α DCs after these conditions. The alteration in ratio is therefore due to cell death. It is normal for DCs to die after activation, however this cell death was not due to generic inflammation as WT cells treated with strong adjuvant, Poly I:C/αCD40, had no alteration in ratio. This is an important observation as others have previously noted a unique role for STAT1 signaling in dendritic cells to control cell death\textsuperscript{150}.

**Figure 4.5: IL-27Rα sustains life of CD8α DCs from Pam3 stimulation.** WT or IL-27RαKO mice were immunized with Pam3/ova (25ug/100ug), Poly I:C/αCD40/ova (50ug/50ug/100ug) or Pam3/αCD40/ova (25ug/50ug/100ug). 18 hours post immunization spleens were harvested and collagenase and DNase digested to liberate DCs, generating a single cell suspension. Splenic suspension was then stained as outlined in Fig 4.2A. A.) Representative contour plot of resting DC distribution between CD11b+ and CD8α+ DC subsets in WT untreated subjects (left panel) and IL-27RαKO mice receiving Pam3/αCD40 immunization (B) ratio of CD11b+/CD8α+ DCs. Data displayed is combined results of two experiments with n greater than or equal to 3 in each experiment. Statistical significance was determine by Student’s t-test.

This process was shown to occur downstream of type I IFN signaling that ultimately upregulated the cytokine signaling dampening molecule SOCS-1. Through its association with JAKs, SOCS-1 can directly modulate STAT1-mediated signals resulting in an enhanced lifespan of dendritic cells. Consequently primed and longer lived dendritic cells greatly increase the magnitude of the resulting antigen-specific T cell response. This observation is
consistent with our model in which IL-27Rα signaling on DCs acts as a surrogate type I IFN signal. In this case, IL27Rα signaling (presumably through STAT1) sustains the life of the most effective cross-presenting and IL-15Rα expressing DCs.

4.6 IL-27Rα is required on BATF3-dependent cells for maximal T cell responses to Pam3/αCD40

The data we have shown thus far strongly implicate a role for IL-27Rα on CD8α+ dendritic cells specifically to induce upregulation of IL-15Rα and preserve DC cell life in response to Pam3αCD40 immunization. To more formerly demonstrate the importance of IL-27Rα on these cells we made mixed bone marrow chimeras with congenically marked IL-27RαKO and BATF3KO bone marrow. In these animals every hematopoetically derived cell type from the IL-27RαKO has been reconstituted alongside WT cells with the exception of CD8α DCs which are largely absent due to their dependence upon the BATF3 transcription factor for development. Previously we have observed that BATF3 deficient animals are impaired in their responses to the dual agonist vaccines. We next assessed the consequence of only having IL-27RαKO CD8α+ DCs in the system by administering both dual agonist immunizations and observing the resulting magnitude of T cell responses 7 days later.

We observed normal responses in the IL-27RαKOxBATF3KO mBMC in response to Poly I:C/αCD40 immunization (Fig. 4.6). This illustrates the ability for IL-27RαKO DCs to rescue T cells responses from deficits imposed by BATF3 deletion. This is likely the result of CD8α+ IL-27RαKO DCs being able to still perceive signals and appropriately respond to the robust type I IFN generated from Poly I:C/αCD40 immunization. For Pam3/αCD40 immunization, no rescue
of the response could be afforded by the IL-27RαKO bone marrow. This reveals that IL-27RαKO deficient BATF3 dendritic cells are insufficient for priming antigen-specific CD8+ T cell responses. Conversely this conclusively demonstrates that IL-27Rα must be present on BATF3 hematopoetically derived populations for fulminate T cell responses to the Pam3/αCD40 vaccine.

![Figure 4.6](image)

**Figure 4.6: IL-27RαKO x BATF3KO mBMC T cell responses to dual agonist immunization.** Mixed bone marrow chimeras were made by reconstituting lethally irradiated WT or IL-27RαKO hosts with 1:1 mature lymphocyte depleted bone marrow from either IL-27RαKO and BATF3KO bone marrow or BATF3KO and WT bone marrow. After 12 weeks post reconstitution mice were checked for chimerism by congenic marker stain of the blood. Chimeric mice were immunized with either Poly I:C/αCD40/ova (50ug/50ug/100ug) or Pam3/αCD40/ova (25ug/50ug/100ug). At the peak of the response, 7 days post immunization spleens were harvested and stained for B220-CD3+CD8+ antigen-specific T cells by tetramer and population expressed as a relative proportion of the same congenic CD8+ T cell population. The data displayed is from two separate experiments. Statistical significance was determined by paired Student’s t-test.

4.7 IL-27RαKO DCs cross presentation and B7-2 expression is functionally intact

Apart from IL-15 presentation, dendritic cells are highly effective at delivering other necessary signals for T cells. We have not yet evaluated whether the deficits in IL-27RαKO CD8α+ DCs might also be attributed to impairments in
these functions as well. For extracellular whole proteins such as those present in the synergy vaccine, one important specialized feature of CD8α+ DCs is their higher efficiency of the process known as cross presentation. In cross presentation extracellular proteins are taken up by endocytic compartments and then released into the cytosol where they can be processed by the proteasome/inflammasome and imported by TAP proteins for presentation on MHC I molecules. The cross-presentation pathway is a critical target for vaccinologist as a means of effectively educating cytotoxic CD8+ T cells against targets administered into the extracellular environment. These cytotoxic CD8+ T cells require recognition on MHC I for initial expansion and differentiation but also utilize cognition of peptide-MHC I for targeted killing.

Other groups have already explored potential roles of IL-27Rα on dendritic cells in response to Poly I:C. They have specifically addressed the issue of cross-presentation and found that cross-presentation is enhanced in the absence of IL-27Rα. To evaluate the effect of IL-27Rα gene deficiency on cross presentation by dendritic cells in our system, we immunized cohorts of mice with either Poly I:C/αCD40 or Pam3/αCD40 with whole ovalbumin to perform a cross-presentation assay. In this assay, after in vivo overnight activation, spleens were harvested, minced and digested with collagenase and DNase. Dendritic cells were then enriched by a combination of magnetic particles and antibodies. Then these enriched dendritic cell populations were seeded in wells at varying numbers. 5 day old primed effectors OT-1 T cells at fixed number were then added into the well with the varied numbers of isolated dendritic cells. These primed OT-1s produce IFNγ over a short 4-6 hour incubation if peptide is presented on the surface of the DCs. The titration of
dendritic cells effects IFNγ production proportional to the amount of cognate peptide, processed from the administered whole ova, on the surface of the DCs. When we performed these assays we confirmed the observations of others and saw no impairment in the IL-27RαKO DCs to process and present ovalbumin to OT-1s with either Poly I:C/αCD40 administration or Pam3/αCD40 administration. In complete agreement with the existing data there appeared to be greater antigen presentation in the absence of the IL-27Rα. Since this negative effect of IL-27R on DCs is already reported, it is not novel, however it does increase the relative importance of other process executed by the CD8α DCs in the presence of the IL-27R that facilitate T cell expansion.

STAT1 binding sites in the promoter of B7-2 give rationale for examining the effect of deficiency of STAT1 utilizing cytokines/cytokine receptors on dendritic cells. The Kedl lab and others have documented an impaired expression of B7-2 in IFNαRKO mice in response to Poly I:C/αCD40 administration\textsuperscript{117}. Similarly through examination of surface marker expression we routinely observe a decrease in B7-2 expression in the CD8α+ DC compartment of IL-27RαKO mice, specifically in response to Pam3/αCD40. To test the physiological relevance of this lowered expression we constructed IL-27RαKO x B7-1/2KO mixed bone marrow chimeric mice. When these mice were challenged with both dual agonist vaccines, the IL-27 competent T cell compartment was fully capable of generating robust antigen-specific T cell responses. We have already shown previously (Fig 1.6.2), using these same B7-1/B7-2KO mice, that signal 2 is absolutely required for synergy immunization. The successful vaccination of the IL-27RαKOxB7-1/2KO bone marrow chimeras therefore argues that sufficient B7-1 and B7-2 signals must be provided from cells
descendent of the IL-27RαKO bone marrow. This tells us while the B7-2 levels in IL-27RαKO mice might be lower they must be sufficient, or otherwise compensated for by other molecules (B7-1), to allow for adequate T cell priming. Collectively this argues that neither antigen presentation (signal 1) nor costimulation (signal 2) can be the chief impairment on IL-27RαKO CD8α+ dendritic cells prohibiting them from rescuing BATF3-deficient DCs in response to Pam3/αCD40 immunization.

4.8 Discussion

In review (Fig 4.8), we have demonstrated clearly that IL-27 acting on T cell IL-27R has an important role in programming T cell function and long term effector/memory polarization. This IL-27 instigated programming determines the magnitude of the primary and subsequent secondary responses. Part of these differences seem to be mechanistically attributable to the noted decreases in CD122 expression in IL-27RαKO T cells as many characteristics of these T cells are recapitulated in an IL-15 deficient environment. Additionally we propose an unreported and unappreciated role for IL-27 to serve as an innate, type I IFN – like pro-inflammatory cytokine acting on dendritic cells. This role is restricted to CD8α+ DCs, as proven by the IL-27RαKO/BATF3KO mixed bone marrow chimera, and furthermore relevant only in the context of Pam3/αCD40 immunization.

We also demonstrate an obligate role for STAT1 on non-T cell populations in both Poly I:C/αCD40 and Pam3/αCD40 immunizations. Furthermore we documented deficiencies of IL-15Rα expression and DC survival within the CD8α+ DC compartment of IL-27RαKO mice in response to Pam3/αCD40. Coincidentally these are both features that are classically
attributed to type I IFN signaling through STAT1 in response to IFN generating innate agonists. The importance of these deficiencies is enhanced by ruling out impairments induced by IL-27R deficiency in other important dendritic cell functions, namely provision of signals 1 and 2. For Pam3Cys like vaccines, IL-27 seems to be playing a non-redundant critical role in DC activation. For Poly I:C like adjuvants IL-27 is likely compensated for by type I IFN and therefore dispensable or even disadvantageous.

Serendipitously our investigations on the role IL-27 plays in dendritic cells took a familiar turn and revealed connections to both cell life and IL-15 signaling. Signaling through IL-27Rα insures maximal IL-15Rα expression and retention of CD8α DCs during the priming phase of vaccination instigated by Pam3/αCD40. In response to IL-27, DCs express more IL-15/IL-15Rα complexes and survive longer thereby enhancing critical IL-15 signals to cognate T cells. All of these dendritic cell findings are preliminary investigations into a potential and specialized role for IL-27 to augment IFN like signals in IFN poor adjuvants. While these experiments provide a firm footing for hypotheses, many more experiments are required to formally demonstrate the mechanisms proposed. Some future experiments should include mixed bone marrow chimeras between WT and IL-27RαKO to insure the effects on dendritic cell life and IL-15Rα expression observed are not compounded by an impaired T cell response. Furthermore IL-15Rα floxed mice are available and would clearly demonstrate the importance of this molecule in the dual agonist vaccine platform. When these IL-15Rα deficient mice are combined in chimeras with IL-27RαKO mice we will be able to further elucidate the relatedness/redundancy of these two pathways.
Along those lines mixed bone marrow chimeras utilizing the BATF3KO will further hone the regional importance of IL-27R and IL-15Rα expression.

4.8 Model of the IL-27 involvement in dual agonist vaccination. IL-27 acting on T cells (blue) through STAT1 and STAT3 signaling upregulate the transcription factor Eomesodermin (Eomes). Eomes subsequently promotes CD122 expression. Signaling induced by IL-15 through CD122 on the T cell is proposed to drive T cell survival and expansion. IL-27 acting on dendritic cells does so through STAT1. STAT1 upregulates SOCS-1 increasing dendritic cell survival, and IL-15/IL-15Rα expression, providing support for T cell expansion. For Pam3/αCD40, this role of IL-27 acting on dendritic cells is unique whereas in Poly I:C/αCD40 these roles can be similarly fulfilled by type I IFN.
CHAPTER V
IL-15/IL-27 AND T CELL SURVIVAL

5.1 Introduction

In our earlier examination of the diminished T cell numbers in IL-27RαKO OT-1s, we pointed to a late survival defect on the grounds that cell numbers and proliferation profiles were unaltered from those of WT through day 3 of activation. Furthermore, days 3 and onward show identical percentages of Ki67+ T cells, indicating the proportion of actively proliferating cells was the same between WT and IL-27RαKO. A reasonable alternative to cell death would be that WT T cells either accelerate their division from day 3 onward or conversely IL-27RαKO T cells slow down rates of division. To begin to address which of these possibilities is responsible for the reduced numbers of IL-27RαKO T cells at the peak of the response we first looked to see what is already known about IL-27 and IL-15 in T cell survival or rate of cell cycling.

5.2 IL-27R and the extrinsic apoptotic cell death pathway

Others have documented a similar defect in survival of activated IL-27RαKO T cells in models of homeostatically induced colitis. In that system the mechanism proposed was an observed decrease in cFLIP expression and a corresponding increased susceptibility to Fas-mediated cell death. In vitro this process was attributed to alterations in STAT3 signaling downstream from the IL-27R. In what is dubbed as the extrinsic apoptotic pathway, ligation of the TNFR family members (notably Fas/FasL, CD40, DR3,DR4, DR5, TNFR1) results in receptor multimerization that recruits and binds the adaptor molecule FADD154 (Fas Associated Death Domain). FADD is then able to bind and activate Caspase 8. This protease then propagates the death signal through numerous
targets including the executioner caspase, Caspase 3. cFLIP is an inhibitor of Caspase 8-induced cell death through direct interaction with both FADD and Caspase 8. cFLIP binding prevents Caspase 8 activation\textsuperscript{155–157}.

To evaluate what role this extrinsic apoptotic cell death pathway may have in the observed impairment of T cell numbers, we used the co-adoptive transfer OT-1 system to evaluate differences in expression of the Fas, Fas-L, and active Caspase 8 at multiple time points through the peak of the primary response. For examining caspase activation throughout our investigations into cell death pathways we employed caspase specific FLICA molecules. FLICA stands for FLuorescent Inhibitors of Caspase Activation. FLICAs peptides are highly specific to particular caspases and passively taken up by the cell and staying within the cytosol. With specific caspase activation, FLICAs perform two very important roles. First once the non-fluorescent FLICA substrate is cleaved by its specific caspase the resulting product becomes fluorescent, allowing fluorescence to be an indicator of caspase activity. Secondly, when cleaved, these FLICA products become specific inhibitors of caspases as well. This is critical because it stops the cell death process allowing for the observation and enumeration of cells that would have otherwise undergone apoptosis and been rapidly cleared by neighboring cells.

Using the adoptive co-transferred OT-1 system, we were unable to observe significant increases in expression over wild-type for Fas, Fas-L or active Caspase 8 (Fig. 5.2A,B,C). In fact, IL-27R\textsubscript{α}KO OT-1 T cells had lower amounts of Fas (Fig. 5.2A) and Fas-L (Fig. 5.2B) at various time points, suggesting that the cells would be less able to instigate Fas-induced cell death against one another and less able to receive Fas signals. Seeing as there was no
evidence for a role of the extrinsic pathway we turned our attention towards other mechanisms of cell death.

Figure 5.2 IL-27RαKO OT-1s and the extrinsic cell death pathway. Equal numbers of congenically disparate WT and IL-27RαKO OT-1s were co-transferred into WT B6 mice. The next day mice were immunized with Poly I:C/αCD40/ova (50ug/50ug/150ug). At the days indicated, spleens were harvested and prepped into single cell suspensions for flow cytometric staining and quantification of extracellular expression of (A) Fas and (B) Fas-L, as well as intracellular caspase staining for one hour at 37°C by passive uptake of Caspase 8 specific (C) FLICA. To the left of every bar graphs is a corresponding representative histogram for that molecule. Gates were set based upon FMO, isotype and unstained cells. Gray bars and gray histogram represent antigen non-specific biological control which is the endogenous CD8+T cell population at the indicate time point for the bar graph and day 1 for the histogram. Black-lined histogram and black bars are WT OT-1s at the indicated time point, and day 5 for the histogram. IL-27RαKO OT-1s are shown in the hatched lines for the bar graphs. Mice for days 3-5 received 3000 of each OT-1 and were processed normally for flow staining. For days 1 and 2 mice were transferred with 3x10^5 of each OT-1 and spleens were collagenase and DNase treated to recover highly adherent early activated T cells prior to flow staining. Data are from one experiment representative of two experiments with n=4 each.
5.3 IL-27R and intrinsic apoptosis

Cells unable to compensate with metabolic stress will leak Cytochrome C from their mitochondria initiating another cell death cascade known as the intrinsic apoptotic cell death pathway. Cytochrome C directly interacts with the protein scaffold APAF1. Apaf 1 recruits and organizes proteins to form the apoptosome which cleaves Pro-Caspase 9 into active Caspase 9. Like Caspase 8, Caspase 9 in turn activates Caspase 3 leading to the cleavage of multiple cellular proteins resulting in cell death. Since the intrinsic pathway revolves around mitochondrial balance; cytokines and proteins that alter the balance of the pro or anti-apoptotic molecules of the BH3/Bcl2 family are of particular interest.

These Bcl2 family members can be divided up into three basic subgroups. Anti-apoptotic family members, which include Bcl2, Bcl-XL and Mcl1 exert their function by binding an inhibiting the function of pro-apoptotic family member Bak and Bax. Bak and Bax meanwhile are key death effector molecules. Upon activation they mediate the generation of mitochondrial pores that allow for leakage of mitochondrial proteins into the cytosol, most notably cytochrome C. The third class of molecules are termed “BH3 only” family members and they are Bim, Bid and Bad. These molecules have no inherent effect on pore formation but instead bind the anti-apoptotic proteins directly. This frees the pro-apoptotic molecules to carry out their cell death inducing functions. The overall cell death process is dictated but the summation of the active form of these three constituents. Active levels as well as well overall levels of these proteins are influenced by multiple signaling pathways (TNFR, cytokine, etc). These signals regulate the BH3/Bcl2 molecules by altering RNA transcription.
(splice isoforms), translation (relative abundance) and phosphorylation (active and inactive states).

In regards to the pathways we have shown important for success of the dual agonist vaccine platform, IL-15 has been demonstrated to rescue superantigen instigated cell deletion through over-expression of Bcl2 and the capacity to regulate pBIM levels\(^{160,161}\). Others have documented however that overexpression of Bcl2 in CD122-depressed cells is not sufficient to rescue OT-1 cells from TLR agonist-primed immune responses\(^{122}\). To examine what role the intrinsic cell death pathway may have on disparities in T cell number between WT and IL-27R-deficient antigen-specific T cells, we again employed the co-adoptive OT-1 transfer system and examined levels of active Caspase 3 and the anti-apoptotic proteins Bcl2 and Bcl-xL. Since no perturbations were witnessed in the extrinsic pathway between WT and IL-27R\(\alpha\)KO OT-1s, most notably no difference in levels of active Caspase 8, any differences we see in the activation of Caspase 3, which is the shared component between the extrinsic and intrinsic pathways, can therefore be attributed to the intrinsic pathway. While we were clearly able to observe upregulation of these molecules throughout the primary response, we were unable again to see any differences between WT and IL-27R\(\alpha\)KO OT-1s for Bcl2 (Fig. 5.3A), Bcl-xL (Fig. 5.3B) or active Caspase 3 (Fig. 5.3C).
Figure 5.3 IL-27RαKO OT-1s and intrinsic apoptosis. Equal numbers of congenically disparate WT and IL-27RαKO OT-1s were co-transferred into WT B6 mice. The next day mice were immunized with Poly I:C/αCD40/ova (50ug/50ug/150ug). At the days indicated, spleens were harvested and prepped into single cell suspensions for flow cytometric staining and quantification of intracellular expression of (A) Bcl-2 and (B) Bcl-xL, as well as intracellular caspase staining for one hour at 37°C by passive uptake of Caspase 3 specific (C) FLICA. To the left of every bar graphs is a corresponding representative histogram for that molecule. Gates were set based upon FMO, isotype and unstained cells. For (A) and (B) gray bars and gray histograms represent antigen non-specific biological control which is the endogenous CD8+T cell population at the indicate time point for the bar graph and day 1 for the histogram. Black lined histogram and black bars are WT OT-1s at the indicated time point, and day 5 for the histogram. IL-27RαKO OT-1s are shown in the hatched lines for the bar graphs. For (C) the gray histograms represents fresh naïve bulk splenocytes stained Caspase 3 FLICA before (1hr) treatment with freshly prepared Splenda (10ug/mL in media, black line histogram) as a positive control for Caspase 3 activation. Mice for days 3-5 received 3000 of each OT-1 and were processed normally for flow staining. For days 1 and 2 mice were transferred with 3x10^5 of each OT-1 and spleens were collagenase and DNase treated to recover highly adherent early activated T cells prior to flow staining. Data are from one experiment representative of two experiments with n=4 each.
There are several reasons why we may not be able to observe differences in apoptotic signaling. First of all apoptotic cells are quickly cleared from the body. For this reason our technique may simply be unable to capture an image of the cells that are dying before they are being rapidly cleared. The alterations observed in caspase levels however argue against that likelihood. Caspase levels are altered throughout the time course suggesting we are “catching” cells as they are headed toward cell death. While the levels of caspase are clearly changing there are no disparate observations between WT and IL-27RαKO T cells. Another possibility exists that the cells are succumbing to cell death in processes other than the classical extrinsic and intrinsic apoptotic pathways. Finally it is still a possibility the cells are ceasing to divide as rapidly as WT cells at day 3.

5.4 IL-27R in necroptosis and pyroptosis

At one time it was thought cell death that resulted in cell lysis and expunging of intracellular contents into the extracellular space was a passive form of cell death. This “necrotic” cell death is disparate from apoptosis in morphology as well as its contribution to the tissue microenvironment. Recently it has become appreciated that instead of being passive, some forms of necrosis are also programmed responses employing unique effector proteins for regulating cell death\textsuperscript{108,154,162}. Consequently these forms of necrosis have adopted the “ptosis” moniker to reflect the programmatic elements of these forms of cell death. Two broad classifications of this type of cell death are pyroptosis, and necroptosis.

Classically, apoptosis is thought of as programmed cell death employed to induce tolerance and aid in the resolution of inflammation. As indicated by its name, pyroptosis, while also a form of programmed cell death, enhances tissue
inflammation. The hallmark feature of this form of cell death is the dependence upon Caspase 1 to induce cleavage of the zymogens of IL-1β and IL-18\textsuperscript{163}.

Consequently when a pyroptotic cell dies it also introduces the active forms of IL-1β and IL-18 into the extracellular milieu, exaggerating the pro-inflammatory response. To evaluate whether this specialized form of necrosis was responsible for the decreasing numbers of antigen-specific IL-27Rα deficient T cells we looked at the expression of active Caspase-1 during the primary response through that adoptive transfer system (Fig. 5.4). Once again we could see no preferential expression of active Caspase-1 in IL-27RαKO T cells at any point during the primary T cell response.

**Figure 5.4 Active Caspase 1 in WT and IL-27RαKO OT-1.** Equal numbers of congenically disparate WT and IL-27RαKO OT-1s were co-transferred into WT B6 mice. The next day mice were immunized with Poly I:C/αCD40/ova (50ug/50ug/150ug). At the days indicated, spleens were harvested and prepped into single cell suspensions for flow cytometric staining and quantification of intracellular expression of active caspase 1 through incubation for one hour at 37°C and passive uptake of Caspase 1 specific FLICA. Black dots represent WT OT-1s, while white dotes represent IL-27RαKO OT-1s. Mice for days 3-5 received 3000 of each OT-1 and were processed normally for flow staining. For days 1 and 2, mice were transferred with 3x10^5 of each OT-1 and spleens were collagenase and dnase treated to recover highly adherent early activated T cells prior to flow staining. Data are from one experiment representative of two experiments with n=4 each.
Apart from pyroptosis, there are at least three different necroptotic pathways with varying dependence upon the kinases RIPK1 and RIPK3\(^{164-166}\). The pathways converge on activation of the pseudokinase protein MLKL. MLKL once recruited into the so called “necroptosome”, oligomerizes and associates with the plasma membrane. Once there MLKL induces cell death through destabilization of Ca\(^+\) balance and direct disruption of plasma membrane integrity through interactions with membrane phosphatidylinositol phosphates PI\(5\)P and PIP\(2\)\(^{164,167-169}\). For our considerations, other key features of necroptotic pathways are the direct inhibition of necroptosis by active Caspase 8 and the extrinsic cell death pathway, as well as it is enhancement as the preferred mechanism of cell death when cells have a relative abundance of reactive oxygen species (ROS). The elevated ROS levels immediately implicate an alteration in cellular metabolism. We decided to further explore the link of cellular metabolism to cell death in response to our dual agonist immunization.

5.5 Cell death and metabolism

From what is described about necroptosis above it is difficult to overlook the presence of two critical features of cellular metabolism; PIP2 and reactive oxygen species. PIP2 is a direct target of PI3K, the kinase central to regulation of cellular metabolism through AKT activation of glycolysis\(^{170}\). Reactive oxygen species on the other hand is a byproduct of the electron transport chain downstream of the TCA cycle. Bob Dylan once wrote, “He not busy being born is busy dying.” The same might be said for leukocytes, although more accurately phrased in this case, if you’re not living, you’re dying, if your living you’re breathing and metabolizing.

In recent years there has been a growing appreciation for role of cellular metabolism in programming the effectiveness and diversity of the immune
response. In our previous examinations of effector vs memory differentiation we pointed to hypermorphic phenotypes of these cell fates which lead generally to either an overactive, rapidly expanded, exhausted phenotype or conversely for memory, a long lived ineffective, non-proliferative phenotype (trivial memory). Along these lines it has been well documented that pharmaceutical alterations of metabolism during early activation of T cells dictates the magnitude and effector/memory T cell fate. Specifically, strong T cell priming induced by plate-bound CD3 + CD28 or by chronic LCMV (clone 13), can be more effective in the clearance of cancer cells and chronic virus respectively, if during the priming a phase a low dose of the mTOR inhibitor, rapamycin, is present.

Expanding from these observations, many have demonstrated that the generation of longstanding functional memory comes with a controlled regulation of glycolytic metabolism either through pharmacological manipulation or transcription factor regulation. Reports have clearly demonstrated that memory cells have a lower glucose uptake and therefore seem to be powered by conventional glucose metabolism or beta oxidation of fatty acids through the TCA cycle. Conversely it has been appreciated for some time that rapidly dividing cells such as cancer cells and antigen-stimulated effector T cells employ an alternative metabolic programming to meet their energetic needs.

The classical overview of cellular metabolism dictates that the most “efficient” means of generating ATP is the conversion of glucose to pyruvate in process knowns as glycolysis. The pyruvate generated by this process is then utilized by mitochondrial metabolism through the TCA cycle to generate NADPH and FADH2. These molecules generate proton gradients to fuel ATP generation through the proton motive force and ATP Synthase via a set of protein
complexes known as the electron transport chain. The TCA/Krebs cycle requires oxygen and also generates reactive oxygen as a byproduct. This process is reinforced by a transcription factor known as Foxo1. Foxo1 is a transcription factor negatively regulated by activated PI3K and AKT signaling. In its non-phosphorylated form, Foxo1 facilitates the metabolic programming of naïve and memory T cells by positively upregulating the expression of TCA-ETC proteins as well as anti-oxidants to prepare the cell for increased generation of reactive oxygen species that comes along with a TCA-biased metabolism\(^{183-185}\).

In the absence of oxygen, TCA cannot move forward towards the ETC and instead glycolysis shunts pyruvate away from entrance into the TCA cycle. Instead of entering the TCA cycle, lactate dehydrogenase (LDH) converts pyruvate to lactic acid which can then be recycled in the form of glucose through the process known as gluconeogenesis. Furthermore when oxygen is not present several operations of the TCA cycle (a series of catabolic reactions) can operate in reverse. One example of this is the generation of fatty acids through the anabolic reverse pathways of fatty acid beta oxidation. The enzyme responsible for this is acetyl CoA carboxylase and is upregulated by an active PI3K/AKT/HIF1\(\alpha\) pathway\(^{186}\). This generation of fatty acids from Acetyl CoA further reinforces the shutdown of the TCA cycle.

Cancer cells and rapidly dividing T cells seem to be unable to meet their energetic and building block needs through TCA-mediated aerobic metabolism. Consequently, despite the presence of oxygen these high energy demand cells switch their metabolism to favoring glycolysis, fatty acid synthesis and lactate generation in the presence of oxygen. This process is known as aerobic glycolysis and termed the “Warburg effect”.
Curiously along with this decision of metabolism comes along the choice of death. If energetic needs are still not met by the programmed metabolic responses or if additional stresses are exerted upon the cellular system for which its metabolism cannot compensate, cell death will occur. The particular form of death undergone is somewhat determined by intracellular facilitators of death that were upregulated by that specific metabolic program. Necroptosis utilizes reactive oxygen species to instigate cell death and is therefore favored in conditions in which the TCA cycle is functioning. On the other hand without reactive oxygen species, as is the case in aerobic glycolysis, classical apoptotic cell death pathways are favored\textsuperscript{187,188}.

While both naïve and memory T cells have a biased reliance upon TCA-mediated metabolism, recent reports have illustrated that memory T cells require an additional processes, known as autophagy, to remain alive while dependent upon TCA metabolism. The perceived purpose of this “self-eating” process is to preserve cell life through recycling of cellular components through vacuoles. The intracellular trafficking of these components feeds mitochondrial metabolism and allows for maintenance of cell life. Autophagy and necroptosis obligatorily operate in conditions favoring TCA metabolism however in opposition to one another. Molecules that program the necroptotic process like RIPK3 actively impair that autophagy pathway. When autophagy is impaired, this stop gap in the necroptotic pathway is impaired, the efficiency of TCA-mediated metabolism is likewise reduced and the cells die by necroptosis. The bias towards necroptotic cell death in this circumstance is thought to be consequent of another role of autophagy in controlling ROS production by mitochondria through the recycling of mitochondria themselves\textsuperscript{189,190}. 
When successfully balanced on the knives edge between autophagy and necroptosis, surviving memory T cells, when re-engaged by antigen or inflammatory cytokines, are able to emerge from their TCA-biased metabolism and upregulate aerobic glycolysis necessary for clonal expansion and effector functions.

**5.6 Antigen-specific IL-27RαKO T cells are biased towards TCA**

Thus far we have been unable to conclusively address the mechanism of reduced cell number experience by both IL-27RαKO antigen-specific T cells and T cells primed in an IL-15 deficient environments. The inability to illustrate cell death by the extrinsic, intrinsic and pyroptotic pathways under these conditions suggests the use of alternative death pathways. Considering CD122/PI3K/mTOR pathways are known to promote glycolysis, we hypothesized that the impairment of CD122 signaling by either IL-27Rα or IL-15 deficiency leads to metabolic imbalance favoring the TCA cycle and therefore favoring necroptosis.

To explore this hypothesis we first set about determining whether any alteration in metabolism was consequent of the observed cytokine pathway deficiencies. Several groups have employed a fluorescent glucose analog (2NBDG) to determine glucose uptake by cells. Utilizing this system groups have isolated the lowest and highest 7.5% glucose consuming populations from a normal distribution of in vitro stimulated naïve T cells and illustrated that they have differential outcomes with regard to memory and effector differentiation. Specifically 2NBDG hi cells were able to provide short term protection in tumor models and displayed a terminal effector T cell phenotype. The 2NBDG low cells conversely illustrated features of functional “stem cell” memory. One significant
limitation to this system is that it evaluates metabolic balance on the basis of only one area of metabolism, glucose uptake.

Conversely other groups have utilized a superoxide cleavable fluorescent substrate known as Mitosox red (MSR) to characterize the magnitude of mitochondrial electron transport chain function\textsuperscript{191,192}. Mitosox red preferentially associates with mitochondria and upon cleavage by superoxide results in a fluorescent signature. This biosensor is acted upon specifically by superoxide and cannot be activated by other reactive oxygen or reactive nitrogen species. Consequently the fluorescence observed is directly proportional to the generation of superoxide from the electron transport chain downstream of mitochondrial respiration. This molecule is employed often to characterize mitochondrial function and oxidative potential of innate lymphocytes.

By combining these two reagents we can simultaneously examine both the uptake of glucose as well as the relative function of mitochondria, allowing us to elucidate the relative balance of either metabolic pathway. The novel importance of simultaneously combining these two reagents in this assay (2NBDG/MSR) is emphasized by considering the result of observing an increase in glucose uptake (increased 2NBDG) alone. Increased glucose uptake can be reflective of aerobic glycolysis in which glucose breakdown to lactate is enhanced and TCA cycle is actively being blocked. Alternatively, glucose uptake rates can increase while proceeding more rapidly through classical glycolytic pathways in which pyruvate feeds forward into the TCA cycle through enzymatic processing into Acetyl CoA. Each of these processes at varying rates could have equal glucose uptake yet have distinctly different effector/memory T cell outputs. Distinguishing between these two possibilities is therefore enabled by
determining the upregulation of glucose with consideration to mitochondrial function, determined by MSR signal.

Similarly, an MSR signal can be generated by an increase in glucose uptake or also an increase in one of the many catabolic process that feed into the TCA cycle. The most common compensatory pathway for TCA cycle in the absence of an abundance of glucose is the β-oxidation of fatty acids which happens early in the TCA cycle. The rate limiting step of this process is the transfer of long chain fatty acids across the mitochondrial membrane. This process is enzymatically facilitated by carnitine palmitoyltransferase.\textsuperscript{136,193,194} Action of this enzyme provides the substrates for the TCA cycle when sufficient pyruvate is unavailable. One dimensional consideration of metabolism by examining MSR signature alone may result in two cellular metabolisms looking identical when in reality they are distinct. Proportional increases in both glucose uptake and superoxide generation are expected downstream of early initial T cell engagement, however TCA cycle with reduced glycolysis is a signature of autophagy, necroptosis and memory formation.

Using this metabolically informative system (2NBDG/MSR) we co-adoptively transferred equal numbers of WT and IL-27RαKO OT-1 T cells into wild type B6 mice. At the peak of the response to the dual agonist immunization (day 5) we harvested spleens and subjected the cells to interrogation by the 2NBDG/MSR assay. After 4 hours incubation at 37°C, we could observe two distinct T cell populations within each of the antigen-specific T cell pools (Figure 5.6). Approximately 30% of the IL-27RαKO OT-1 T cells at the peak of the response where relatively low for 2NBDG but high for MSR compared to 8% for the WT. Considering the strength of the OT-1 transgenic T cell for its antigen as
well as the robustness of the dual vaccination system in generating large primary responses and functional memory immune responses, we characterize the distribution and fluorescence of these metabolically distinct cells (the metabolic profile) of the WT OT-1s as idealized for subunit immunization. In comparison to this idealized profile we observed that the IL-27RαKO OT-1s have more cells that we would predict would result in a memory population (high for MSR while low for 2NBDG). This is in fact predictive and consistent with what we have demonstrated earlier of IL-27RαKO antigen-specific T cells. Furthermore it depicts an increased frequency of T cells that produce superoxide, a substrate for cellular activation and necroptosis.

From these data alone we cannot infer the metabolic state of the cells that have already disappeared prior to the peak of the T cell response. With this methodology in our molecular tool box we are well equipped to begin interrogating T cells at various times throughout the priming process to determine when metabolic augmentation first occurs. Evidence from multiple reports illustrates that either naturally occurring or pharmacologically induced modifications to glucose uptake during the initial priming phase (first 48 hours) epigenetically programs long term fate. This window of timing fits well into the range we expect to see the most dynamic changes in IL-27 and IL-15Rα expression. We look forward to employing the 2NBDG/MSR system to see if differential innate metabolic programming exists between WT and IL-27RαKO T cells in both IL-15 sufficient and deficient environments. Furthermore future studies in our lab should set out to recapitulate the fate mapping of metabolically distinct subsets determined by our 2NBDG/MSR system and compare those outcomes to what has been previously observed in other labs that only utilize
2NBDG profiles. The commonly used assay system that does account for both branches of metabolism is known as a Seahorse analyzer. The Seahorse apparatus, monitors media pH as an output of Lactate Dehydrogenase and glycolytic flux, while simultaneously monitoring oxygen consumption to determine aerobic rates of respiration (TCA cycle). These observations are done on a per well basis meaning the results of every cell are averaged out based upon phenotypic frequency.

By employing flow cytometry our system gives a metabolic profile for every individual cell. The advantages to this approach are numerous. First of all populations need not be sorted before the assay is performed saving; time, money and also increasing the likelihood of capturing a metabolic profile most akin to the in vivo state. Secondly, visualization by flow readout allows for phenotypic characterizations of heterogeneous cell populations. We have yet to test whether the clear profile depicted above (whose biologically relevant phenotype has already been ascribed) would even be detectable by the Seahorse assay. Lastly the rapidity of this assay, the clear separation of the populations as well as the temporary nature of the detection reagents is well suited for flow sorting allowing for future applications of cells in adoptive transfer experiments and molecular biology interrogations. Such future experiments will explicitly demonstrate the significance of these distinct populations.
Figure 5.6 IL-27RαKO OT-1s have elevated frequency of MSR high cells.
3000 congenically disparate WT and IL-27RαKO OT-1s were adoptively co-transferred into WT B6 mice. The following day mice were immunized with Pam/αCD40/ova (25ug/50ug/100ug) i.v. Day 5 post immunization, at the peak of the primary response, spleens were harvested and preloaded in 5μM MSR/PBS solution for 10 minutes at 37°C. Cells were then washed and resuspended in complete RPMI medium containing 10% FCS and 10μM 2NBDG. Cells were incubated for 4 hours at 37°C and 5% CO2. During the last 30 minutes of incubation fluorescently conjugated antibodies were added for CD8+ T cell and congenic discrimination. Cells were washed, kept on ice and run on a flow cytometer. (A) Representative 2NBDG/MSR contour plot of WT (left panel) and IL-27RαKO (right panel) OT-1s at the peak of the primary response, day 5. (B) Enumeration across the experiment of the relative distribution of 2NBDG high and MSR high populations. Graph is from one experiment representative of greater than 3 individual experiments, each with an n greater than or equal to 3. Statistical significance was determined by paired Student’s t-test.
5.7 Discussion

In the preceding sections we have examined the potential causes of the decreased cell numbers in IL-27R-deficient antigen-specific T cells evoked by the dual agonist immunization platform. No obvious deleterious alterations in the extrinsic apoptotic, intrinsic apoptotic or pyroptotic cell death pathways were observable between WT OT-1s and IL-27RαKO OT-1s. We explored alterations in metabolism and reactive oxygen species generation through a novel assay (2NBDG/MSR). The results of this assay demonstrated a higher frequency of cells with increased reactive oxygen species generation with less glycolytic flux in IL-27RαKO OT-1s at the peak of the response. This output is consistent with the previously described “trivial” memory phenotype observed in the IL-27RαKO antigen-specific T cells. This is also consistent with a recent report in dendritic cells demonstrating the ability for IL-27 to facilitate PI3K/mTOR signaling190.

Based upon existing literature, the phenotype ascribed to these IL-27R-deficient cells would strongly argue for necroptosis as the preferred avenue for cell death. However, this phenotypic analysis has all been done on cells that have survived to the peak of the primary response. To explicitly demonstrate the mechanism of cell death, efforts will have to be made to alter necroptosis closer to the day 3 time point when WT and IL-27RαKO antigen-specific T cells begin to display a disproportionate alteration in cell number. Necrostatin-1s is a specific inhibitor or RIPK1. However its short half-life of 1-4 hours in vivo prohibits its use for in vivo confirmation of necroptotic cell death pathways which span a 72 hour window of interest in our system. Future molecular biological studies should focus on the cellular distribution of MLKL and activation states of the RIPK1 and RIPK3 molecules in the WT and IL-27RαKO OT-1s at various time points during
the primary response. Alternatively experiments are designed but yet to be carried out to ablate cell death by neutralization of reactive oxygen species and augmentation of cellular metabolism.

The goal for the use of antioxidants is to neutralize the reactive oxygen species known to facilitate necroptosis. There are several available anti-oxidants (NecroX, MnTBAP) that can be administered to mice on a regular basis\textsuperscript{195,196}. Generally speaking, these anti-oxidants are indiscriminate to which reactive nitrogen (RNS) or reactive oxygen species (ROS) they neutralize and which cells they function on. While there is depth to the literature illustrating their efficacy at reducing overall inflammation, it also acknowledged that these ROS and RNS serve as chemical messengers for coordinating an immune response. It is therefore likely that neutralization of these molecules might stave off necroptotic-induced cell death while simultaneously impairing the ability for the immune system to mount the synergistic T cell response characteristic of the dual agonist platform.

Another approach to confirm the role of metabolically programmed cell death pathways is to alter the metabolic programming itself. While there are a number of pharmaceuticals that can block the glycolytic pathway, with Rapamycin the most commonly used, there are very few reagents available for in vivo administration that can block the TCA cycle without inducing death. Etomoxir is an inhibitor of carnitine palmitoyltransferase, the key enzyme for facilitating fatty acid oxidation to supply the critical molecule Acetyl-CoA to the TCA cycle in conditions of lowered pyruvate generation\textsuperscript{193,194}. Shut down of this pathways both impairs the rate of mitochondrial respiration as well as upregulates the rate of glycolysis. Etomoxir is well tolerated in vivo and its effects on shifting metabolism to favoring glycolytic flux have been well documented.
While preliminary experiments are underway and further experiments planned, we have yet to extensively explore the use of this inhibitor. The rationale for use of this inhibitor is the assumption that switching of the metabolic profile early on will generate a greater glycolytic metabolic program (Warburg effect), that will both support the unique metabolic demands of effector T cell differentiation as well as reduced the generation or ROS that favor induction of necroptosis.

All of these approaches and techniques when employed with the CD122 OT-1, IL-27RαKO OT-1 and IL-15KO mice should provide definitive answers as to whether IL-27 and IL-15 play any role or share a common pathway in T cell metabolism and cell death. It is still possible however, the T cells we observed in lower frequency are lower in number because their rate of cell cycle is less than that of WT. To address this concern more complicated and mathematically intensive modified Bromodeoxyuridine (BrdU) pulse-chase experiments could be devised that determine the numbers of cells actively dividing at a given time and the rate of that division in vivo.
CHAPTER VI
DISCUSSION

6.1 Overview model of the role of IL-27 in subunit vaccine elicited cellular immunity

Throughout this dissertation a role for IL-27 and IL-15 in vaccine elicited cellular immunity has been clearly demonstrated by a dramatic alteration in both the magnitude and character of antigen-specific T cells in environments deficient in either of these cytokine signaling networks. The model we propose to coalesce all of these observations is the following. Ligation of IL-27Rα is required on T cells for upregulation of transcription factors that alter T cell function and fate and for the upregulation of CD122 that modulates responsivity to IL-15. While several features of IL-27R signaling on T cells have been reported with regards to classical transcription factor regulation and signal 3 cytokine programming, this is the first report to draw a connection to IL-27R with modulation of CD122 directly and illustrated the consequences of IL-15 deprivation in subunit immunization. Both the classically understood role of IL-27 and this new role for programming sensitivity to IL-15 undoubtedly contribute to the final outcome of IL-27Rα deficient T cells. Furthermore we draw the connection between IL-27Rα T cell deficiency and metabolism through the known effects of IL-15 in driving PI3K. (Fig 4.8). Consistent with the literature we propose the IL-15 drives glycolytic balance to TCA cycle. Removal of this signal we suggest alters the effect of early metabolic programming resulting in an enhanced useless memory population and ultimately results in increased cell death through necroptosis consequent of the TCA-biased metabolism. This
hypothesis is supported by a lack of observable differences between WT and IL-27RαKO OT-1s in the extrinsic, intrinsic and pyroptotic cell death pathways.

Apart from the role of IL-27 acting on T cells we also illustrate two roles for IL-27 acting on dendritic cells for a subset of TLR adjuvants. We show that IL-27R is necessary for maximal IL-15Rα expression as well as for enhanced CD8α+ DC numbers during the priming phase of T cell responses. Both of these processes have been documented to be mediated by STAT1 previously and we suggest that IL-27’s ability to propagate cytokine signaling by both STAT1 and STAT3 uniquely positions IL-27 for this role in non-type IFN biased immune responses, such as those to Pam3. Importantly, we also document that the combined synergy platform results in a greater expression of the bioavailable IL-15 components then either adjuvant in isolation.

Collectively, this uniquely robust synergy subunit immunization platform utilizes IL-27 to reinforce CD122/IL-15 signaling by modulating maximal CD122 expression on antigen-specific T cells and simultaneously elevating bioavailable IL-15 through the upregulation of IL-15Rα expression and retention of dendritic cells most capable of IL-15 trans-presentation to these T cells. This both confirms and expands upon previously reported data highlighting the importance of IL-15 in subunit immunization.

6.2 The unifying theory of subunit vaccine elicited T cell metabolism

By identifying the dependence on IL-27 and IL-15 for subunit immunization and furthermore placing these dependencies central to the efficacy of the dual agonist vaccine, several previously documented yet poorly understood characteristics of this vaccine begin to fall into place. In the following sections attention is turned to addressing these previously confounding realities
Figure 6.2 IL-15, CD27 metabolism and cell death. T cell metabolism in response to the dual agonist vaccine is supported by two well-balanced pathways. IL-15/IL-15Rα complexes presented on the surface of priming DCs (green) activate CD122/CD132 signaling on the adjacent T cell (blue). Among many other signaling pathways CD122 critically activates PI3K and mTOR to drive aerobic glycolysis. One downstream target of mTOR activity is the upregulation of functionally active HIF1α which targets Pyruvate Dehydrogenase Kinase and Acetyl-CoA Carboxylase α, which stop the citric acid cycle and actually cause the metabolic pathways to reverse. CD70 upregulation by the dendritic cells interacts with its receptor CD27 on T cells, providing two critical signals. First, through the classical TRAF2/NFκB signaling pathway CD27 upregulates Bcl-2 anti-apoptotic family members and cFLIP. Collectively these actions block both the intrinsic and extrinsic apoptotic cell death pathways respectively. In the absence of IL-27 this biases cells to die by necroptosis. In the presence of IL-27 these factors facilitate life and expansion supported by aerobic glycolysis. CD27 also signals via another pathway through direct engagement with PIM1 kinase. We propose this pathway supports classical aerobic metabolism by increasing glucose transport and pyruvate generation for feeding into the TCA cycle (mitochondria in red/pink).

6.2.1 CD4+ T cell Independence of Synergy Immunized CD8+ T cell Responses

Classical coordinated T cell activation in response to either pathogen challenge or vaccination ascribes an essential helper function to CD4+ T cells in
programming the memory response of CD8+ T cells. This is achieved through cytokine production by T cells and CD154 engagement with dendritic cells. Furthermore it has been demonstrated that IL-2 specifically is required during T cell priming for CD8+ T cell memory. This is not true however for the dual agonist vaccine. CD8+ T cell responses are completely independent of CD4+ help.197

A substantial body of evidence illustrates that while the expression pattern of the alpha chain greatly varies between the functional IL-2 receptor and IL-15 receptor, if the presence and magnitude of the cytokines and functional receptor is equalized, IL-2 and IL-15 signal virtually interchangeably on a given cell population. Here we have documented that synergy immunization uniquely upregulates the magnitude of functional IL-15 presented during the early stages of vaccine priming. This elevated and early expression of “innate” IL-15 could conceivably replace the reliance upon IL-2 generated from CD4+ T cells. The administration of CD40 agonist, an essential component to the synergy-mediated immune response, further makes CD4+ T cell interactions dispensable by replacing the CD154 component of CD4+ help. Traditionally IL-15 and IL-2 cytokines as well as their unique cytokine receptor alpha subunits are temporally disparate in their expression. Classically speaking in CD4-dependent CD8+ T cell responses, IL-2 is generated from cognate CD4+ T cells at substantial levels starting 6 hours post TCR engagement and continuing beyond 48 hours but waning as inflammation resolves. Unlike IL-2, IL-15 is constitutively made by many cells of the body and therefore exists at low but physiologically relevant levels as an important homeostatic cytokine for memory T cells and NK cells. In this homeostatic state the present but limited IL-15/IL-15Rα is sufficient for its well characterized role in the preservation of memory CD8+ T cell life in an environment devoid of IL-2.
The unique and dramatic reliance upon IL-15 for the primary response in the dual agonist platform compared to pathogen challenge may be informative of IL-2/IL-2R dynamics in response to synergy. The heavy reliance for IL-15 would predict the dual agonist platform, despite the successful recruitment of large numbers of antigen-specific CD4 T cells, does not instigate a sufficiently robust innate IL-2 response or initiate the CD25 upregulation necessary to utilize IL-2 to compensate for IL-15 deprivation. This has yet to be formally tested.

6.2.2 Complementing Roles for IL-27 and CD27 in Cell Death

Earlier we had discussed the critical reliance upon the TNFR family member CD27 as another unique feature of the dual agonist platform. In documenting this dependency, the magnitude and kinetics of CD70 (the ligand for CD27) on dendritic cells was different between the dual agonist immunization compared to either of the single agonists\textsuperscript{117}. The importance of this unique CD70/CD27 dependence comes into light when we consider the cell fate IL-27R\textsubscript{α}KO antigen-specific cells are relegated to. As we have document above, in the absence of IL-27, antigen-specific T cells responses although fewer in number showed no observable increase in the intrinsic or extrinsic classical apoptotic cell death pathways. TNFR family members have been demonstrated repeatedly to oppose cell death by these means\textsuperscript{198}. Furthermore it has been demonstrated that upregulation of cFLIP or other antagonism of apoptotic cell pathways will drive unbalanced cells towards death via necroptosis. So while the deficiency of IL-27 drives cells to commit suicide by metabolic insufficiency, CD70 engagement prevents this from occurring through apoptosis. Unfortunately this complements the cells by pushing the cells towards a pathway of cell death enhanced by TCA function, necroptosis.
As bad as this CD27 facilitated necroptosis may appear to be in the absence of IL-27, in the presence of IL-27, the engagement of CD27/CD70 would be ideal. With reinforcement of the aerobic glycolytic metabolism through PI3K via IL-27/IL-15, cells will be programmed by metabolism to be less likely to die by necroptosis overall and therefore CD70 engagement would serve to thwart apoptotic cell death that might be more likely favored by glycolytic activity. Collectively IL-27/IL-15 and CD27 signals would be staged well to balance one another and support T cell persistence.

6.2.3 Dual Agonist Appears Like Rapamycin Treated LCMV – Another Role for CD27

We have previously discussed data illustrating that a pharmaceutically (Rapamycin) induced metabolic balance in T cell response is critical to combating chronic viral infection. Many of the characteristics of this T cell response mimic the phenotypes observed in Poly I:C/αCD40 immunization platform. Microarray data comparing Poly I:C/αCD40 to Listeria challenge from our lab illustrate an enhanced central memory phenotype. Based upon the paradigms of others, this bias should be reflected in the cellular metabolism of the antigen-specific cells favoring a more TCA centric immune response.

While the primary and secondary immune responses to subunit immunization are uniquely IL-27 dependent we have illustrated that IL-27 is unlikely to be responsible for evoking this central memory/TCA-biased response. As we have demonstrated above, interrogation of the remaining antigen-specific IL-27RαKO T-cells in fact illustrated a greater bias towards trivial memory and TCA-mediated metabolism. Therefore some other components unique to the
dual agonist vaccine must be responsible for instigating or supporting this TCA bias.

A closer examination of the unique dual agonist dynamics of CD27-CD70 engagement revealed the critical reliance upon CD70 occurred during the first 48 hours of T cell priming. Afterward, CD70 blockade had a dramatically decreasing impact on the overall T cell response. This is curious as both in the synergy and in pathogen challenge systems CD27KO antigen-specific CD8+ T cells remain equal in number until 72 hours post primary challenge. For pathogen challenge this time point correlates closely with the kinetics of CD70 upregulation. It is logical in that case that deprivation of CD27 would have no effect prior to the natural engagement of its ligand. For the dual agonist system however CD70 arises early and goes away yet death occurs in the CD27KO cells later. This illustrates that signaling necessary for survival through the peak of the effector response is footprinted on the initial engaging cells and subsequently cloned into the expanded progeny post 48 hours of activation.

As mentioned previously CD27 is known to signal in a manner analogous to most classical TNFR, through TRAF2-associated NFκB activation. Through this pathway CD27 has been well documented to upregulate the anti-apoptotic molecules Bcl2, Bcl-xl and Mcl1. We hypothesized earlier that is the employment of this anti-apoptotic function that in fact reinforces the necroptotic cell death presumed to be responsible for decreased cell numbers in IL-27RαKO T cells. Less appreciated, however well documented, is another pathway downstream of CD27 that results in the activation of PIM1 Kinase47,72,199. Through this pathway CD27 has been shown to transmit mTOR-independent signals that increase cellular metabolism. What form of cellular metabolism is called into question. The
reports documenting CD27 and PIM1 mediated effects in metabolism have reported a role for these molecules in promoting glycolytic flux. From this they mostly infer a contribution of CD27 to aerobic glycolysis. This might very well be however increases in glycolytic flux also serve to fuel pyruvate generation to feed the TCA cycle. This highlights the importance of the simultaneous interrogation of both glycolysis and TCA.

As the data currently stands there is no way to definitively ascribe a role for CD27 in supporting a particular form of cellular metabolism. Upon closer examination there is a body of evidence that strongly suggests that CD27 can facilitate traditional oxidative phosphorylation. As mentioned earlier the PIM 1-mediated pathway instigates an mTOR-independent glycolytic flux. Rapamycin has been used extensively to interrogate and alter the balance between oxidative phosphorylation and aerobic glycolysis. mTOR activation is critical to initiation of true aerobic glycolysis which includes the active inhibition of the TCA cycle\textsuperscript{200}. Rapamycin blocks mTOR activation and thereby relieves the active inhibition of TCA cycle. Many reports have illustrated that in the presence of rapamycin there is an overall increase in glucose uptake as GLUT1 (glucose receptor gene) expression is actively impaired by mTOR signaling\textsuperscript{201}. Once again reinforcing the relative rates of metabolism as being key in the dictating the cellular outcome. As described here it seems mutually exclusive that engagement of CD27 and subsequent PIM1 activation could upregulate aerobic glycolysis side stepping an effect on mTOR. The fact that mTOR is left unperturbed suggests more strongly that the increase in glycolytic flux observed is likely consequent of an increased rate of glycolysis and TCA cycle.

Furthermore studies with CD27KO mice have demonstrated a role for CD27 in actively increasing the proportion of cells expressing CD127, the IL-
CD127 is tightly associated with development of central memory T cells, those that are known to be obligated to TCA-biased metabolism. Furthermore, one study went on to show that more than regulating the expression of CD127Rα directly, CD27 was responsible for retaining a less differentiated T cell subset capable of differentiating into central memory in response to infection\textsuperscript{203}. Reinforcing this notion, observations in our lab have shown the proportion of KLRG1 high expressing antigen-specific T cells (associated with terminal effector differentiation) is increased in the absence of CD27. Collectively these data predict a role for CD27 in promoting a TCA-biased metabolism and central memory phenotype. This role of course is not in opposition to CD27's well documented classical TRAF2 NFκB-mediate anti-apoptotic function. By virtue of these two pathways CD27 is well situated to facilitate the generation of robust, long term T cell responses. Along these lines we have demonstrated that CD70 -/- mice do not develop antigen-specific memory in response to dual agonist immunization. Preliminary experiments have also shown the ability of low dose rapamycin treatment to partially rescue T cell defects in a CD27KO host. More exhaustive and formal experimentation is necessary to concretely test this hypothesis.

6.2.4 Resolution of the IL-15 Paradox

With the rising appreciation of the role for metabolism in dictating effector/memory T cell fate there has been an unaddressed paradox of dependency. Metabolically all the data clearly shows that memory T cells are dependent upon TCA-mediated metabolism for long term survival. Before the interest in metabolism however there existed a large body of literature clearly illustrating a role for IL-15 signaling in memory T cell function. As has been
discussed at length, IL-15 drives both a PI3K, glycolytic pathway as well as a STAT5 driven pathway. At the present time there is no clear connection between these metabolic and IL-15 dependencies.

To account for this paradox, I propose a metabolic balance model in which constitutive IL-15 is necessary for the sustaining of functional memory. This process would be fundamentally different than a naïve T cells metabolic reliance upon TCA, which can be passively maintained by the most basal expression of the glucose receptor. For memory cells this model would implicate an active tonic balance of both metabolic pathways. In this model the absence of IL-15 (and its associated elevated glycolytic metabolism) would cause memory cells to either die by necroptosis or differentiate into useless memory. Evidence for this argument arises from the previously presented and discussed data in which CD122 signals are not as necessary for trivial memory cells from either our in vivo vaccine model or in vitro stimulations of OT-1s\textsuperscript{122}. By using congeneric and easily identifiable T cell populations via the TCR transgenic T cells or examining endogenous T cells from a robust immunization, we are able to discriminate between cell survivability and cellular inability to re-expand (trivial memory). Accurately discerning between these two possibilities will be critical in validating this model and reconciling the role of IL-15 and TCA-mediated metabolism in memory cell survival.

6.2.5 - Repertoire Selection Through Selective Cell Death

One of the curious observations during this thesis work has been the greater impact of IL-27R\(\alpha\) deficiency on the numbers of antigen-specific T cells of high affinity compared to those of low affinity. Several features are known to be disproportionately more important for the survival of high affinity T cells after stimulation. The first is TCR downregulation. Several groups have reported the
abortive effects on T cells if they chronically experience high levels of TCR stimulation. It is easy to see how if too much of a good thing, is bad, then too much of a better thing (even stronger TCR engagement compared to low affinity clones) would be worse. This argues that one strategy for circumventing cell death is signal attenuation.

Another feature shown to rescue T cell death from strong TCR stimulation is coincidentally enough IL-15. Signal one generated by TCR agonism from either anti-CD3 alone or super agonism of TCR through engagement by a superantigen, leads to tolerance by cell death. However this is ameliorated by the presence of IL-15 or engagement by CD28 costimulation. This is intriguing as either of these signals would not serve to impair signaling (as is the case of TCR downregulation) but instead to enhance signaling, notably through mTOR activation. In both of these cases, PI3K has been shown to upregulate Bcl-xl or Bcl2 or suppress pBIM levels\textsuperscript{34}. Alterations of these death molecules were supposed to mediate resistance to cell death. Groups have shown however that over-expression of Bcl2 specifically is not always sufficient to overcome impairments of IL-15 signaling\textsuperscript{122,158}. At the time of these reports no consideration was given to how IL-15/IL-2 or CD28 might also be altering cellular metabolism to meet the unique need of a high affinity TCR signaled demand.

Coincidentally and curiously, this observation of IL-27 deficiency augmentation of affinity repertoire mirrors that observed in CD27KO antigen specific T cells. We have observed and others have reported that CD27KO animals generate lower numbers of antigen-specific T cells. The fewer CD27KO T cells that do survive are of higher affinity. We have already discussed how these two 27 deficiencies result in opposing phenotypes of residual cells with regard to effector/memory phenotypes. It’s curious that those phenotypes carry
along with them differences in affinity and potentially metabolism as well. Correlatively, these observations posit that high affinity T cells require greater support in the form of aerobic glycolysis during primary expansion, where as low affinity T cells perhaps can make do with metabolic reinforcement through the TCA cycle alone so long as mitochondrial integrity is maintained by Bcl2 family members.

If this hypothesis were to be true it might also shed light on a feature of T cell selection known as affinity maturation. After initial priming and contraction the residual memory pool will be a spectrum of antigen-specific affinities. Upon subsequent challenges, the pool will become more and more enriched for the high affinity antigen-specific clones. If high affinity T cells are programed to be more reliant upon IL-15, they would logically also be more capable of fully utilizing its signals. During the resting memory stage there would likely not be any advantage afforded to either affinity or metabolism as TCA/glycolysis are actively maintained in balance. However once inflammatory IL-15 or IL-2 are generated in the early phase of the subsequent challenge one would suppose that the CD122-dependent cells would best employ these cytokines for expansion. In the model proposed here, this process would be impaired in IL-15KO, which we know already generate a low affinity pool post the primary, or impaired in CD122 low expressing cells of high affinity. TCR transgenic cells, adoptive transfers, varied levels of CD122 OT-1s, IL-15KOs and altered peptide ligands are available with which to definitively address this hypothesis in the future.

6.3 Sources of IL-27 and IFNαR independent αGalCer adjuvant

Having established the importance of IL-27 in subunit immunizations, we have sought to better understand the dynamics of IL-27 production. We would
like to determine when IL-27 is being made and by whom during subunit immunization. IL-27 is a heterodimeric cytokine composed of the unique IL-27p28 subunit paired alongside the shared subunit EBI3\textsuperscript{204}. Microarray data have shown EBI3 to be broadly expressed in multiple cell types and is present at biologically relevant levels in the steady state. In contrast gene expression of IL-27p28 is restricted to hematopoietic lineages and the protein is produced only after stimulation. The populations that have been well documented to generate IL-27 are monocytes, macrophages, dendritic cells and NK T cells\textsuperscript{205}.

The reported observation of NK-T cell production of IL-27 after engagement by $\alpha$GalCer is of particular interest as it may shed light on an unanswered question of dual agonist vaccine biology. As discussed previously, the dual agonist system has a variable dependency on the type I IFNAR on antigen presenting cells. This dependence tracks well with the ability of the TLR agonist to generate type I IFN, with one exception $\alpha$GalCer. The Kedl lab has reportedly previously that while $\alpha$GalCer is known to generate robust amounts of type I IFN, when utilized in combination with $\alpha$CD40 there is no observable dependence upon the type I IFN receptor (IFNAR). This has led us to wonder what cytokines are critically produce by $\alpha$GalCer to facilitate the synergistic dual agonist response. The data presented earlier illustrated a dependence upon IL-27R$\alpha$ for the generation of maximal T cell responses to the single adjuvant utilization of $\alpha$GalCer. This supports the hypothesis that in combination with the CD40 agonist, cognate interactions of NK-T cells with its ligand results in the release of IL-27 that operates on T cells, and perhaps dendritic cells, to facilitate a synergistic T cell response to the dual agonist vaccine. This is an intriguing and easily testable hypothesis. For other robust IFN generating agonists we have
shown IL-27 to be unimportant and in fact deleterious to dendritic cell priming. If IL-27 is shown to be critical in this dual agonist response it is puzzling as to why when αGalCer is combined with αCD40 the hierarchy of cytokine dependence is reversed. Ultimately we would like to observe the timing and location of IL-27p28 generation. For this we have embarked upon the generation of an IL-27p28 fluorescent protein transgenic reporter mouse, based upon the following construct design (Fig. 6.3).

Figure 6.3 Construction of IL-27p28 transgenic reporter mouse. The 8139 bp sequence directly upstream of the ATG of the mouse IL-27p28 gene was placed in frame with the ATG start codon of enhanced green fluorescent protein (eGFP) utilizing the pRED-ET lambda phage recombineering approach. Likewise a short modified simian virus long poly-A sequence (SVLPA) was attached to the 3’ end of eGFP to provide mammalian mRNA stability. Once made, the plasmid was transfected into RAW 264.7 cells utilizing Lipofectamine LTX with Plus reagent and supplemental sucrose. No fluorescence was observed without innate immune receptor perturbation. With the addition of Poly I:C/αCD40 (10ug/ml/20ug/mL) fluorescent cells could be found (pictured above in black and white and fluorescence only images.)
6.4 Vaccine T cell priming analogy – The antigen-specific T cell survival safari

Apart from observing the magnitude of T cells generated consequent of a given immunization, one of the objectives behind cellular immune vaccine research is establishing molecular correlates of protection. From these signatures one would hope to predict the efficacy of a given vaccine long before having to characterize the strength of that T cell response to a secondary challenge. Collectively the observations above illustrate the complexity of attempting to assemble a model to predict T cell fate.

A simple threshold model where by signal 1 and signal 2 are summed together and a “Goldilocks" range of signal summations are deemed acceptable for both T cell activation and persistence is completely out of the question. The inaccuracy of this model has been reinforced by in vitro and in vivo experimentation in both mouse and man. The literature clearly illustrates that above a certain minimum, there is no predictability for T cell efficacy based upon these two signals. That is to say signal 1 and signal 2 are deemed necessary but not sufficient to dictate T cell fate. The signal 3 model allows further qualitative considerations of T cell differentiation and acquisition of effector/memory function. Even still this model does not give the power of predictability because it does not include any metric for how the inflammatory milieu subverts or supports these signal 3 T cell programs.

Based upon personal bias, I would like to claim that quantification of IL-27 and CD27 could contribute in some way to a predictive model. For simple or combined TLR or CD40 agonist adjuvants there appears to be a general requirement for these signals for robust primary and secondary responses. In terms of global T cell activation correlates however, pathogen challenges have
revealed a varied dependency on these signals. Therefore at the present time, unfortunately I cannot shed light on how to consolidate all of this information into a clear predictable model of T cell activation and fate. To conclude this thesis I would like to highlight the difficulty of such a pursuit by way of analogy.

Imagine a collection of diverse Olympic athletes on a photo safari. These swimmers, high jumpers, sprinters, long distance runners, shot-putters, ice skaters, etc. represent the specialized differentiated states of antigen-specific T cells. These Olympians share the same goal from this experience, to have a good time and then return home to raise the next generation of athletes. The open air vehicle they are traveling in pulls down close to a body of water. By the water’s edge animals gather, grouped based upon species.

While observing these animals the most peculiar thing happens. The driver of the vehicle grips his chest and falls out of the cab dead. No cell phone, no backup, nothing. Worse yet the animals that were previously viewed as majestic and tranquil have revealed their wild nature. Upon seeing the vehicle stranded the lions and bears start licking their chops and the antelope start sharpening their horns. To do nothing is to seal your fate. The mere presence of people in the vehicle compels the animals to take action against them.

The Olympians notice that scattered all about are small reinforced animal blinds offering security. Each blind however, can hold only one person. Each Olympian picks a target blind and at the count of three all of the Olympians jump out of the car and make a break for a blind. Simultaneously the movement spurs the animals to set out after the Olympians. One sprinter is pursued by a lumbering crocodile. The sprinter is a safe distance away from the crocodile but to increase his safety and distance increases to his max speed. As he tires he realizes he has misjudged the distance to the blind and almost immediately he
falls over with a cramp. There are no trainers and no Gatorade to assist him with his cramp. Slowly but surely the lumbering crocodile gains ground on the sprinter and lunch is served. Chomp. A similar fate, although over shorter duration, overcomes the distance runner who was sure he could outsprint the lion to a blind.

Various pairings of athlete, animal and terrain have resulted in a now smaller number of athletes taking shelter in various blinds for a rest. Sitting in the back of the truck just a few minutes before, who would have picked the shot-putter or rhythmic gymnast to have made it this far? Although not the fastest or most aerobically enhanced individual the shot-putter was correctly given the tortoise to compete against and the closest blind to make it too. This represents good pairing of skill and environment. The rhythmic gymnast on the other hand lucked out. While being pursued by a bear, who was gaining ground quickly the gymnast benefitted by the fortuitous intercession by his sequined costume. While being besieged by the bear, light reflecting from the sequins blinded the bear, the texture of the sequins was found to be unappealing to the claws of the bear and a lose sequin lodged in the throat of the bear causing the bear to gag profusely. All of this bought the gymnast the time he needed to safely scramble into the blind. He had thwarted certain death.

Resting in their blinds, the light begins to fade as dusk approaches. Regardless of their Olympic training or the location of their blinds, or how they made it this far, all of the remaining athletes are 200 meters away from the safety of their hotel. In that last distance however remains a 100m wide river infested with hippos followed by a 100m field of tall grass populated by venomous snakes. Who will make it through to live the life all had envisioned, the white picket fence back home to raise the next generation of athletes?
This model is undoubtedly oversimplified but establishes a conceptual platform for future explorations and depicts many of the difficulties before the field of cellular vaccinology today. Reliably predicting T cell outcomes is predicated upon being able to correctly assess the capabilities of the individual athlete as well as the unique challenges they are sure to face. For the vaccinologist there is always the complication of having to select the athlete that will overcome two unique sets of challenges, the vaccine itself and the pathogenic condition for which the vaccine was administered. Like the metaphor depicts, there are selective pressures from the vaccine that have deleterious effects in selecting the most capable competitor against that particular pathogen. This reality has been known for many decades and is known as “original antigenic sin.” As athletes know all too well, there is no such thing as cross training.

Despite this decades-long realization, vaccinologist have been trying to figure out how to generate the superhuman Olympian. The athlete that can compete the best in all events. Now that we know we the influences of metabolic and effector/memory programming we should accept that such a superhuman T cell is transcriptionally impossible. In the light of this reality we are left with two approaches to vaccine design. The first is to molecularly understand the vaccine and its milieu as well as the disease to which you hope to employ it. In this approach there are no universal correlates of protection and no shortcuts for determining vaccine efficacy. You simply try and try again until the appropriate pairing between vaccine and pathogen is made. This approach is well suited for prophylactic vaccines and in existing conditions in which sterilizing immunity is possible and a static pathogenic state is observed. The second approach is to bet on the decathlete.
The reigning world decathlon champion is given the honorary title as the
“World’s Greatest Athlete”. These people however are not superhuman. In fact
their careers are made by striving for mediocrity. In any individual event these
athletes are nowhere near the world’s elite. Facing a barrage of disparate
challenges however, they are the most likely to perform the best overall.
Immunologically, this concept has been gaining traction with the idea of “stem
cell” memory T cells. These T cells, while not the best effectors retain the
plasticity necessary for a spectrum of functionality allowing them to respond to a
multitude of future challenges. For cancer in particular, this decathlete approach
maybe of greater importance since the likelihood of sterilizing immunity is slim
(therefore a long-term antigen-specific T cell response is required) and the
cellular microenvironment is continuously changing in response to vaccination,
tumor burden and treatment. The tradeoff for this flexibility is a lowering in
cytotoxic function. To compensate for this loss in functionality a greater number
of antigen-specific cells is required.

Earlier we discussed that the only true correlate to protection thus far for
cancer and chronic viral infection was indeed the magnitude of the T cell
response. Chimeric antigen receptor therapy relies heavily on the infusion of
super physiologic numbers of antigen-specific T cells. As it stands today, it is
financially impractical to perform these infusions for every cancer patient, in part
due to the cost and time required to grow the requisite numbers of cells. In the
vein of all that has been discussed and presented, the hope is that by learning
how IL-27 and IL-15 influence the T cell response, one could reduce the number
of T cells required for clinical efficacy by increasing the proportion of
appropriately suited T cells. In this way augmentation of CD27 and IL-27 signals
provide experimental tools for tailor making T cells for a particular intended
application be it a highly specialized T cell or the generation of stem memory T cells from a variety of adjuvants.
CHAPTER VII
MATERIALS AND METHODS

7.1 Mice

Female C57BL/6 mice were obtained from NCI (CD45.2) or Jackson Labs (B6 Ly5.2 – congenic CD45.1). B6 IL-27RαKO mice were obtained from Genentech. STAT1KO mice were obtained from Jackson Labs on the 129 background and back breed 10x to B6. CD45.1+ OT-1, IL-27RαKO CD45.1+ OT1, CD45.1+ Vβ5, and IL-27RαKO CD45.1+ Vβ5 mice were bred and maintained at the Biological Resource Center at National Jewish Health and Vivarium at University of Colorado Denver Anschutz Medical Campus. STAT3 fl/fl x CD4-Cre mice were provided by Charles Drake (Johns Hopkins, Baltimore, MD). STAT1 fl/fl mice were kindly provided by Matt Frieman (U of MD) with permission from Lothar Hennighausen (NIH, Frederick, MD) and back bred to in STAT3 fl/fl x CD4-Cre mice to generate T cell conditional STAT1, STAT3 single KOs and STAT1/3 double knockouts. IL-15KO and IL-15 reporter mice (TE20) were kindly provided by Philippa Marrack (National Jewish Health, Denver, CO). BATF3KO mice were obtained from Jackson Labs and by material transfer agreement with Ken Murphy (Washington University, St. Louis, MO). All mice were age matched within 10 days for experiments and used at 5+ weeks of age. The Institutional Animal Care and Use Committee at National Jewish Health, and the University of Colorado Anschutz Medical Campus approved all animal experimental procedures and housing conditions according to guidelines provided by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).
7.2 Mixed bone marrow chimeras (mBMC)

Recipient mice received 1000 Rads and were subsequently rested 4 hrs before intravenous (i.v.) reconstitution with magnetic antibody depleted (anti-CD3, NK1.1, B220, CD19, CD4, CD8) bone marrow of equal cellularity from congenically disparate donors as designated in figures. 12 weeks post reconstitution ratios of CD45.2/CD45.1 was determined prior to use in experiments. Only mice with relatively equal engraftment were utilized for experiments. Donor bone marrow was isolated from WT, IL-27RαKO, STAT1KO, STAT1fl/flxSTAT3fl/fl +/-CD4cre, BATF3KO.

7.3 Immunization and pathogen challenge

Mice were immunized via tail vein injections with 2-50ug (as indicated) innate receptor agonist in 200ul 1xPBS containing 100-150ug/mouse of detoxified (LPS free as determined by limulus assay) whole chicken ovalbumin (Sigma). Where indicated, mice were administered 50ug/mouse of αCD40 antibody clone FGK-45 (BioXcell). For pathogen exposure, female mice were injected i.v. with either 1 x 10^7 p.f.u./mouse Vaccinia Virus Western Reserve Strain (Vv) or the same strain recombinantly expressing chicken ovalbumin (Vv-ova), or 2000 c.f.u./mouse *Listeria Monocytogenes* (Lm) expressing either whole Ovalbumin (Lm-ova) or the Vaccinia Virus epitope B8R (Lm-B8R). For secondary Lm-ova challenges 250,000 c.f.u. were delivered i.v.. Innate immune receptor agonist were obtain from the following sources and used in the following doses: Pam3-Cys 25ug/mouse (Invivogen), αGalCer (purchased from Alexis Biochemicals, cat #306-027-M001) 2ug/mouse, Flagellin was prepared from homogenate of S. typhi used at 15ug/mouse (InVivogen, FLA-ST Ultrapure), Poly I:C 50ug/mouse (GE HealthCare).
7.4 Lm-ova protection assay

Congenically disparate WT and IL-27RαKO Vβ5 TCRβ transgenic mice were directly immunized with Poly I:C/αCD40/ova and allowed to rest for 35+ days. Spleens were harvested and CD8 T cells enriched by magnetic negative selection (Miltenyi) to a purity of greater than 90%. The percent tetramer+ was used to calculate the numbers of antigen-specific T cells transferred to congenically disparate naïve WT hosts. The following day animals were challenged with a lethal dose (250,000 cfu) of erythromycin-resistant Lm-ova. 5 days later spleens were harvested and supernatants plated out on erythromycin (5ug/mL) containing BHI agar plates incubated for 36 hours at 37°C to count splenic bacterial burden.

7.5 OT-1 adoptive transfer

WT and IL-27RαKO OT-1 T cells were purified from spleen by magnetic negative selection for CD8 (Miltenyi). Populations were always > than 90% pure. WT and IL-27RαKO OT-1s were mixed 1:1 and transferred i.v. The following day mice were immunized as described for each figure. For CFSE labeling cells were incubated at 2x10^7/mL with 10uM CFSE in PBS for 10 minutes at 37°C, then quenched with equal volume of heat inactivated FBS.

7.6 Flow cytometric staining and determination of antigen-specific T cells

Animals were euthanized at day 7 post immunization (days post immunization, dpi) for all endogenous T cell subunit vaccinations and 8 dpi for Vaccinia and Listeria primary infection. Antigen-specific T cell responses in spleen and blood were determined by tetramer staining as previously described. Analysis of tetramer staining was performed as previously described. Black line in figure 2.2.1.2D denotes limit of detection for tetramer all tetramer experiments
(0.07%) established by tetramer staining of unimmunized animals and use of irrelevant tetramers in immunized mice. Flow cytometry data were obtained using a DakoCytomation CyAN ADP flow cytometer and analysis was performed utilizing FlowJo Software (Treestar, PC version 7.6.5). The following cell surface antibodies and clones were used: Brilliant Violet anti-mouse B220 (Biolegend, clone:RA3-6B2), APC-AF750 anti-mouse CD8a (eBioscience, clone 53-6.7), PerCP-Cy5.5 anti-mouse /human CD44 (Biolegend, Clone: IM7), APC-anti-mouse CD3e (Biolegend clone 145-2c11). For bmc experiments congenic CD45 marker was determined by using APC labeled anti-mouse CD45.1 (Biolegend, Clone:A20). Kb SIINFEKL tetramers coupled to fluorochromes BV421 (Biolegend) –and PE (Prozyme) were generated from baculovirus as previously described. Peptides were synthesized at University of Colorado Denver Anschutz Medical Campus Peptide Core facility. I-Ab 2W1S tetramer was kindly provided by John Kappler and Pippa Marrack (National Jewish Health). For transcription factors cells were stained as described above and fixed with 1% PFA-3% sucrose-PBS (fixation buffer) for 10 minutes then washed with 1x eBioscience Foxp3 perm buffer. Cells were then resuspended in eBioscience Foxp3 perm buffer and transcription factor antibodies added for 2 hrs, washed fixed and run on flow cytometer. For intracellular cytokine staining procedures were followed as described previously. Briefly aliquots of splenic suspensions were restimulated with 1ug/mL of SIINFEKL peptide in the presence of 3ug/mL Brefeldin A for 4+hrs. Surface stains were then added. Cells washed fixed and stained for intracellular cytokine utilizing the eBioscience Foxp3 perm buffer followed by washing and fixation. gMFI on graphs of flow acquired data indicates the geometric mean fluorescence intensity of the indicated cellular subset for the stated parameter as determine by Flow Jo analysis software.
7.7 Calculation of total tetramer + cell number

Calculation of total antigen-specific cell numbers was performed by determining the relative % of tetramer positive cells in a splenocyte sample multiplied by the total splenocyte count as determined by Vi-Cell automated cell counter (Beckman Coulter).

7.8 Recombinant IL-27

Plasmid for generation of recIL-27 was generously provided by Dr. Xiaoqing Wei (Cardiff University, UK). The plasmid encodes for the expression of the dimeric components of IL-27 covalently linked to one another and attached to an Fc backbone (7). The plasmid was transfected by use of Lipofectamine Ltx (Invitrogen) into HEK293T cells and supernatants collected. RecIL-27 was isolated through affinity chromatography across a protein G column. RecIL-27 free of Fc was generated through enzymatic digestion by overnight 37°C incubation with papain 0.05mg/mL. Purified recIL-27 free of Fc was separated from papain digested reagents by dialysis with 30KDa cutoff. Recombinant IL-27 (250ug per injection) was administered i.v.

7.9 IL-10R blockade

Antibody (clone 1B1.3) recognizing the unique IL-10R (CD210) was purchased from Bioexcell and used at 250ug i.p. per injection. Antibody was administered at day 3 and day 4 post Poly I:C/αCD40 immunization as to not interfere with innate IL-10 signals and to blockade T cell generated IL-10. This also precedes the alteration of WT/IL-27RαKO OT-1 observed in figure 2.2.3.2D.

7.10 Tetramer dissociation assay

WT Vβ5 and IL-27RαKO Vβ5 cohorts were utilized for the elevated naïve precursor frequency of ova antigen-specific CD8 T cells and immunized as
described in Lm challenged methods. At day 7 peak of primary response, splenic suspension were stained first with Kb-SIINFEKL-PE tetramer and then with antibodies for immune subsetting. Samples were thoroughly washed from unbound antibodies and tetramer. Aliquots for a 0 time point were then removed and fixed with 1% PCHO-3% sucrose. 25D1.16 antibody (eBioscience) against Kb-SIINFEKL was added at a final concentration of 20µg/mL. Aliquots were removed and fixed at time points of 0.5, 2, 4, 6, 8, 10 and 20 minutes. Geometric MFI (gMFI) of tetramer positive cells was obtain by flow cytometry and plotted for each mouse. Data were plotted by Microsoft excel and fit to a second order polynomial resulting in the displayed R2 line fit values. From the equations of the line the time for each time 0 sample to have half of the tetramer competed off by 25D1 was determined and plotted by Prism (Graph Pad), reflecting the average TCR affinity for ova for each sample. Statistical significance was determined by Student’s t-test.

7.11 Statistical analyses–data displayed

Paired (bone marrow chimeras) and unpaired statistical analyses were made between experimental populations or groups using Student’s t-test or across time or dose by two way anova with GraphPad Prism Software (version 5). All experiments were performed independently at least twice with a minimum of three mice per group. Figures and figure legends detail replicates of experiments and numbers within each experiment. For figures p-value are coded as follows * p=<0.05, ** p=<0.01, *** p=<0.001. All error bars depict the standard error of the mean (SEM).

7.12 Dendritic cell staining

To characterize splenic DC populations, spleens were harvested in EHAA (Invitrogen, Click’s Media) at the indicated time points. Spleens were minced to
fine pieces with forceps and incubated in 2 mL EHAA medium with a final concentration of 0.5mg/mL collagenase D (Roche Diagnostics, Indianapolis, IN) and 50ug/mL DNase (Worthington, Lakewood, NJ) for 30 minutes at 10% CO₂ and 37°C. 0.1 M EDTA in PBS was added to a final concentration 20uM and cells incubated for an additional 10 minutes before being homogenize against at 100uM strainer with the back end of a 3mL syringe. Cells were spun down at 450g for 5 minutes and resuspended in 2.5% BSA in 5mM EDTA for flow staining. The preferred dendritic cell stain set utilized CD11c (clone N418) in APC-Cy7, CD11b in Pacific Blue, B220/CD3e/NK1.1 in AF488, IL-15Rα in PE (eBioscience, clone DNT15Ra), CD8α in APC, MHCII (I-A, I-E) in PE-Cy5.

7.13 Active caspase staining

Active Caspase 1,3, and8, were stained from directly ex vivo spleens harvested at the indicated times. Spleens were collected in ice-cold PBS and maintained in ice-cold PBS or ice-cold assay buffer until the indicated incubation. Active Caspase 1 kit was obtained from Immunochemistry Technologies, Bloomington, MN. Caspase 3 and 8 kits (CaspGLOW) were obtained from eBioscience affymetrix. Loading of cells with fluorescent inhibitors of Caspase was done as dictated in the product manuals. After loading of the dye, cells were incubated in 10% FCS containing RPMI CTM for 1 hour at 37°C and 10% CO₂. The last half hour of incubation cells were stained with the appropriate CD8 T cell and congenic marker antibodies as described in flow methods.

7.14 2NBDG/MSR metabolic assay

Spleens were harvested and processed into ACK-lysed single-cell suspensions as described in flow cytometry methods. Cell were subsequently washed 2x in 1xPBS and resuspended in PBS at a concentration 1x10⁶
cell/100ul/well containing freshly prepared Mitosox Red (Invitrogen) superoxide reactive dye at a final concentration of 5uM and incubated at 37°C and 10% CO₂ for 10 minutes. Equal volume of 10% FCS – RPMI CTM was added to quench MSR loading. Cells were washed with 10% FCS – RPMI CTM and subsequently resuspended in 10% FCS – RPMI CTM containing 10uM 2NBDG. Cells were incubate for 4 hours at 37°C and 10% CO₂. During the final 30 minutes of incubation surface stains delineating the CD8+T cell lineage and congenic markers were added. At 4 hrs, cell were washed 2x with cold PBS-2% BSA and resuspend in 2% BSA-PBS+5uM EDTA and kept on ice while being immediately run on the cytometer.

7.15 IL-27p28 eGFP reporter construct

The 8139bp sequence directly upstream of the ATG start codon of the mouse IL-27p28 gene was placed in frame with the ATG start codon of enhanced green fluorescent protein (eGFP) utilizing the pRED-ET lambda phage recombineering approach. The bacterial backbone was obtained from Addgene (pUCBB-eGFP). Bacterial Artificial Chromosomes containing the mouse chromosomal region of IL-27p28 were obtained from the Childrens Hospital of Oakland. Likewise a short modified simian virus long poly-A sequence (SVLPA) was attached to the 3’ end of eGFP to provide mammalian mRNA stability. Vector designed and recombineering methodologies were assisted by Jen Matsuda and Bicheng Zhang (National Jewish Health). Once made the plasmid was transfected into RAW 264.7 cells utilizing Lipofectamine LTX with Plus reagent and supplemental sucrose.
REFERENCES


