ROLE OF INDOLEAMINE 2,3-DIOXYGENASE IN GENERATION OF MEMORY RESPONSE TO INFLUENZA AND DEVELOPMENT OF HETEROLOGOUS IMMUNITY TO 2009 PANDEMIC H1N1 INFLUENZA

by

LEO KO SAGE

(Under the Direction of Ralph A. Tripp)

Influenza viruses are a major pathogen of humans, domestic animals, and wild animals which constantly mutate and cause devastating disease in their hosts. Understanding the immune response and eliciting protective immunity against influenza is critical to minimize disease and transmission. This study examines inhibition of indoleamine 2,3-dioxygenase (IDO) as a potential method to modulate robust immune response against influenza. IDO inhibition results in an enhanced T cell response with reduced pathology upon influenza challenge. This would suggest that the inclusion of IDO inhibitors in vaccination may serve as a way to enhance memory T cell responses and reduce the damage from influenza infection. The importance of memory T cells in maintaining effective heterosubtypic immunity is also examined in the context of the 2009 pandemic H1N1 influenza virus. This study showed that the pandemic H1N1 virus evades memory T cells generated against pre-pandemic H1N1 influenza and emphasizes the need for a diverse T cell repertoire to prepare populations against novel influenza challenges.

INDEX WORDS: Influenza; Indoleamine 2,3-dioxygenase; Memory T cell; Vaccine

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# CHAPTER 1 INTRODUCTION

Influenza virus is a pathogen of major concern major to humans, domestic animals, and wild animals. While immunocompetent, healthy human adults usually overcome influenza virus infection as a self-limiting disease, it can be a serious problem in debilitating, elderly, and young children, as infections predispose patients to opportunistic diseases such as bacterial pneumonia and exacerbate respiratory ailments such as asthma [1-3]. The economic toll from lost labor, deaths, and agricultural loss is devastating and affects domestic and international trade through biosecurity efforts. As vaccination is the most cost-effective way to decrease transmission and disease caused by influenza virus, boosting the memory immune response in vaccines is of great interest to decrease disease burden and transmission.

A member of the *Orthomyxoviridae* family, influenza type A has 16 subtypes of hemagglutinin (HA) and 9 neuraminidase (NA) molecules which are expressed on the surface of virion and function in viral entry and escape, respectively. Influenza viruses can possess any combination of HA and NA. This can make protection afforded by antibodies unreliable when animals are challenged with influenza viruses with a different combination. Thus, generating a robust immune response which is able to recognize a wide variety of influenza virus would be critical to reduce disease from challenge by these diverse subtypes [4, 5]. New strains emerge constantly by a combination of point mutations (antigenic drift) and reassortment of gene segments (antigenic shift). This highly unpredictable nature of influenza necessitates that the immune system be able to respond with a broad, cross-protective (heterosubtypic) immunity.

Because B cells and antibodies are generally unreliable for protection against heterosubtypic challenges, T cells are of paramount importance. Unlike B cells, T cells function by reacting against epitopes derived from a variety of conserved, internal proteins inside the influenza virion and so is more likely than B cells to be able to recognize epitopes which are

shared across different subtypes of influenza [6-8]. Consequently, memory T cells are important for rapid virus clearance and decreasing morbidity against various influenza virus subtypes [9] and allow the host to survive challenges which would be lethal in a naïve host without memory T cells [10]. Memory T cells can be the difference between life and death. Therefore, it would be of great interest to generate a high frequency pool of memory T cells which can cross react with a variety of influenza epitopes which will react rapidly upon challenge. Unfortunately, the current inactivated influenza vaccines induce negligible level of memory T cells [9] so a way to boost memory T cell immunity in these situations is highly desirable.

One way to potentially augment the cross-reactivity of memory T cell response may be to decrease the expression and/or activity of the immunomodulatory enzyme, indoleamine 2,3-dioxygenase (IDO). IDO is the first and rate-limiting enzyme in the Tryptophan catabolism pathway [11, 12] which is expressed in response to inflammation to minimize immunopathology. However, overexpression of IDO when the virus-specific effector T cells are mounting a response to influenza may be detrimental due to excessive suppression. Thus, decreasing IDO activity during induction of anti-influenza virus immunity may be a way to achieve a robust, cross-reactive memory T cell response.

IDO's effects on T cell mediated immunity have been studied in various disease models including autoimmune, graft-versus host disease, tumor growth [13, 14], allergies [15], and various infectious models. Although expression of IDO has been verified during influenza virus infection [16], the specific effects of IDO on memory T cells in the context of influenza infection have not been assessed. Various steps in immunity which pave the way towards developing a robust memory T cell response may be affected by IDO. This may be starts at the level of antigen presenting cells [12, 17, 18], regulatory T cells (Tregs) [19], and immunomodulatory cytokines [20]. It is possible that one or more of these mechanisms can be manipulated to optimize the memory T cell response.

Specific antiviral parameters of memory T cell immunity which are correlates of protection include increased Th1 (CD4+ IFNγ+) and Th17 (CD4+ IL-6+) response and higher number of functional, virus-specific CD8+ T cells. This combination of a pro-inflammatory cytokine environment and efficient CD8+ T cell response is important because influenza infection can recruit large numbers of T cells which are not virus specific [5]. Higher proportion of virus-specific T cells in the midst of infiltrating leukocytes is generally a more efficient response. These antiviral parameters were boosted as a result of IDO inhibition on the primary response to influenza [21, 22]. Because higher clonal burst size is correlated with increased memory precursor frequency, IDO inhibition may increase virus-specific memory precursors [23, 24].

This led to the central hypothesis that the memory T cell response against influenza challenge would lead to a higher Th1 cell and virus specific CD8+ T cell response. These findings would be of paramount importance because understanding the memory T cell response is critical to vaccine designs. Vaccines are designed to optimize the memory precursors for expansion and recall during influenza challenge. The antiviral parameters that are boosted by IDO inhibition during vaccination may be a potential strategy to counteract the challenges facing influenza. IDO inhibition during vaccination against a tumor antigen reduced the Treg/Th17 ratio and improved anti-tumor immunity [25]. This same principle is hypothesized to take effect to boost influenza vaccination immunity.

While initially studying the effects of IDO on memory T cells, a peculiar observation was made which led to the study to evaluate memory T cell cross reactivity and responsiveness. Mice were primed with a pre-2009 seasonal influenza virus (cH1N1) was then challenged with 2009 pandemic H1N1 (pH1N1). The epitopes against cH1N1 was similar enough to pH1N1 that a cross-reactive memory response was expected. Upon challenge, a significant lack of cross-reactivity was observed, and consequently, the pH1N1 persisted much longer. This led to the hypothesis that subtle but key mutations in the pH1N1 was sufficient to evade major subsets of

heterologous memory T cells, allowing the virus to persist and could be a contributing to the spread and establishment of pH1N1 in the human population.

This dissertation illustrates the role of IDO inhibition in the T cell response to influenza and how this strategy can be translated for potential incorporation in influenza virus vaccines. Finally, the relationship between the immunity generated by pre-pandemic influenza virus against the latest pandemic virus, pH1N1 will be explored. To summarize, this dissertation addresses the following hypotheses:

- **IDO inhibition during primary response to influenza enhances the memory T cell response**

  This study outlines the effects of IDO inhibition on memory T cell response and how specific memory antiviral immune parameters are influenced by IDO.

- **IDO inhibition during vaccination to influenza enhances the memory cell response**

  Building on the previous hypothesis, this study evaluates the application of the changes in the memory response as a result of IDO inhibition to assess the potential benefits of IDO inhibition during vaccination.

- **2009 pandemic H1N1 influenza evades subsets of memory T cells mounted against pre-2009 H1N1 influenza**

  This study evaluates the extent of heterosubtypic immunity conferred by memory T cells induced by pre-pandemic influenza virus against pH1N1 influenza and emphasizes the importance for a broad, cross-reactive T cell response

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# CHAPTER 2 LITERATURE REVIEW

## Challenges Facing Influenza

Influenza virus is a member of the *Orthomyxoviridae*, a family of enveloped, negative sense segmented RNA virus with types A, B, and C. While types B and C infect a variety of species and are relevant, this review will focus on type A. Influenza A warrant extensive investigation as they are a major source of infectious disease of humans, domestic and wild animals [1].

Influenza is primarily a respiratory pathogen in mammals. Disease is caused by cytopathology, immunopathology, and secondary bacterial pneumonia [2] and also exacerbate pre-existing respiratory ailments such as asthma [3-5]. The resulting disease causes over 5 million illnesses, 2.4 million outpatient visits, 32,000 hospitalizations, and over 600 deaths in the United States (U.S.) alone [6]. In addition, the loss in productivity in the workforce results in economic cost of nearly 9 billion dollars in the U.S. annually [6]. The damage to agriculture is just as devastating; the main group of animals of concern being poultry. These avians serve as hosts which can develop and occasionally transmit highly pathogenic avian influenza (HPAI) which has a high case fatality rate in both humans and birds. Hundreds of millions of poultry have been culled worldwide due to HPAI [7]. All animals which are affected can potentially benefit from vaccination to protect from disease.

Although spontaneous mutation is common in RNA viruses, influenza experiences various selection pressures that favor some mutations over others. The selection pressure comes largely from the virus' ability to infect and sustain themselves in the host population [8]. In all hosts, immune driven antigenic drift is one of the primary factors driving its evolution, namely antibody mediated selection [9-11]. Influenza possessing mutations in the surface proteins that

can evade antibody neutralization is selected for and persists. Although antibodies that can confer protection against multiple subtypes of influenza, such as those against matrix 2 (M2) exist [12-14], there are limitations as it will not protect against all viruses [15]. In addition to antigenic drift, there exists a fundamentally different class of mutations induced antigenic shift, which does not depend on point mutations but on the influenza virus' ability to reasssort its segmented genome. Influenza has 16 known subtypes of Hemagglutinin (HA1–HA16) and 9 Neuraminidase (NA1–NA9), and can possess any combinations of these genes and participate in the specifying the infection of host cells [16, 17]. Antigenic shift has been responsible for the emergence of virus with new HA and NA combinations, such as pH1N1 and 1918 H1N1 [18]. In addition to HA and NA, genes for internal proteins may also shuffle and generate novel virus, even though the HA and NA may be the same. These mechanisms allow influenza to reproduce with slight or drastically different variations within one replication round which can render host immunity upon encounter. Although antibodies that target specific combinations of HA and NA can protect the host from infection [19], infection with different subtypes would be recognized as a novel virus from an antibody/B cell perspective. Even subtypes of the same designation can also lack cross reactivity. For example, pH1N1 and cH1N1 are both H1N1 viruses but the antibodies do not cross react well [20, 21], rendering most of the population naïve with respect to the pH1N1 and can make protection afforded by antibodies unreliable. Although antibodies against the internal proteins of influenza exist [22-25], they do not confer protection because the virion does not expose these proteins and they are not necessary for entry into host cells.

A combined effort by T and B cell mediated immunity against conserved viral proteins can control for antigenic drift and shift mediated escape [10]. In contrast to B cells, T cells react against the internal epitopes of influenza and become indispensable in challenges against different subtypes [26-28] where B cells fail to cross react (Figure 2.1). Memory T cells are important to promote early virus clearance and decrease morbidity against influenza variants [29]. While

sterilizing immunity is not afforded by memory T cell response, host can survive challenges against otherwise lethal challenges if memory T cells are present and can make a difference between life and death [30]. Internal proteins such as PA, PB1, and M1 are recognized by virus-specific CD8+ T cells in mice and humans [31, 32] and so acts as a target for optimum protection against circulating and novel influenza virus strains which will invariably arise in the future.

Unfortunately, inactivated influenza vaccines induce negligible levels of T cell response. In contrast, live attenuated influenza vaccines (LAIV) such as FluMist® can confer protection against influenza strains beyond that of the vaccine strain [33]. However, the memory T cell resposne induced by LAIV are lower than that generated by a challenge and may not be sufficient to confer adequate protection against subsequent challenges by novel influenza subtypes [34, 35]. One way to boost the memory T cell response in response to infections and vaccination may be to suppress the activity of an immunomodulatory enzyme, indoleamine 2,3-dioxygenase (IDO).

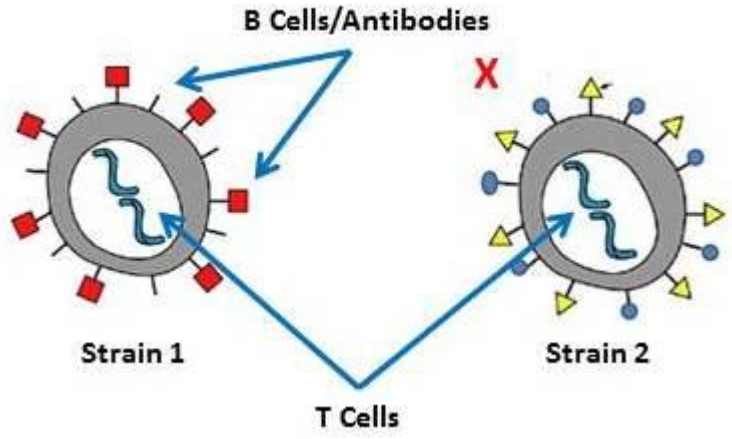


Figure 2.1

B cells and antibodies generated against HA and NA of strain 1 fails to cross react with the HA and NA of different subtypes. In contrast, T cells which recognize internal proteins of the virion cross react between both strains 1 and 2.

## Indoleamine 2,3-dioxygenase

IDO is the first and rate-limiting enzyme in the Trp catabolism pathway and possess immunomodulatory properties [36, 37]. IDO is expressed in various immune and non-immune cells that generally function to downregulate inflammation, DC activation, and expansion of effector T cells (but not regulatory T cells) [38-40]. The depletion of Trp and production of metabolites attenuate T cell responses and reduce inflammation and drive immunity towards a tolerogenic response [37]. Thus, it is expressed in areas where tolerance and inflammation is carefully controlled, including the brain [41], intestine, epididymis [42], gingiva [43], nasal mucosa, and placenta [44].

IDO is upregulated in response to pro-inflammatory cytokines such as IL-1 [45], Type I (α, β) IFN [46] , Type II (γ) IFN [46, 47], Type III (λ) IFN (unpublished results), and tumor necrosis factor (TNFα) [45-47]. Stimulation of various Toll-Like Receptors (TLR) can also directly induce IDO [43, 45, 48]. IDO has a variety of mechanisms to exert its anti-inflammatory and tolerogenic effect. IDO's effects on cell mediated immunity have been studied in various non-infectious diseases such as autoimmune, graft-versus host disease, tumor growth [49, 50], and allergies [51]. Infection by various pathogens are accompanied by IDO expression. These include *Plasmodium* [52], Human Immunodeficiency Virus (HIV) [53], *Neospora caninum* [54], *Toxoplasma gondii* [55], *Rhodococcus equi* [56], and *Mycobacterium tuberculosis* [57]. Note that these are all (facultative) intracellular pathogens and may function by depleting Trp to retard growth of pathogens inside cells. IDO metabolites such as picolinic acid may also hamper pathogen growth [57].

IDO is upregulated in response to influenza virus infection in mice lungs [58, 59], likely in response to the pro-inflammatory cytokines and TLR stimulation. The peak activities of IDO correlate with the peak T cell response and continues to be expressed well after virus clearance when memory T cells are being generated [60]. Only the correlation between IDO expression and

immune responses has been established and few studies have addressed the relationship between IDO and T cells during influenza infection. In addition, few studies have examined the effects of IDO on cell mediated immunity and its effect on memory cell formation in infectious disease models. Understanding and modulating the effects of IDO can greatly benefit in controlling the pathology and generation of memory cells and complements the challenges facing influenza infections, particularly T cell mediated heterosubtypic immunity.

One final point to consider is the two different isoforms found in human and mice (IDO1 and IDO2). They perform similar biochemical functions but have very different sequences and are thought to have evolved independently [61]. Although IDO2 has similar function to IDO1, their expression patterns are different [42] as well as their susceptibility to the different enantiomers (L and D) of the pharmacological inhibitor, 1-methyl-tryptophan (1MT) [62]. The enantiomers show preferential inhibitory activity towards IDO1 and IDO2 [63]. 1MT is an attractive option due to its widely established use for IDO inhibition and low toxicity [64]. The studies which are cited in this dissertation almost exclusively use 1MT as an inhibitor. Now, the antiviral immunity against influenza will be outlined and how IDO can affect these processes.

## Innate Immunity to Influenza

### *Pattern Recognition Receptors, Cytokines, and Chemokines*

The innate immunity to influenza is essential for survival of the host and induction of adaptive immunity (reviewed in [65]). There are three main class of Pathogen Recognition Receptor (PRR) important to the recognition of influenza viruses: TLR; Retinoic acid-inducible gene 1-like helicase (RLR) [66, 67], and nucleotide-binding domain and leucine-rich-repeat-containing proteins (NLR) [68]. All three pathways participate in the antiviral inflammatory response against influenza [69]. Surfactants and mucosa in the airway and lower respiratory tracts serve as initial obstacles for viral infection of cells [70], but influenza can manage to bypass these barriers. There

are various APC which possess the PRRs, including macrophages, plasmacytoid dendritic cells (pDC), and myeloid dendritic cells (mDC). TLR recognizes of viral RNA during its replication: TLR3 recognize dsRNA [71] and TLR7 recognize ssRNA [72, 73]. This array of TLR on resident leukocytes is critical for downstream immune effects; lack of adequate TLR stimulation can drive an immune response towards tolerance [74].

T cells are affected downstream of TLR stimulation. TLR 9 stimulation in DC can block Treg development and stimulate IL-6 production, decreasing the Treg/Th17 cell ratio [75]. On the other hand, TLR9 also stimulates IDO expression which counteracts the effects of IL-6 [76]. In addition, different APCs express different arrays of TLRs and so IDO can exert profoundly different roles depending on the type of APC responding to virus. The inflammatory context can drive the immune response either way (inflammatory versus tolerogenic) and is important for maintaining a balanced immune response to maintain normal respiratory physiological functions. The upper respiratory airway is coated with benign commensals, so IDO functions to limit excessive inflammation and clearance of these organisms. The degree of TLR stimulation and the pre-existing cytokine environment can determine the course of IL-6 production and IDO activity [43].

Stimulation of PRR leads to the production of Type I IFN [77, 78]. Type I IFN has pleiotropic effects by activation of interferon stimulated gene. These effects include arresting the metabolism of infected and neighboring cells to limit virus replication. IFN also activates antiviral factors such as nuclease and surface molecules like MHC, selectin, and integrin ligands to recruit circulating leukocytes [79]. NOD2, a subtype of NLR induce IFN-β production in response to influenza ssRNA [68]. RIG-1 stimulation by influenza also stimulate Type I [80] and Type III (λ) IFN [81-83]. Thus, PRRs work in concert to mount an optimum response against influenza, so immunotherapy and vaccines should consider stimulation of multiple PRR pathways to produce a synergistic, immunogenic effect. These signals are often amplified downstream in

the form of cytokines. In addition to Type I IFN, other cytokines are produced by various cells in response to viral insult. These antiviral cytokines include: CXCL8/IL-8 [84]; TNFα [85]; IL-12 [86, 87]; IL-1 [88]; and IL-18 [89].

IL-8 is involved in neutrophil recruitment [84]. In addition to IL-6, IL-8 protects and promotes neutrophils from premature death and drives them to infected sites for viral clearance [90]. IL-6 promotes a balanced effector memory CD4+ T cell response, reducing tolerance against antigen [91] and antagonizes expression of IDO in DC. IL-6 has a downstream effect of a higher T cell activation capacity [92]. IL-1β is critical for activation of CD4+ immunity and antibody response [88] and synergizes with IL-12 to support a Th1 response [86, 87]. IL-18 augments the NK cytotoxicity and IFNγ expression [93], which again, supports a Th1 response. The activity of cytokines is critical for a balanced innate immune response as well as induction of appropriate adaptive immunity and shaping recall of memory responses. Influenza infection induces the production of chemokines to recruit leukocytes also play a critical response [94, 95].

Initially, CCL2/MCP-1 and CCL5/RANTES are secreted by infected alveolar epithelial cells and recruit monocytes and activate macrophages [94, 96, 97]. If memory T cells are present, CCL2 also functions to recruit memory T cells and DC [98]. Concurrent neutrophil recruitment is mediated by IL-6 and IL-8. In turn, activated alveolar macrophages produce CCL3/MIP-1α which recruit more T cells [99]. In addition to cytokines and chemokines, the last important soluble factors which participate in innate immunity (and enhanced by adaptive immunity) are complements. Complement C3b aid in B cell activation to antigen. Cross-linked antibodies on the infected cell surface induce antibody dependent cytotoxicity by macrophages [100]. Finally, the classical [101] and mannose-binding lectin pathway [102] work together clear influenza.

**Adaptive Immunity: Dynamics of Effector T cell response against Influenza**

There are two distinct lineage of T cells: γδ and αβ. γδ T cells react to stereotypical products such as isoprenylpyrrophosphates and are important in mucosal immunology [103, 104]. However, the

role of IDO has been studied most extensively with αβ T cells and will be the focus of the review. αβ T cells have been assigned various names according to functions, lineages, surface markers, and other distinguishing features. However, it may be appropriate to conceive of the T cell spectrum as analogous to a color wheel, where there is no distinct color, but rather a gradient where two T cells may share the expression of the same surface markers but the function differs slightly such as cytokine profile and effector mechanisms. Some may be a darker shade of a specific color, in which some T cells are simply more active and exert a higher degree of a specific function such as cytotoxicity.

IDO may function to turn the color wheel slightly (shift the Th1/Th2, Th17/Treg) or to change the shade of a same color (e.g., transcription factor, cytokine production). Eos, for example is a transcription factor which mediates the degree of suppression/effector activity along with Foxp3 in Tregs [105]. It is possible that IDO shifts this axis of cytokine and transcription factors to some degree. Even a slight shift can have a profound effect downstream in the memory response, as one memory cell can give rise to thousands of clonal cells. The effect is amplified by the development of secondary and tertiary memory cells from infections and vaccinations.

*Antigen Presentation*

Once APCs from influenza infected sites reach the local draining lymph node, they present antigen to lymphocytes. T cells recognize immunodominant antigens in the context of MHC Class I or II for CD8+ and CD4+ T cells, respectively. At this stage, the APCs which arrive at secondary lymphoid organs may express IDO to drive the course of the immune response. IDO+ macrophages decrease CD8+ T cell proliferation in HIV infections [106]. IDO+ DC and Tregs downregulate effector T cell activation and effector function [37, 107, 108]. DC of various lineage and subtypes have been described but all of them (except follicular dendritic cells) show

evidence of IDO expression, including myeloid DC [109], plasmacytoid DC [110], and splenic DC of B-cell lineage (CD19+ DC) [111].

Besides those described for Tregs, IDO have several additional mechanisms to downregulate T cell activation and expansion. The deprivation of essential amino acid tryptophan is detrimental to actively mitotic cells (expanding T cells), specifically by decreasing the mitochondrial bioenergetics [43, 112] and initiation the GCN2 kinase pathway [110, 113-115]. Activating T cells and NK cells are particularly sensitive to tryptophan deprivation and Kyn production [115, 116]. In addition to Kyn, another metabolite, 3-hydroxyanthranilic acid, inhibits PDK1-mediated activation of NF-κB in T cells [117]. IDO's function during antigen presentation is very important and is perhaps the most critical in applications such as vaccination. IDO is expressed at constitutively high levels in myeloid dendritic cells in the draining lymph node (LN) of nasal mucosa [118]. Consequently, when IDO is inhibited in the nasal mucosa, the balance shifts away from tolerance to immunity [118]. While the nasal mucosa is not the primary site of influenza replication, it is the primary site of induction of immunity against LAIV such as FluMist® [119].

*Regulatory T cells and Influenza*

Treg is an important subset of CD4+ T cells which plays an important role in response to antigen and is affected by IDO. There is enormous potential to shape Treg responses due to their functional plasticity. Currently there is no definitive way to measure Tregs which will definitively show how Tregs act in all situations. For example, natural Tregs (nTregs) are capable of expressing proinflammatory cytokines including IFNγ, while at the same time expressing transcription factors T-bet (Th1), GATA-3 (Th2), or RORγt (Th17) and suppressing Th2 and Th17 cells [120, 121]. Tregs are highly variable in their immuophysiology and there is a tremendous amount still to be learned. One Treg can suppress hundreds of neighboring cells and

therefore has a tremendous potential to shape the immune response [122] and must be considered in the immunity to influenza virus.

During influenza infection, Tregs can downregulate virus-specific CD8+ T cell expansion [123]. Tregs that are activated during influenza vaccination compromise B cells [124] and CD4+ T cell response [125]. Conversely, inhibition of Tregs during vaccination against influenza virus increases vaccine efficacy [126]. Tregs disrupt CD4+ T cell help via IL-2 snatching with its high affinity IL-2 receptor, CD25/IL-2Rα [127]. They also directly lyse target cells by perforin and granzyme A/B [128]. Finally, expression of inhibitory cytokines: IL-10 [129]; TGF-β [129]; and IL-35 [130] can suppress distant targets and along with IDO+ DC. Being antigen specific, Tregs do not exhibit equal suppressive activity towards all target cells: they selectively suppress the expansion of CD8+ T cells depending on their immunodominance/epitope specificity and therefore Treg modulate the immunodominance hierarchy [131].

IDO serves as a conduit between DC and Treg to support each other's functions and suppress effector T cell activation and expansion through multiple mechanisms. PD-1 expression is upregulated in IDO+ DC [132] which stimulates Treg suppression [110]. In turn, activation of the aryl hydrocarbon receptor in Tregs by Kyn induce IDO1 and IDO2 in DC in lungs and spleen, both of which are important sites of effector lymphocyte induction in response to influenza [133]. Kyn also upregulates CTLA-4 on Tregs which suppresses target cells in a contact-dependent manner [134] [129, 135]. Treg's CTLA-4 upregulates IDO expression even further in DC by ligating to DC's B7 through a positive feedback mechanism [136, 137] (Figure 2.2). Phosphorylated IDO in pDC acts as a signaling molecule by upregulating suppressive and tolerogenic genes [138]. Disrupting this cycle would be of great interest to maximize effector T cell response. Once Tregs express cytokines, TGF-β can continue to upregulate IDO in DC [139]. TGF-β synergizes with IL-2 and retinoic acid to drive differentiation of other T cells to become Treg. However, in the presence of TGF-β and IL-6, Th17 differentiation is favored [140]. pDC is

a key mediator in the production of IL-6 to promote Th17 differentiation [141, 142]. IL-6 induces the expression of Suppressor Of Cytokine Signaling factor 3 (SOCS3) which forms a complex with IDO. IDO/SOCS3 complex becomes ubiquinated and targeted for proteasomal degradation [143]. Increased IL-6 decreases IDO concentration to promote Th17. This is corroborated by the shift in Th17/Treg seen with an increase in IL-6. Lysis of IDO+ DC increases pro-inflammatory cytokines TNFα and IL-6 with a concurrent decrease in IL-10. The Th17/Treg ratio shifts towards Th17 and increases the number of virus-specific T cells [144].

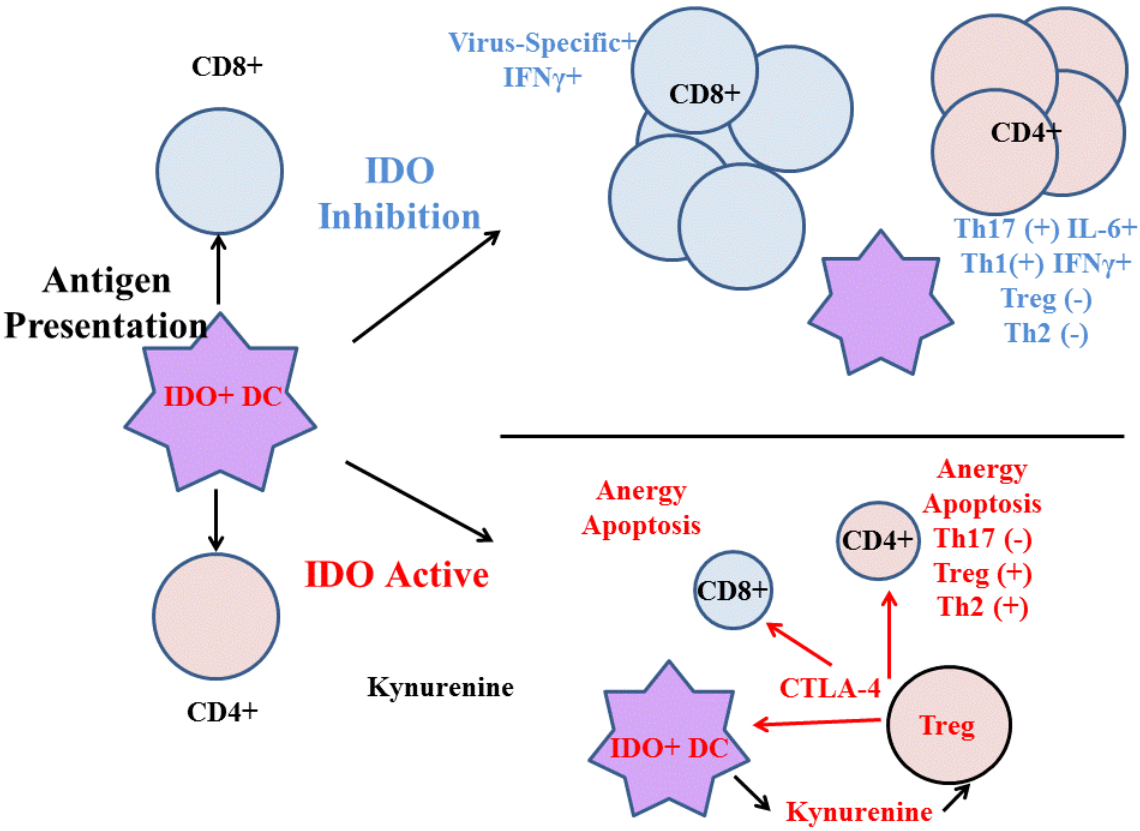


Figure 2.2 Proposed model of IDO's effect on T cells at antigen presentation

IDO expression by dendritic cells promotes anergy and apoptosis of CD8+ and CD4+ T cells during activation. CD4+ T cells are also directed to develop to Th2 and Treg over Th1 and Th17, respectively. Tregs which develop in response to kynurenine also upregulate expression of CTLA-4 which promotes further IDO expression in DC and suppress effector CD4+ and CD8+ T cell development by contact-dependent inhibition.

*Effector Function of CD4+ and CD8+ T cells*

Effector T cells undergo activation and expansion from antigen presentation and activation and will exhibit a myriad of phenotypic changes associated with their functions. These changes include shedding CD62L/L-selectin [145, 146] and CCR7 [147] which allows them to bypass secondary lymphoid organ in high endothelial venules. They also increase expression of CD44 which binds to hyaluronate, a component of the extracellular matrix [148]. Collectively, these changes allow effector T cells to home in to the infected inflammatory sites, namely the airways and lung in influenza infections [149]. These surface markers have thus been used to identify effector T cells and will be important for identifying the effect of IDO on the generation and migration pattern of these cells ($CD62L^{lo}$ $CD44^{hi}$). In addition to the ligands expressed on T cells, chemokines once again play a role in T cell trafficking. CCR5 and CXCR3 directed localization of T cells within infected tissues and regulate antigen encounter and differentiation towards memory cells [150]. Whether IDO affects chemokine mediated differentiation of effector and memory T cell response is unknown in the context of viral infections as there is no evidence either way at this time. Since IDO is upregulated in response to TLR signaling, IDO may affect the chemokine mediated T cell trafficking and memory differentiation and be a contributing factor to T cell memory development and recall. This is particularly important because influenza infection is accompanied by recruitment of non-virus specific T cells [151, 152].

Upon expansion and migration to the infection site, virus specific CD8+ T cells kill infected target cells by recognition of viral epitopes on MHC Class I on target cells by perforin/granzyme and Fas ligation [153]. This not only kills infected cells and reduce spread of virus, it also limits escape variants of influenza [154]. It is here again IDO downregulates CD8+ T cell response by decreasing production of cytolytic factors perforin and Granzyme A/B [112]. There is no evidence of IDO mediated downregulation of Fas, but IDO may function to skew the

killing mechanism towards a Fas-FasL approach which may affect the generation of escape mutants and affect virus clearance and transmission.

In addition to cytolysis, effector CD8+ T cells also produce IFNγ which supports their own functions in an autocrine manner [155]. In addition to the formation of memory Th1 cells [156], and activate macrophages to facilitate phagocytic killing. In turn, IFNγ from NK cells [157] and CD4+ T cells support memory CD8+ T cell formation [158]. Thus, CD4+ and CD8+ T cells complement each other's functions and memory cell development. During expansion in secondary lymphoid organs, CD4+ T cells also provide IL-2 [159] to drive differentiation towards a robust memory response [160] [161]. Clearly, cytokine signaling by Th1 cells is important. Th2 cells function to improve antibody quality by cytokines and CD40-CD40L interaction [162-164]. However, stimulation of CD40L on DC also increases IDO expression [40] and may affect the course of the response of remaining T cells which are activated later in the immune response. The depletion of NK cells may also affect the cytokine environment (namely IFNγ) and dampen the Th1 development. In addition to performing its helper functions by cytokine, influenza-specific CD4+ T cells also mediate direct cytotoxicity against infected target cells [164].

*Memory T Cell Generation and Recall*

Upon infection, memory T cells clear influenza virus faster by several days compared to naïve host without memory T cells [165]. Boosting the memory CD8+ T cell response can provide protection against lethal challenges [26]. Consequently, it would be of great interest to boost the memory T cell response which could be aided by IDO inhibition. Since memory T cell precursor development against influenza is largely determined by clonal burst size [166, 167], and burst size is largely influenced by early priming events, IDO inhibition would be a prime target to boost memory precursor frequency. IDO inhibition may accomplish this is by decreasing Treg

mediated suppression of effector T cells. Tregs are induced earlier in the immune response compared to effector CD4+ and CD8+ T cells [168] so suppression of early effector T cell priming and expansion may drastically affect T cell burst size [123], and consequently, the memory precursor frequency. After antigen clearance in the primary response, the memory precursors are seeded through the body as reserve, including the liver [169], spleen [170], as PBMCs, and as IELs in lungs and airways [147, 171, 172].

In addition to precursor frequency, a major issue to consider is recall and trafficking of the memory T cells. No matter how many virus specific T cells are generated, they are useless if they do not migrate to the site of infection. The trafficking pattern of responding memory cells is affected by various factors including antigen load [165], CD62L and IL-7Rα expression [173-176], the location of the memory T cell at the time of reinfection [177, 178], and the cytokine environment. The cytokines which affect recall include IL-6 for memory CD4+ T cells [91] and for CD8+ T cells, IFNγ [179, 180] and IL-15 [159]. Higher Th1 and Th17-type cytokine expression (IFNγ and IL-6, respectively) as a consequence of IDO inhibition may aid in recall of memory T cells. Finally, the size of the memory precursor pool directly affects clonal burst size like the primary response [175]. This is important because influenza virus is an antigen that is encountered several times through the life of many individuals. Therefore, the cumulative role that IDO exerts over the lifetime of the host should be considered, which brings forth the issue of Original Antigenic Sin.

*Original Antigenic Sin in B and T cells*

Antibody induction is the main objective of most influenza vaccines. However, antibodies are limited in their ability to confer heterosubtypic immunity and in some cases, even homosubtypic immunity. Antibodies do not always provide immunity, and in some cases may even hinder the production of new antibodies against other strains, a phenomenon known as Original Antigenic

Sin (OAS). OAS is characterized by the production of high affinity, neutralizing antibodies against one virus strain which cross-reacts but at a suboptimal level towards subsequent viruses encountered by the host. This loose antibody-antigen interaction may effectively hide the new virus from recognition by APCs and fail to induce high affinity, neutralizing antibody against the new virus and delay virus clearance [181].

OAS also applies to T cells in the form of peptide-MHC (pMHC) – TCR interaction. However, the dynamics are different compared to BCR to TCR. Like antibodies, TCR of different avidity to pMHC are generated against the same antigen. CD8+ T cells with high-avidity CD8+ T cells dominate by expansion in the presence of low-avidity T cells [182, 183]. In addition, the nature of the effector mechanism of T cells is such that extremely high avidity may not be advantageous but actually detrimental. Although antibodies function by being indefinitely bound to an antigen, T cells must disengage their TCR from pMHC so that they can move to the next target cell. Permanent fixture of TCR to a target cell would be detrimental to controlling a rapidly spreading viral infection.

OAS can lead to Antibody Dependent Enhancement where non-neutralizing antibodies facilitate virus entry into FcR bearing cells. T cells with low avidity TCRs may have decreased cytotoxic capacity [183-185], but would not enhance viral entry into cells. The virus would have to be inside the cells to begin with, although it may decrease virus clearance time [184]. The issue of epitope specificity and avidity's consequence on the T cell response bring to light the importance of immunodominance.

Understanding the epitope specificity of T cells to engineer a broadly protective memory lymphocyte pool is of great interest in vaccine design, particularly through LAIV and peptide vaccines [186]. This allows for a higher proportion of the population receiving vaccines to mount an equally effective response, independent of the individuals' MHC restriction. Consequently, it is of great interest to be able to manipulate the T cell epitope specificity against influenza.

*Immunopathology*

Why some infections lead to exaggerated lung pathology is determined by a combination of host and viral factors. Since alveolar macrophages are major sources of cytokines, they play important roles in the coordination of the early immune response. Alveolar macrophages are susceptible to influenza infection but are not permissive. Nevertheless, the viral protein and RNA production in these cells stimulate cytokine release through PRR stimulation. Large numbers of infected macrophage and overstimulation of PRR can lead to excessive cytokine production. In addition to resident macrophages, neutrophils also play a critical role of infection and may either exacerbate or ameliorate disease by phagocytosis and collateral damage against respiratory structures, respectively [187, 188]. The airways is a delicate environment for an immune response (reviewed in [189-191]); the immune response must be carefully controlled and coordinated to minimize immunopathology. Various factors, including virus dose [192], aerosol size at of the virus inoculum [193], and the cytokine environment such as excessive IL-6 [194-196], IL-1 [88], TNFα [196], IL-15 [196, 197], Type I and II IFN production affect pathology. This massive cytokine release is accompanied by pulmonary infiltration with monocytes and neutrophils and is often associated with pulmonary edema and alveolar-capillary damage [198]. Infection of Type II pneumocytes also exacerbate pathology [199-201] and infection of tracheal epithelial cells compromise the mucociliary escalator and bacterial clearance such as pnemococcus [202].

IDO activity during influenza infection increases pneumococcal burden as a result of increased IL-10 expression [203]. The effect that IDO has on cardiopulmonary physiology must also be considered. Kynurenine has vasodilatory effects [52] which may affect dyspnea and lung perfusion during pneumonia. In addition, the activity of IDO accompanies the production of nitric oxide, another vasodilator and antibacterial factor produced by macrophages [204]. Stimulation of iNOS activity by IFNγ and TNFα shares a common pathway like IDO, so these factors may share a common pathway in their effector mechanisms. IDO expression in lung tissues also

reduce endothelial cell apoptosis and enhance mitochondrial function by protection from oxidative stress [205]. This is likely due to IDO's use of superoxide radicals as a substrate, which protects the microenvironment from oxidative damage. [286][42]. Despite being detrimental to T cell expansion, IDO metabolites are powerful antioxidants any may aid survival of cells which are not actively dividing and be minimally affected by changes in Trp concentration [206]. Mitochondrial integrity in the immune cells has been gaining increasing attention as a major contributor to balancing immunity and regulation of innate immune cell apoptosis [65, 207] and T cells [208, 209].

Collectively, excessive cytokines, inflammation, massive cell infiltrates, and secondary bacterial pneumonia contribute to high morbidity and mortality associated with severe cases of the 1918 H1N1 [210, 211], pH1N1 [196], and HPAI H5N1 [212-214] influenza infections. The expression and context of these cytokines are highly important. Moderate levels of IFNγ ameliorates lung pathology [215, 216] and supports the Th1 and CTL responses. TNFα is another classic example of dose-dependent model of a diminishing return. High TNFα levels can develop severe lung lesions [217] and lower levels decrease gross pathology and mortality [218]. At moderate levels, TNFα can attract neutrophils, stimulate macrophage phagocytosis, and exert direct antiviral effects [219].

Before the induction of adaptive immunity, cytokine induction is solely determined by the innate immune system, provided there are no resident memory cells such as IEL in the airways. IFNγ from NK T cells [220] and macrophages are largely responsible for induction and maintenance of a Th1 response. If IDO expression overwhelms the effects of IFNγ cytokines, an exaggerated Th2 response may dominate and dampen macrophages activation. IDO activity also potentiates the Th2 response in the airways, and in turn, promotes eosinophilia [221] and exacerbate lung pathology [222]. Eosinophils also express IDO and function to maintain a Th2 response and eosinophilia while inducing apoptosis of Th1 cells through Trp deprivation [223].

Suboptimal chemokine expression early in infection may exacerbate both disease and pre-existing respiratory ailments such as asthma [97, 224]. Even subtle defects in the initial response result in decreased monocyte recruitment and induction of T cells. It is in the best interest from both a therapeutic and vaccination standpoint that a dominant but balanced Th1 response is induced. DCs in the presence of high IFN environment are less susceptible to infection and may present viral antigens in a different manner when processing antigen by virion phagocytosis compared to infection [73]. This is particularly important as IFN response accelerates recruitment of effector T cells which reduces pathology [225].

## Adaptive Immunity: Immunodominance of T cells

### *Basics and Importance of Immunodominance*

During antigen presentation, large proteins can yield a very large number of different linear peptides which can fit in the context of MHC that T cells can potentially react against. However, only a select few of these epitopes are represented, composing the immunodominant epitopes. It may be advantageous to manipulate the immunodominance hierarchy during vaccination because if the immunity is broad and directed against multiple epitopes, it decreases the chance that influenza can generate escape mutants which have mutations in all the epitopes that T cells are generated against [226]. This is similar to using multiple antibiotics against bacterial infections to minimize the risk of emergence of antibiotic resistant strains. Indeed, many peptide and DNA vaccines are designed to translate the epitope of interest to induce T cells against specific epitopes.

Vaccine's delivery can alter the hierarchy. The locale of initial antigen presentation can activate different kinds of APC. For example, if a vaccine included interferons and was administered intravenously, pDC may be more responsive to the vaccination due to their presence in lymphatics and responsiveness to interferon, compared to a subcutaneous vaccination which

would activate mDC and macrophages in the interstitium. IDO activity in these cells may shape peptide presentation and immunodominance pattern.

*CD8+ T Cell Immunodominance*

Various factors affect immunodominance and can be divided broadly into 3 categories: host MHC, host T cells, and the virology of the infecting influenza virus. Host MHC first and foremost dictates what peptides can be presented, because no matter what peptides are available, it must fit in one of the available MHC haplotype. In response to influenza infection, C57BL/6 mice has 22 high affinity epitopes for presentation on H2-$D_b$ and H2-$K_b$. However, only six compromise a significant part of the CTL response against influenza X31/PR8 [227]. Of these, three were the most prominent [227, 228]: $NP_{366–374}$, H2-$D_b$ [229, 230]; $PA_{224–233}$, H2-$D_b$ [231]; and $PB1_{703–711}$, H2-$K_b$ [232]. Peptide affinity for MHC drives immunodominance [233] but beyond a certain threshold of affinity, it does not significantly impact the CD8+ T cell response [234]. The peptide is prepared by the host antigen processing system. Anything from cross presentation [235] to immunoproteasome processing impacts immunodominance [236] [234]. In response to proinflammatory factors such as IFNγ, APCs increase the expression of immunoproteasomes [237]. The composition of the proteasome complex can modify the ratio of immunodominant $NP_{366}$ to the subdominant PB1-$F2_{62}$. Therefore, the inflammatory environment and IFNγ indirectly modifies immunodominance [238]. The role that IDO plays to shape immunodominance is unknown. Few changes in the peptide sequence of the epitope outside the key residues can still fit in the MHC and activate the TCR. A low-avidity TCR-MHC contact may also result in shorter contact time between the T cell and target cell. This may be beneficial in that the T cell can perform its effector function: i.e., “peel off” the target cell, and move on to the next target cell [239].

In this way, T cells themselves may also shape immunodominance through their TCR. TCR is relatively stable and is not easily manipulated across different infections and vaccines [240] as the immunodominance pattern must conform to compatible MHC [241]. Lacking high pTCR-MHC interactions for some immune response lead to suboptimal CD8+ T cell responses [242]. Low pTCR-MHC avidity have various effects, including decreased cytokines including IFNγ [243], TNFα [243], and IL-2. In addition to TCR avidity, germline TCR repertoire also shapes immunodominance [244] [245]. In immunodominant CD8+ T cells, the Vβ region within the CDR3 loop drives selection and expansion of $NP_{366}$ specific T cells [246]. In contrast, TCR usage is flexible and CDR3 repertoire against $PA_{244}$ is much more diverse [247]. The diversity of the TCR constituents in subdominant CD8+ T cells may be a form of functional compensation as subdominant T cells exhibit higher expression of granzyme A [248] and thus may exhibit higher cytotoxicity. CTLs against the dominant epitopes $NP_{366}$ and $PA_{224}$ exhibit comparable levels of cytotoxicity on a per-cell basis [249], but subdominant epitope specific cells may have higher cytotoxic capacity. The cytotoxicity of these T cells also influence APCs infiltrating the lymphoid organs and influence the generation of CD8+ T cells that develop later [250]. For example, $NP_{366}$ specific CD8+ T cells dominates the response in the primary phase but subdominant epitopes become more prevalent in the late memory response, namely $K^b$ $PB1_{703}$, $D^b$ $PB1\text{-}F2_{62}$, and $K^b$ $NS2_{114}$ [251] [252]. This tempo of antigen presentation and activation is heavily influenced by the nature of the virus.

The virology of the influenza also contributes to immunodominance. Particularly, the replication pattern affects the antigen dose [243, 253] and stability [236]. The replication pattern of influenza is such that potential peptides compatible with available MHC are present in unequal stoichiometric ratios, so generally the most abundant epitopes would have the greatest chance to be loaded on to MHC and gain a greater chance for being immunodominant [253]. Practically, an attenuated vaccine may induce a broader immunodominance than a natural challenge [254] and

affect the memory precursor formation from vaccination [255]. Some mutations in the virus may decrease the avidity with the TCR to the extent that the recall response is compromised [233], especially if the mutation is at a TCR contact residue [256]. Escape mutants may evade antibody and T cell responses delay virus clearance and exacerbate disease [257]. This brings up the consequences of immunodominance in primary and memory cells.

The immunodominance pattern in the primary versus secondary response varies, particularly with the ratio of $PA_{224}$ to $NP_{366}$ specific CD8+ T cells [235, 250, 258]. The numbers are approximately equal in a primary challenge, but $NP_{366}$ becomes dominant in the secondary response [235]. Antigen presentation [259], cytokine responses, and other factors which affect immunodominance are different in secondary responses and must be taken into account for vaccination of individuals with history of exposure to influenza. Cells which are infected and the APCs presenting antigens affect this response. For example, $PA_{224}$ seems to be presented primarily by influenza infected DC in the primary response, whereas it is presented by infected epithelial cells in the secondary response [250]. In contrast, $NP_{366}$ seems to be effectively expressed by both dendritic and non-dendritic cells in both primary and secondary challenges [235]. Antigen presentation may be a critical point where IDO could be driving the activation of specific CD8+ T cell clonotypes. These factors become especially important in vaccine designs as some vaccine formulations may skew immunodominance towards specific epitopes [260]. For example, heat-aggregated/inactivated influenza particles exhibits different patterns compared to infections [254].

*CD4+ T Cell Immunodominance*

Immunodominance exists in CD4+ T cells as well, but the dynamic is less understood compared to CD8+ T cells. It is known that epitope specificity is generally broader than against CD8+ T cells and that various clonotypes are generated against various epitopes derived from HA [261].

Nevertheless, the hierarchy is established by various factors, including availability of the HA peptides generated by proteasome cleavage. Peptide stability of HA is largely restricted to conserved C and N-terminal flanks and constitute a large proportion of the peptides presented [262]. In addition, TCR repertoire of the precursor CD4+ T cells against HA is a contributing factor [263]. Thus, there may be overlapping factors of varying importance which drive immunodominance in CD4+ and CD8+ T cells. Their specificity is important to investigate as CD4+ T cells provide a critical function in the clearance of influenza and the formation of memory lymphocytes. Novel influenza strains such as pH1N1 are cleared by CD4+ T cells when they escape CD8+ T cell recognition [264] and may confer protection when other factors fail to cross-react. Now that the possible role of IDO has been integrated into the immunity against influenza, the last section will briefly discuss the role of IDO in the primary response.

**Releasing the Brake: IDO's Function in the Primary T Cell Response to Influenza**

This section will summarize the findings of the effects of IDO on the primary immune response to influenza [265] and how it pertains to the main body of the dissertation. IDO was inhibited by 1-MT in C57BL/6 mice during influenza infection. The endpoints measured were: Kyn/Trp ratio to assess IDO activity during infection; virus clearance in the lungs; and analysis of the immune cells responding to infection. Flow cytometry was used to assess the populations of: functional CTL (CD8+ granzyme B+ IFNγ+) [266]; Th1 (CD4+ IFNγ+); Th2 (CD4+ IL-4+); Th17 (CD4+ IL-6+); effector T cells ($CD44^{hi}$ $CD62L^{lo}$); and the TCRVβ usage of CD8+ T cells.

IDO inhibition results in increased T cell numbers with different cytokine profiles. Specifically, Th1 and Th17 responses were higher but Th2 were unaffected. CD8+ T cells were also modified: higher numbers of virus specific CD8+ T cells infiltrated the airways and had a broader immunodominance profile (i.e., higher proportion of CD8+ T cells reactive against subdominant epitopes). In addition to being virus specific, IFNγ and granzyme B expression of

the virus specific CD8+ T cells. Surprisingly, these changes did not correlate with faster virus clearance. The higher cell number observed may be due to increased burst size due to reduced level of apoptosis of the T cells which may affect memory formation [166]. In other words, CD8+ T cells which would otherwise undergo apoptosis were rescued by IDO inhibition (Figure 2.3). Changes in the CD4+ profile could be attributed to IDO inhibition in DC which enhanced Type 1 cytokine production such as IL-12, leading to bias towards Th1.

In addition, Treg suppression could be decreased by IDO inhibition. There was a lower number of Tregs expressing granzyme B in 1MT treated mice (unpublished observation), suggesting that IDO upregulates Treg suppression in a contact-dependent manner. IDO is not known to affect Treg granzyme expression and may be a novel way that IDO upregulates Treg suppression. This could be the mechanism for modification of the immunodominance hierarchy [131]. Further investigation is needed to better understanding of both the relationship between influenza, IDO, effector T cells, Tregs, and memory cells.

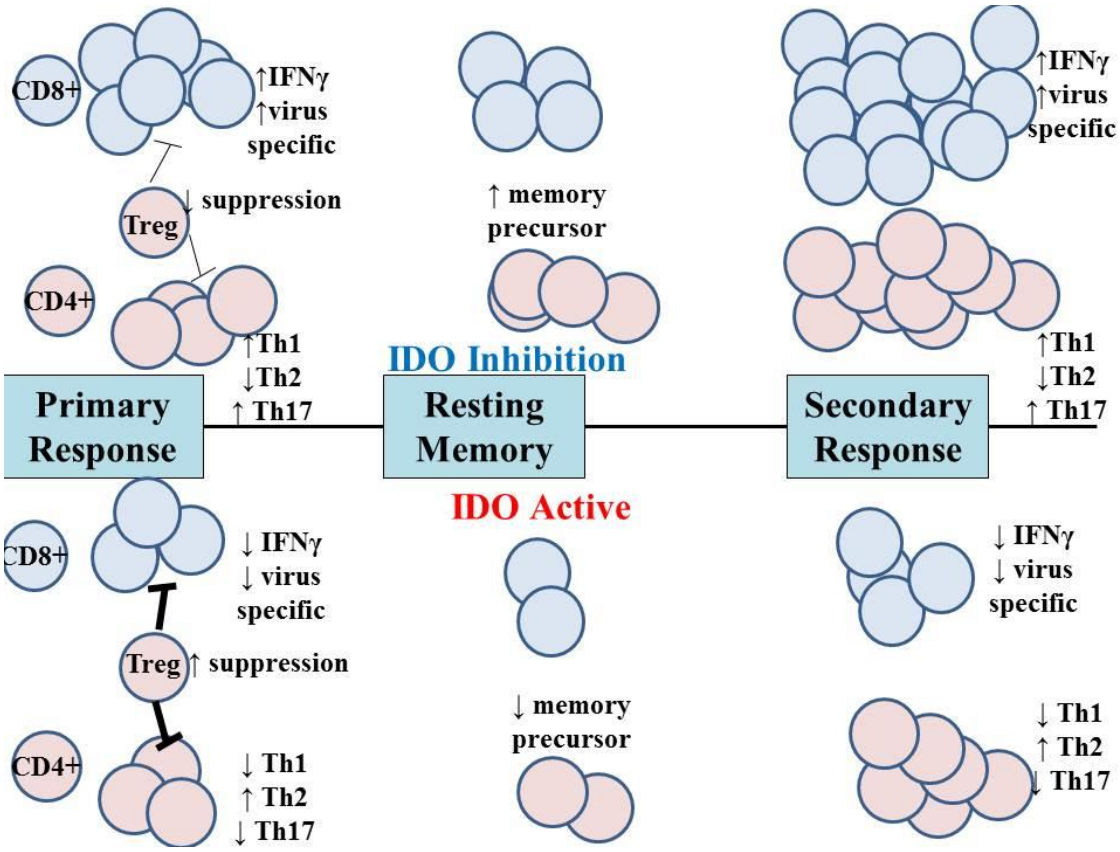


Figure 2.3: Proposed model of IDO's effect on memory T cell formation

IDO inhibition increases Th1 and Th17 while decreasing Th2 and Treg generation which suppresses effector CD4+ and CD8+ T cell generation. This would increase memory precursor formation, and ultimately increased response upon secondary challenge with influenza. The increase in frequency of virus-specific precursors will also result in a higher proportion of virus-specific CD8+ T cell response upon secondary challenge. These changes would be supported by a combination of increased IFNγ, IL-6, and decreased Treg suppression.

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# CHAPTER 3

# IDO ACTIVITY DURING THE PRIMARY IMMUNE RESPONSE TO INFLUENZA INFECTION MODIFIES THE MEMORY T CELL RESPONSE TO CHALLENGE[1]

[1] Sage, L. K., Fox, J. M., Mellor, A. L., Tompkins, S. M., and Tripp, R. A. Submitted to *Viral Immunology*, 10/04/2013.

## Abstract

The generation of heterosubtypic CD8+ T cell responses is important for cross-protective immunity against unrelated strains of influenza virus. Influenza virus infection and the interferon response to infection induce a tryptophan catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO). IDO-mediated depletion of tryptophan diminishes T cell responses. In this study, IDO activity was pharmacologically inhibited with 1-methyl-tryptophan (1MT) during the primary response to influenza virus infection and its effect on the memory T cell memory response was evaluated. 1MT treatment enhanced the CD4+ and CD8+ memory T cell response by increasing the Th1 response and shifted the immunodominance hierarchy to overrepresent subdominant CD8+ T cell epitopes, a feature which may be linked to decreased regulatory T cell function. These changes also accompanied evidence of accelerated lung tissue repair upon virus challenge. These findings suggest that modulation of IDO activity in the context of influenza infection could be exploited in influenza vaccine development to enhance memory T cell responses and reduce disease burden.


## Introduction

Influenza A virus is a worldwide health threat causing seasonal morbidity and mortality [1]. Emerging strains of influenza continually threaten as exemplified by the recent H1N1 influenza pandemic [2], producing illness in at-risk populations [3]. Vaccination can diminish influenza transmission and disease severity although strain-specific antibodies do not provide adequate protection against other strains and is heavily reliant on both memory CD4+ and CD8+ T cells for immunity against novel strains by recognition of conserved viral epitopes (heterologous immunity) [4, 5]. Thus, inducing a robust memory T cell response is important for optimal protection.

Indoleamine 2,3-dioxygenase (IDO) is an immunomodulatory enzyme expressed by antigen-presenting-cells (APCs) in response to proinflammatory mediators such as interferons (IFN) and TNF-α [6-9]. IDO modifies the immune response to several pathogens including HIV [10-12]. APCs including plasmacytoid dendritic cells (pDC) express IDO and induce anergy in effector T cells [13] by depletion of tryptophan (Trp) and production of metabolites such as kynurenine (Kyn) [14], leading to activation of the GCN2 kinase pathway [9] and simultaneously upregulate regulatory T cells (Treg) [15, 16]. IDO also alters the cytokine environment during activation of T cells promoting a Th2 over Th1 [17]. Influenza infection induces IDO [18] which may affect T cell priming and differentiation [19]. Thus, inhibition of IDO results in enhancing the primary T cell response to influenza [20] but its impact on memory T cell response has not yet been evaluated; the hypothesis that IDO also enhances the memory T cell response against heterologous infection was evaluated in this study. IDO inhibition by D,L-1-methyl-tryptophan (1MT) resulted in a heightened memory response characterized by higher IFNγ expression by CD4+ and CD8+ T cells and broadening CD8+ epitope specificity yet not compromising response against immunodominant epitopes.

## Results

*1-methyl-tryptophan reduces IDO activity*

To determine IDO activity in lungs during the primary response to influenza (X31) infection, the ratio of IDO's product to substrate was measured in lung homogenates of infected mice (Figure 3.1A). Where IDO is active, the product [Kyn] to substrate [Trp] ratio increases [21]. X31 infection induced peak IDO activity between days 8 and 12 post-infection (pi), where significant ($p=0.01$) difference between 1MT-treated and control mice was evident at day 10 pi. Lung virus titers were not substantially affected by IDO inhibition (Figure 3.1B) and consistent with virus clearance occurring at day 8 pi (Figure 3.1B) [22] and replicates the condition established to

modify the primary T cell response [20]. The remainder of the Results outlines the impact of these changes on the memory T cell response (secondary challenge with PR8).

*Inhibition of IDO activity increases memory Th1 response*

To assess the effect of IDO inhibition on the memory T cell response to influenza, X31 primed mice treated with 1MT or control were challenged with PR8 28 days later. The total number of CD4+ and CD8+ effector ($CD62L^{lo}$ $CD44^{hi}$) T cells in the BAL and MLN were determined at days 0, 1, 3, 5, 7 and 9 pi with PR8 (Table 3.1). Peak effector CD4+ T cell response in the BAL occurred between day 5 and 7 pi in the control and 1MT group, respectively and at day 5 pi for both groups in the MLN. Effector CD8+ T cell in MLN peaked at day 5 and 7 pi in 1MT and control groups, respectively. BAL also had higher (though not statistically significant) numbers at day 5 pi in the 1MT group. There were no significant differences between the groups with the raw number of effector T cell counts, so specific subsets of both CD4+ and CD8+ T cells were evaluated at the peak response times to assess if IDO has an effect on specific parameters of the memory T cell response.

IDO inhibition has been associated with a greater Th1 (IFNγ+) over Th2 (IL-4+) response [17]. Given the importance of Th1 in the response against influenza [23], and IDO inhibition modifies both CD4+ and CD8+ response in a primary (X31) response to influenza [20], the memory CD4+ T cells at day 5 and 7 pi were dissected to see if IDO affected this axis of Th1/Th2. The frequency of Th1 was significantly ($p$=0.04) higher in BAL from 1MT-treatment to controls at day 5 pi (Figure 3.2A). Th2 response in the BAL was equivalent between the groups (Figure 3.2B), suggesting that IDO inhibition during priming promotes memory Th1.

*IDO inhibition shifts CD8+ T cell epitope specificity and maintains dynamic virus-specific response*

Since IDO inhibition affected CD4+ T cells, CD8+ T cells were examined next. 1MT treatment resulted in higher numbers of memory CD8+IFNγ+ T cells in BAL compared to controls upon PR8 challenge, ($p$=0.07, 0.09 at days 5 and 9 pi, respectively) (Figure 3.3A). However, when virus specificity is taken into account, significant differences in immunodominance ($NP_{366-374}$>$PA_{224-233}$>$PB1_{703-711}$) [24] emerged (Figure 3.3B). The response was equivalent at day 5, but at day 7, there was a decrease in the fraction of CD8+ T cells reactive to PA ($p$=0.03) and NP, but an increase in reactivity against the subdominant epitope PB1 ($p$<0.01). These difference may be small (few percent), but since the response towards subdominant epitopes is normally reduced following challenge [24] (<10%), the changes resulted in a two-fold change in the overall % of CD8+ specificity and is reflective of the changes seen in the primary response due to IDO inhibition [20]; IDO activity during influenza priming affects immunodominance of memory CD8+ T cells at day 7.

To determine if differences in immunodominance were linked to kinetics, the number of CD8+ IFNγ+ T cells against NP, PA, and PB1 was determined at day 5 pi onwards (Figure 3.3 C). NP-specific cells in the BAL of 1MT-treated mice were significantly higher at day 5, 7, and 9 pi ($p$<0.05 for all days) compared to controls. The late resurgence of NP-specific cells at day 9 pi in 1MT treated mice may reflect a second memory T cell population which migrated to the airways, although no substantial differences were detected in the MLN. PA-specific cells in the MLN at day 5 pi ($p$<0.05), and BAL at day 9 pi ($p$=0.06) were higher in 1MT treated mice compared to controls. PB1-specific cells were also over-represented at day 7 pi in BAL ($p$=0.02), and remained higher through day 9 pi in 1MT-treated mice. MLN were also higher at day 7 pi ($p$=0.07), through day 9 ($p$<0.001). The ratio of NP to PA-specific cells were lower in 1MT

group BAL ($p$=0.09; Figure 3.3D) at day 5 and 7 pi, as were NP to PB1-specific cells in both MLN ($p$=0.07) and BAL ($p$=0.09) at day 7 pi.

*IDO inhibition decreases CTLA-4 expression on Tregs*

IDO activity is influenced by Tregs [15, 16, 25]. The number of CD4+/Foxp3+ (Tregs) responding to PR8 challenge was not changed by 1MT treatment (data not shown), so again, these T cells were further analyzed for specific parameters (Granzyme B and CTLA-4) which may be affected by IDO [26]. Fewer proportion of Tregs from 1MT-treatment expressed CTLA-4 ($p$<0.05) (Figure 3.4A) upon virus antigen stimulation, a feature also reflected by CTLA-4 expression on a per-cell basis (MFI; $p$=0.05) (Figure 3.4B). Thus, 1MT results in fewer Tregs expressing CTLA-4, and those that do express it is to a lower degree. No difference in Granzyme B expression was evident (data not shown).

*IDO inhibition accelerates lung tissue repair following challenge*

As enhanced memory T cell responses followed IDO inhibition, its effect on pathology and virus clearance was evaluated. Evaluation of overall, gross pathology at days 5 and 7 pi showed no substantial differences due to 1MT treatment (Figure 3.5A) but specific parameters associated with influenza pathology was affected which may be attributed to the cytokine environment afforded by increased Th1 (IFNγ) response from 1MT-treatment (Figure 3.2). At day 5 pi, control mice's lungs had increased fibrin deposition in the alveoli with substantial neutrophil involvement in bronchioles which can delay tissue repair [27] and at day 7, control mice lungs had increased necrosis, alveolar exudate, and neutrophil recruitment. Excessive fibrin deposition results from the alveolar capillaries to control exudates. Since IFNγ regulates fibrinolysis [28], 1MT treatment may promote a balanced lung repair. Decreased necrosis and

neutrophil recruitment also promote tissue repair, possibly due to IFNγ favoring additional lymphocytes over neutrophil recruitment.

To determine if virus load was also affected by IDO, virus levels in lungs were measured but no differences in infectious virion (Figure 3.5B) or antigen (IHC: data not shown) were evident, which was unexpected but corresponds the equivalent cytotoxicity of CD8+ T cells. These findings indicate that although 1MT treatment is associated with an enhanced memory CD8+ T cell response, the outcome is not associated with detectable differences in virus clearance but may promote tissue repair post-challenge.

## Discussion

Inhibition of IDO activity during the primary response to influenza infection modifies aspects of the memory T cell response to secondary influenza challenge. These changes are characterized by higher numbers of memory Th1 cells, activated virus-specific CD8+ T cells, and pathological parameters associated with repair. These changes may be explained in part by an increased precursor frequency and Th1 (IFNγ) response seen in a primary response due to IDO inhibition [20]. IFNγ promotes T cells which are virus-specific and IFNγ+ [29], and is particularly important as influenza infection recruits a large number of non-specific T cells [30]. This was seen when IDO inhibition resulted in a larger number of virus-specific, $IFN\gamma^+$ CD8+ T cells (Figure 3.3C). Although NP and PA-specific CD8+ T cells still dominate the response, the increase in PB1-specific CD8+ T cells is remarkable and these incremental changes are consistent with broadened epitope specificity and may be a favorable feature for vaccination to promote heterologous immunity [31]. Although there were no significant differences in overall CD4+ and CD8+ T cell numbers (Table 3.1), scrutiny of T cells against antiviral parameters (IFNγ and virus-specificity) revealed profound differences in CD4+ and CD8+ T cells due to IDO activity. Collectively, the changes in the antiviral CTL response suggested that IDO may affect expansion

of CTLs against antigen *in vivo* and have a higher specific killing ability. Thus, the %-specific lysis by memory CD8+ T cell derived from MLN and spleen was assessed in infected target cells, but CTL cytotoxicity was equivalent (data not shown).

It is possible that while the number and epitope specificity of the T cells were different, the ability of individual CD8+ T cells to kill target cells is not affected by IDO. It may be the case that the memory T cell response with or without IDO is very robust to begin with, and thus a threshold is reached where beyond it, even large changes in the immunodominance profile or the higher number of IFNγ+ CTLs does not make a significant difference in the capacity to clear PR8. This is evidenced by the fact that PR8 is already cleared by day 5 (Figure 3.5B), and differences in CD4+ and CD8+ T cells is seen at day 5 onwards. If a different influenza challenge strain which persists longer (day 7 or longer), differences in cytotoxicity and virus clearance may have been more clear. In addition, influenza strains which persist longer in the airways have more opportunity for generating escape mutants which would be better fought off with an immune response with a broadened CD8+ T cell repertoire. In addition to altering the memory T cell response, 1MT-treatment mice had lower pulmonary neutrophil infiltrates which may exacerbate pathology [32] and higher Th1 response may have contributed to the accelerated tissue repair, as Th2 responses promote eosinophilia [33] and occlude airways [23].

These changes may be attributed to priming of memory T cells as early as virus recognition by resident APCs in the airways [34] and stimulation of some TLR which upregulates IDO [35, 36] and is a critical site for IDO inhibition for applications such as vaccination. APCs expressing IDO have been shown to be involved in the regulation of T cell activation, differentiation, and expansion [15]. Tregs are upregulated in response to IDO, and in turn, further upregulate IDO activity in DC [15, 16, 25] by various modes such as CTLA-4 [37] which disrupt virus-specific effector T cell function [38], as well as PD-1 [39] and TGF-β [38] in the context of influenza infections. It is possible that IDO modifies one or more of these pathways during

influenza infections and makes experimenting with IDO inhibitors in influenza vaccines an appealing prospect.

## Materials and Methods

*Influenza, mouse, and IDO inhibition*

Influenza A strains X31 (H3N2; A/Aichi/2/1968 x A/Puerto Rico/8/1934) and PR8 (H1N1; A/Puerto Rico/8/1934) were propagated in 9-day old embryonated chicken eggs and their allantoic fluids were collected and stored at -80˚C until use. Virus titers were determined by plaque assay using MDCK [40]. Eight-to-ten week old female C57BL/6 mice (Charles River, Wilmington, MA) were anesthetized using 2,2,2-tribromoethanol [41] and intranasally (i.n.) infected with $10^3$ plaque forming units (PFU) of X31 in 50μL PBS. IDO was inhibited by oral administration of D,L-1-methyl-tryptophan (Sigma-Aldrich, St. Louis, MO) in drinking water (2mg/mL with 2mg/mL of aspartame) during the primary T cell response [20, 42]. Aspartame was added to increase palatability or used alone in the control group. Both solutions were filter-sterilized and provided to cohorts of mice *ad libitum* 3 days before through 14 days after X31 infection and replaced with a fresh solution every 5 days. 28 days later, mice were i.n. challenged with 10 $LD_{50}$ of PR8 ($10^3$ PFU). 1MT was not administered during secondary challenge. All animal work was approved by Institutional Animal Care and Use Committee of the University of Georgia.

*Cell preparation and flow cytometry*

At various time-points post-PR8 challenge, mice were euthanized, and cells in the airways was collected by BAL. Single cell suspensions were also prepared from the mediastinal lymph nodes (MLN) in PBS following passage through 100μm cell-strainers (BD Biosciences, San Jose, CA). Cell numbers from the tissue samples were enumerated using a Z2-Coulter-Counter (Beckman-Coulter, Brea, CA). These cells were immunophenotyped as previously

described [43]. Cells were stained with antibodies against CD8, CD4, CD62L, CD44 (BD Biosciences), in combination with MHC-Class I Tetramers (Emory University, Atlanta, GA) loaded with influenza peptides: $NP_{366-374}$: ASNENMETM (H-$2D^b$); $PA_{224-233}$: SSLENFRAYV (H-$2D^b$); and $PB1_{703-711}$: SSYRRPVGI (H-$2K^b$). Intracellular IFNγ-staining was performed as previously described [34]. CD4 or CD8 T cells were analyzed by flow cytometry using a BD LSR-II (BD Biosciences) where at least 50,000 events were recorded following gating on T cells (BD FACSDiva, BD Biosciences).

*High Pressure Liquid Chromatography (HPLC)*

HPLC was used to determine IDO activity by measuring the concentration of Trp and Kyn in clarified lung homogenates on a 4.6x50mm reverse-phase C18 column (Restek, Bellefonte, PA) as described [44].

*Influenza Virus Titer by $TCID_{50}$*

Virus titer was measured by $TCID_{50}$ [45]. Briefly, extracted lungs were homogenized using a tissue-lyser (Eppendorf, Hamburg, Germany). The supernatant of centrifuged lysate was diluted in MEM (HyClone) containing 100 μg/mL of streptomycin, 100 IU/mL of penicillin, 250ng/mL of amphotericin B (Mediatech), and 1μg/mL of TPCK-Trypsin (Worthington, Lakewood, NJ) in 96-well plate (Corning) over MDCK cells grown in DMEM (HyClone) with 5% FBS. The plates were incubated for 72 hours and mixed with equal volume of 0.5% chicken erythrocytes in PBS and incubated for one hour and scored for agglutination patterns.

*In Vitro CTL Restimulation and Killing Assay*

Memory T cells generated following X31 infection were expanded *in vitro* [46]. Briefly, spleen and MLN-derived memory T cells were stimulated *in vitro* with syngeneic splenocytes

infected with 1000HAU/mL of X31 and mitotically inactivation with Mitomycin C (Sigma-Aldrich) [47]. Lymphocytes were restimulated for 6 days in RPMI-1640 with 10% FBS, antibiotics, 50μM β-mercaptoethanol (Sigma), and 20U/mL of mouse IL-2. Expanded T cells were co-incubated at various effector-to-target ratios with H-2b-restricted MC57G target cells infected with 100-HAU of PR8 overnight. Target cells were stained with PKH67 (Sigma-Aldrich). CTL and target cells were coincubated 37°C for 4 hours in 96-well V-bottom plates (Corning) and gently centrifuged (200xG for 1 minute) to maximize cell contact. Cell cytotoxicity was analyzed by flow cytometry: MC57G (PKH67+) were gated and assessed for apoptosis as defined by binding of 7AAD+ and/or Annexin V+ but not 7AAD alone [48].

*Histopathology and Immunohistochemistry*

Lungs from infected mice were perfused, inflated, and fixed in 10% neutral buffered formalin (Fisher) followed by paraffinizaiton and sectioned to 5μm [45]. H&E stained sections were evaluated for gross pathology as well as remarkable pathological features as well immunohistochemistry (IHC) by a pathologist blinded to the lung sections' group assignment [49].

*Regulatory T cell Stimulation*

Treg-mediated suppression of influenza-specific CD8+ T cell proliferation was evaluated [50]. Two days after PR8 challenge, spleens and MLN from 1MT or control mice were removed and enriched for Tregs using a Treg-Isolation-Kit (Miltenyi Biotec, Auburn, CA). Tregs from age-matched naïve mice were used to address non-specific suppressive activities. CD8+ T cells were negatively selected from spleens of X31-immune mice without 1MT treatment. The purity of enriched CD8+ T cells and Tregs was >90% by flow cytometry. Enriched Tregs were co-incubated with CD8+ T cells at specified ratios in the presence of X31-infected stimulator cells as

described for *in vitro* restimulation of CTL assay. As a positive control, 2µg/mL concanavalin A (Sigma) was added to a culture of CD8+ T cells only (no Treg). To evaluate Treg activity, 48h after co-incubation with CD8+ T cells, 10µM of EdU (5-ethynyl-2´-deoxyuridine, Invitrogen) was added for 2 hours, and the level of proliferation as determined by EdU incorporation determined for tetramer+ CD8+ T cells. The Tregs from the co-culture were also immunophenotyped by flow cytometry against intracellular Granzyme-B (eBioscience) and surface CTLA-4 (eBioscience). Differences in the rate of CD8+ T cell proliferation in co-cultures with Tregs from the cohorts were used to measure Treg suppression.

*Statistics*

Statistical significance ($p$ value$\leq$0.05) was tested between means of 1MT treated mice and controls using a Student's *t*-test. Exact *p*-values are listed when significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

## Tables and Figures

**Table 3.1**

| Day Post Challenge | Effector CD4+ T Cell Number ± SEM (x1000) | | | |
|---|---|---|---|---|
| | 1MT BAL | Control BAL | 1MT MLN | Control MLN |
| 0 | 1 ± 0 | 2 ± 0 | 151 ± 4 | 169 ± 17 |
| 1 | 5 ± 1 | 4 ± 1 | 169 ± 25 | 181 ± 45 |
| 3 | 11 ± 4 | 9 ± 5 | 263 ± 103 | 208 ± 44 |
| 5 | 73 ± 12 | 48 ± 20 | 602 ± 166 | 496 ± 0 |
| 7 | 50 ± 6 | 84 ± 41 | 486 ± 88 | 473 ± 99 |
| 9 | 32 ± 5 | 33 ± 7 | 354 ± 115 | 287 ± 30 |

| Day Post Challenge | Effector CD8+ T Cell Number ± SEM (x1000) | | | |
|---|---|---|---|---|
| | 1MT BAL | Control BAL | 1MT MLN | Control MLN |
| 0 | 3 ± 1 | 8 ± 2 | 23 ± 1 | 21 ± 4 |
| 1 | 13 ± 3 | 11 ± 3 | 26 ± 3 | 23 ± 1 |
| 3 | 12 ± 3 | 8 ± 3 | 46 ± 17 | 42 ± 9 |
| 5 | 289 ± 120 | 177 ± 69 | 411 ± 82 | 370 ± 0 |
| 7 | 383 ± 72 | 480 ± 223 | 339 ± 85 | 386 ± 81 |
| 9 | 346 ± 51 | 363 ± 51 | 308 ± 83 | 242 ± 53 |

**Table 3.1: Effector T cell Response to Heterologous Influenza Challenge.** Numbers of T cells exhibiting effector phenotype ($CD44^{hi}$ $CD62L^{lo}$) present in airways (BAL) and MLN at day 0 (before challenge) and through day 9 challenge with PR8 of X31-immue mice treated with vehicle or 1MT. Numbers are the average cell numbers ± SEM. Data is representative of three independent experiments.

**Figure 3.1**

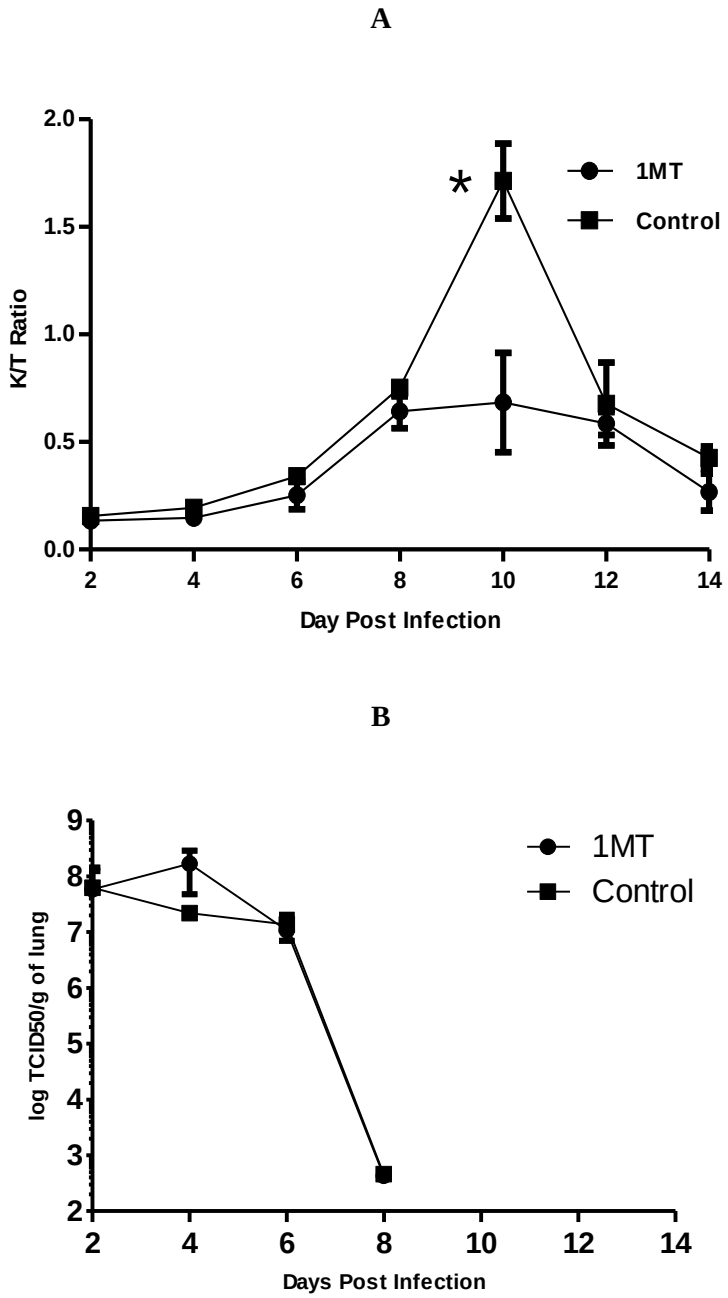
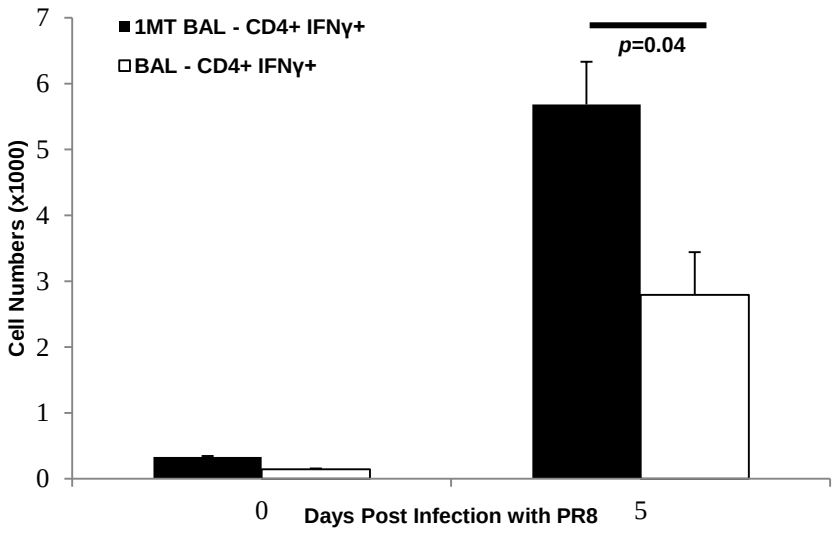



**Figure 3.1: 1-methyl-tryptophan (1MT) decreases IDO activity induced by influenza virus infection** A: [Kyn]/[Trp] ratios ± SEM in lung homogenate was measured using HPLC at the indicated time points post X31 primary infection in mice treated with 1MT (circles) or vehicle control (squared). Data is representative of two independent experiments. Asterisk indicates

statistical significance ($p$=0.01). B: X31 virus titer (log $TCID_{50}$) during the primary infection at which the [Kyn]/[Trp] ratios were assessed. Data is representative of three independent experiments ($n$=3 per experimental group).

**Figure 3.2**

**A**




**B**

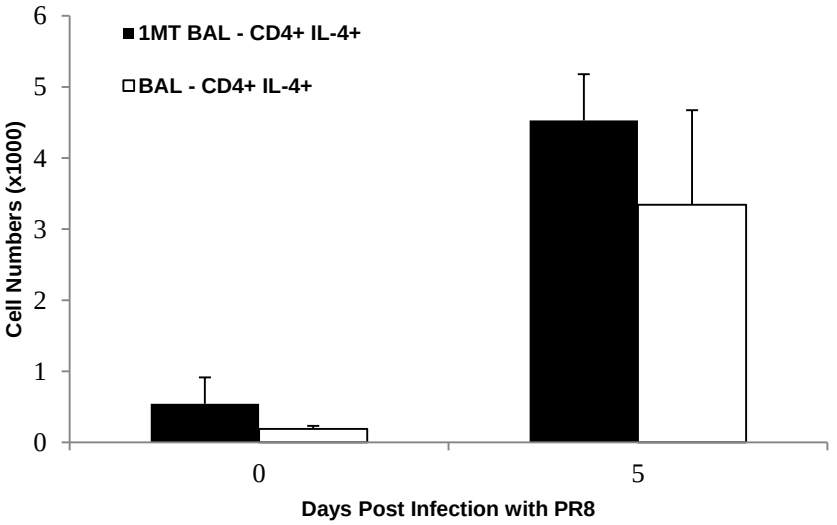
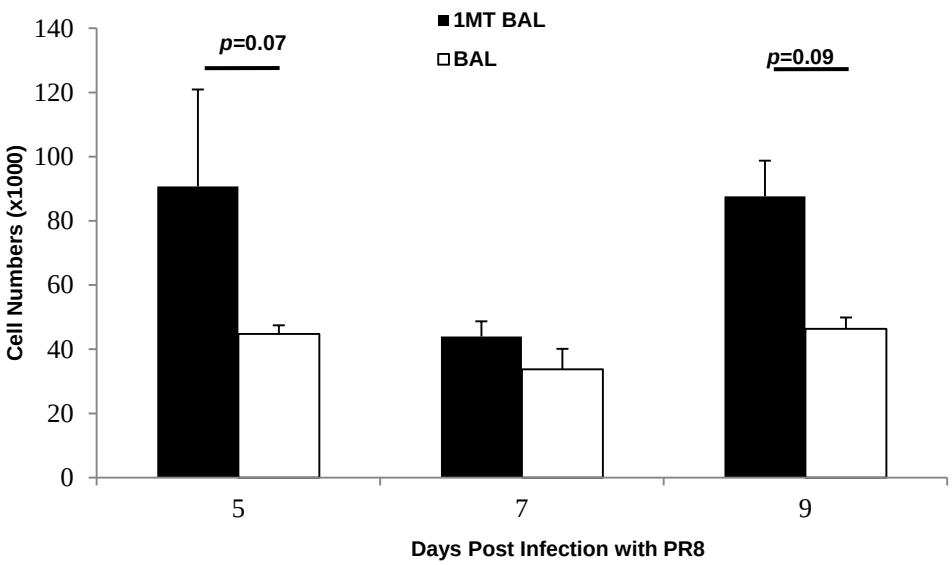
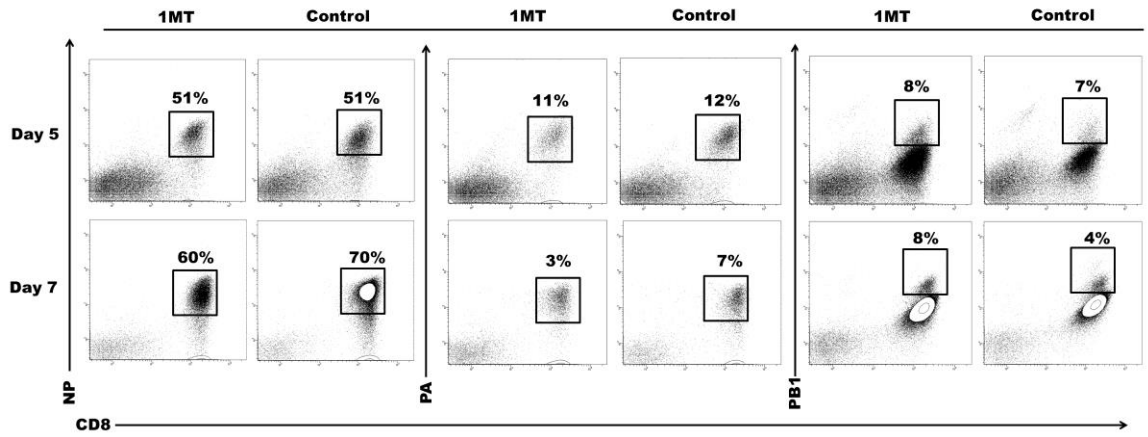


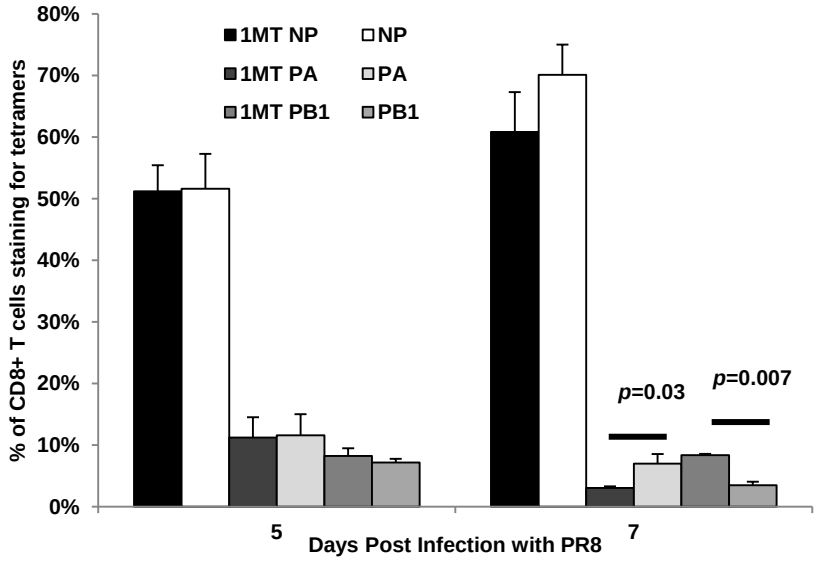

**Figure 3.2: IDO inhibition during primary response increases memory Th1 response to secondary challenge.** Kinetics of CD4+ T cells expressing IFN-γ (A) and IL-4 (B) cells in the airway (BAL) following secondary/PR8 challenge. Numbers are average cell numbers ± SEM. Data is representative of two independent experiments ($n$=3 per experimental group).

**Figure 3.3**
**A**

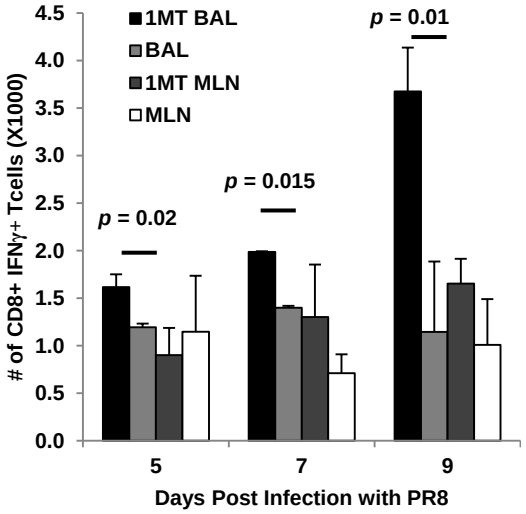

1MT BAL
BAL
p=0.07
p=0.09
140
120
100
80
60
40
20
0
Cell Numbers (x1000)
5
7
9
Days Post Infection with PR8

**B**

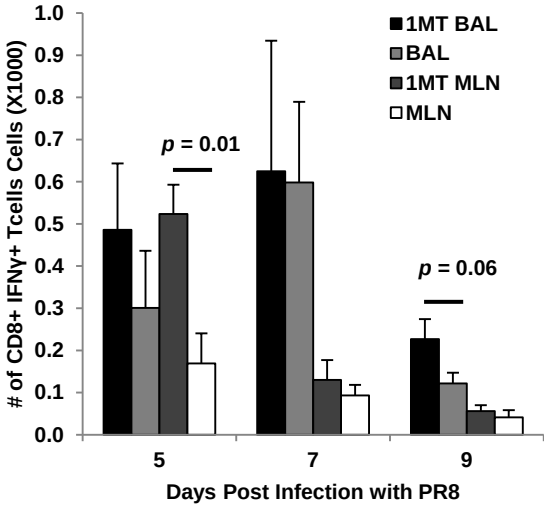

1MT
Control
1MT
Control
1MT
Control
Day 5
Day 7
51%
51%
11%
12%
8%
7%
60%
70%
3%
7%
8%
4%
NP
PA
PB1
CD8

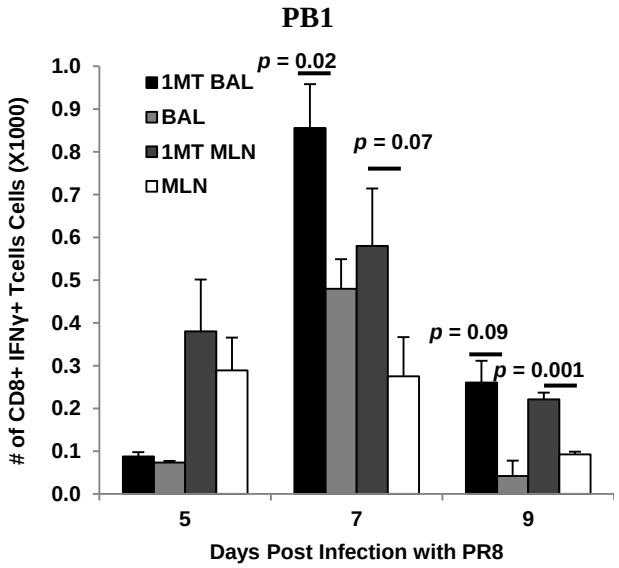

80%
70%
60%
50%
40%
30%
20%
10%
0%
% of CD8+ T cells staining for tetramers
1MT NP
NP
1MT PA
PA
1MT PB1
PB1
p=0.03
p=0.007
5
7
Days Post Infection with PR8

**NP**

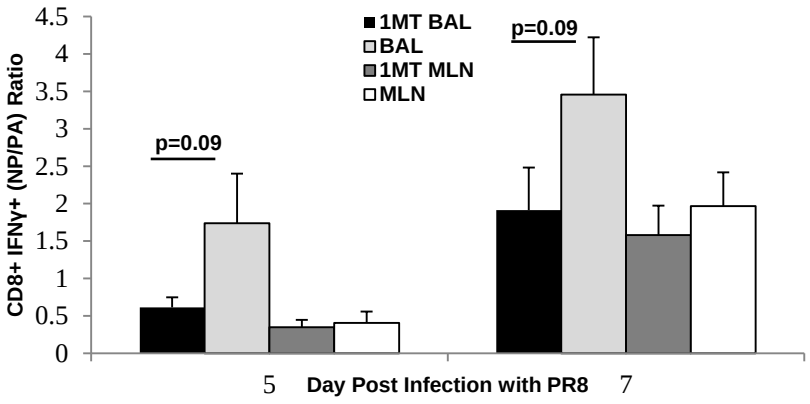

1MT BAL
BAL
1MT MLN
MLN
p = 0.01
p = 0.015
p = 0.02
# of CD8+ IFNγ+ Tcells (X1000)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5
7
9
Days Post Infection with PR8

**PA**


1MT BAL
BAL
1MT MLN
MLN
p = 0.01
p = 0.06
# of CD8+ IFNγ+ Tcells Cells (X1000)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
5
7
9
Days Post Infection with PR8


PB1
1MT BAL
BAL
1MT MLN
MLN
p = 0.02
p = 0.07
p = 0.09
p = 0.001
# of CD8+ IFNγ+ Tcells Cells (X1000)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
5
7
9
Days Post Infection with PR8

**D**


1MT BAL
BAL
1MT MLN
MLN
p=0.09
p=0.09
CD8+ IFNγ+ (NP/PA) Ratio
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
5
Day Post Infection with PR8
7

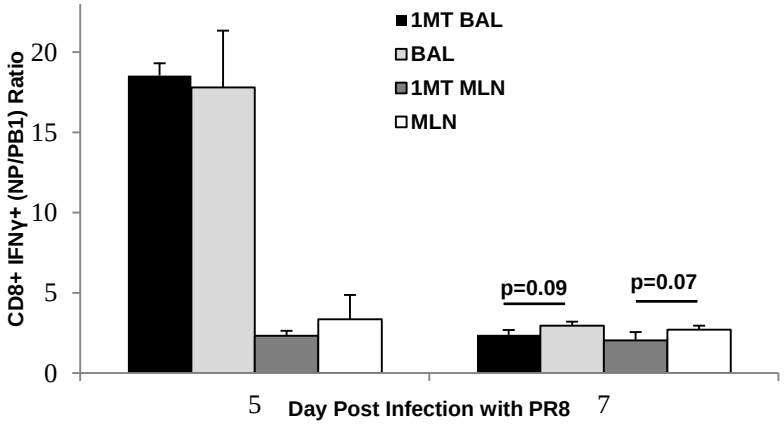

1MT BAL
BAL
1MT MLN
MLN
p=0.09
p=0.07
CD8+ IFNγ+ (NP/PB1) Ratio
20
15
10
5
0
5
Day Post Infection with PR8
7

**Figure 3.3: IDO activity during the primary CD8+ T cell response to influenza modifies immunodominance** In response to secondary/PR8 challenge, the following parameters were assessed: A: CD8+ T cells expressing IFN-γ in the airway (BAL). Numbers are average cell numbers ± SEM. B: Representative contour plots depicting the frequency of CD8+ T cells isolated from the airways of 1MT or vehicle treated mice reactive against NP, PA, or PB1 epitopes at days 5 and 7 p.i. with (indiscriminate of IFNγ expression). Percentages indicate the average frequency (*n*=3) of CD8+ T cells specific for the indicated epitopes ± SEM. C: Number of CD8+ T cells which express IFNγ in the airways and MLN which are specific for NP, PA, and PB1 epitopes ± SEM. D: Average ratio ± SEM of NP-specific CD8+ IFNγ+ T cells to PA and PB1 (top and bottom panels, respectively) specific CD8+ IFNγ+ T cells at day 5 and 7 pi. Data is representative of three independent experiments (*n*=3 per experimental group).

**Figure 3.4**

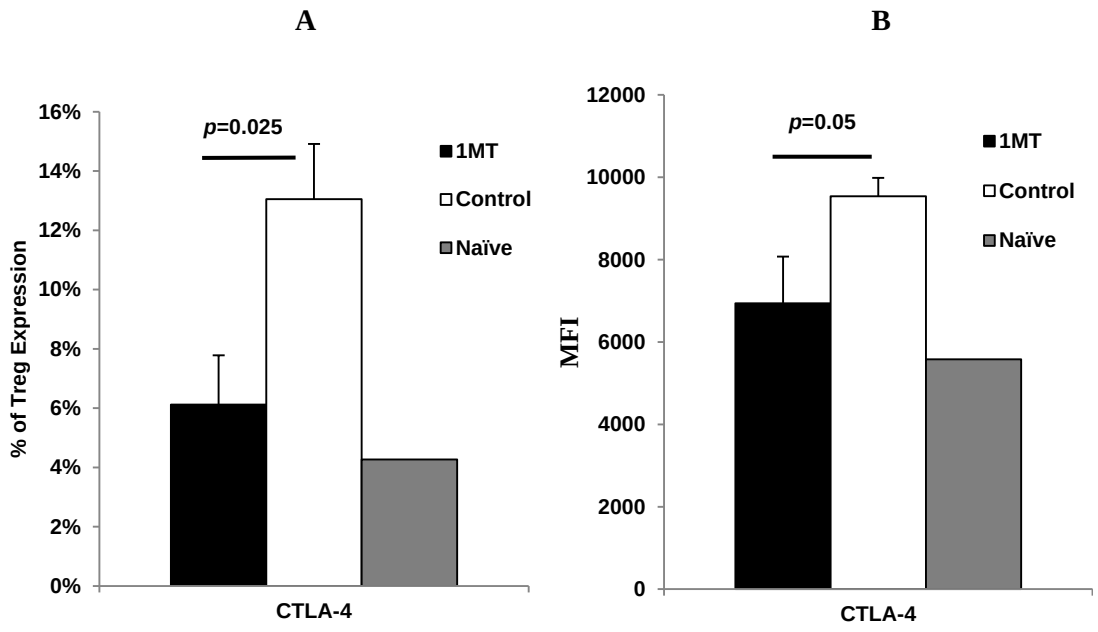


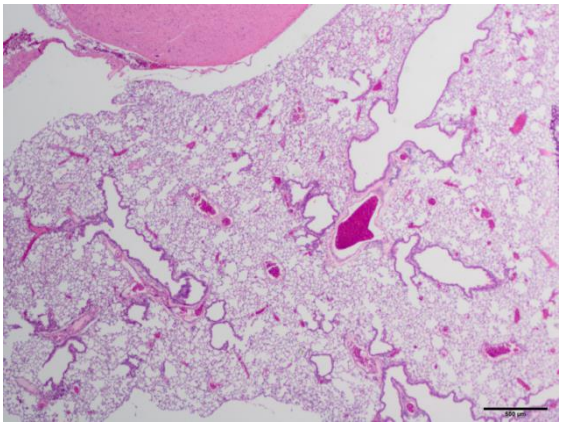

**Figure 3.4: IDO induced Tregs have different expression pattern of suppressive surface molecules.** 2 days after secondary/PR8 challenge, spleens and MLN were extracted from euthanized mice and enriched for Tregs. Tregs were co-incubated with X31 infected stimulator cells at a ratio of 1:1. After 2 days, the co-culture was analyzed for the proportion of Tregs' CTLA-4 expression (A) and their expression level in MFI (B) ($n$=3). Age matched mice which was not primed with X31 (naïve) was used as a negative control ($n$=1). Data is representative of two independent experiments.

**Figure 3.5**

**A**

**Day 0**

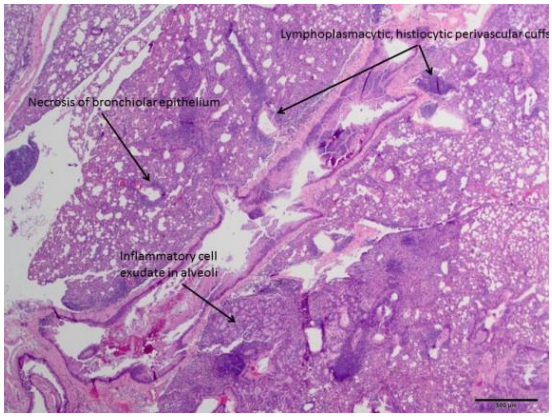
**(1MT, Control)**

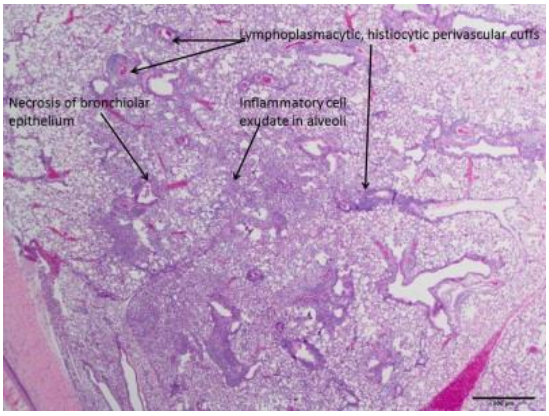


**Day 5**

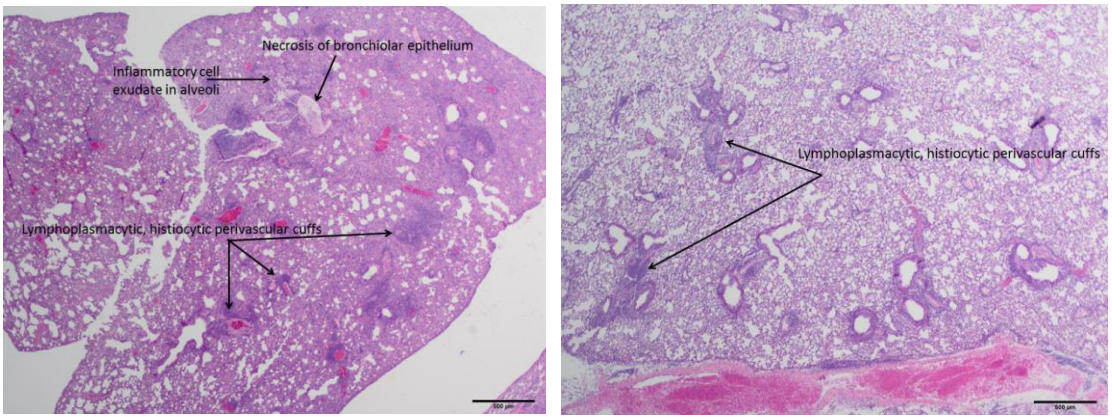
**Control** **1MT**

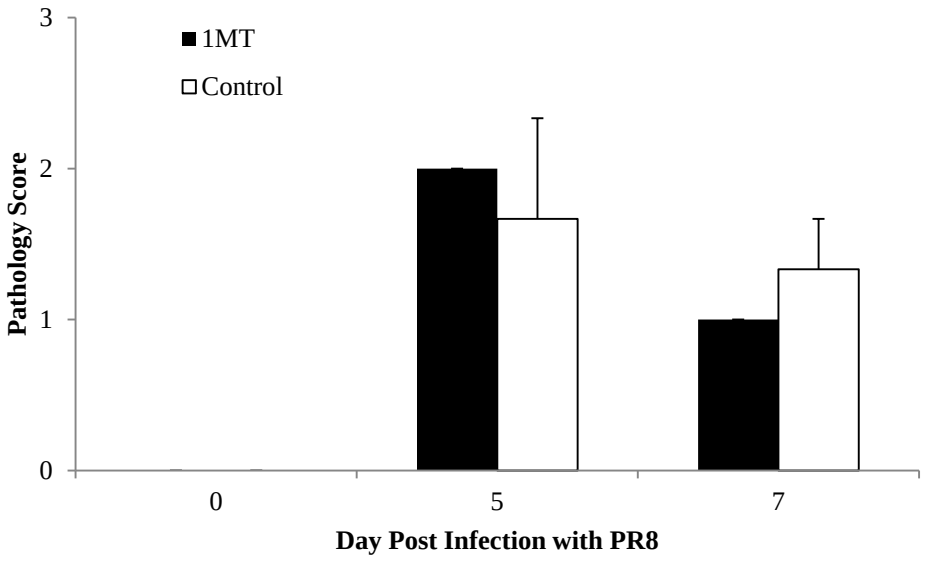
**Day 7**



**Control** **1MT**


Necrosis of bronchiolar epithelium
Inflammatory cell exudate in alveoli
Lymphoplasmacytic, histiocytic perivascular cuffs
Lymphoplasmacytic, histiocytic perivascular cuffs


3
2
1
0
1MT
Control
Pathology Score
0
5
7
Day Post Infection with PR8

**B**

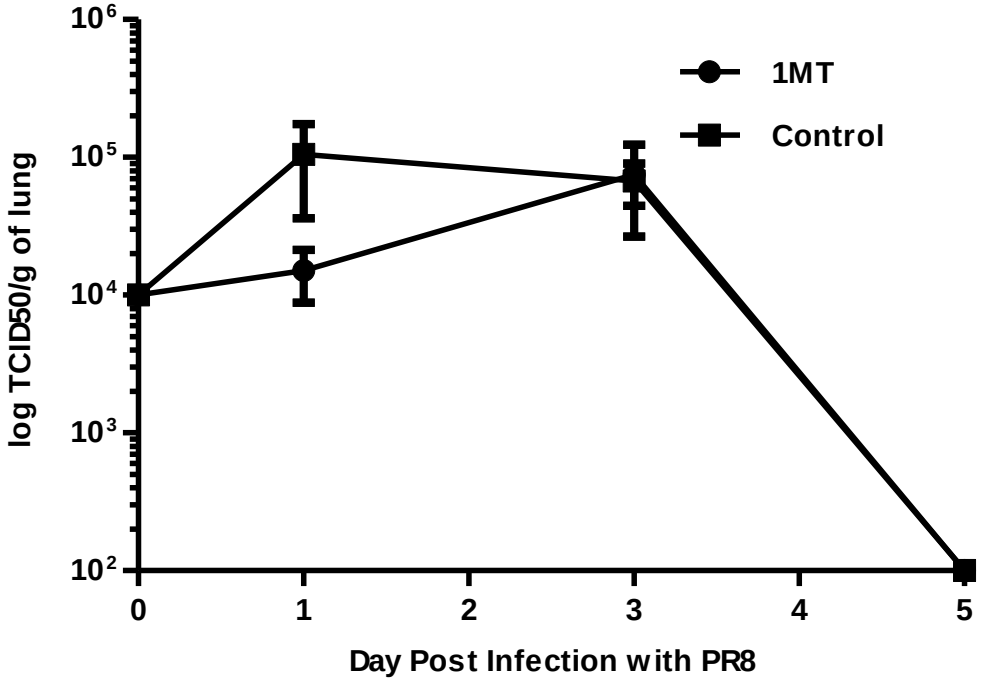



**Figure 3.5: Pharmacological inhibition of IDO during primary immune response against influenza decreases histopathology upon lethal influenza rechallenge independent of virus clearance.** Representative lung sections from mice which received either 1MT (left column) or vehicle control (right column) following secondary/PR8 challenge. Whole lungs were inflated, fixed, sectioned, and evaluated for histopathology by H&E staining by a pathologist blind to the treatments. A: Representative H&E stains of lung sections prior to challenge (day 0, representing both 1MT and control treatment). Specific pathological parameters are noted in each image. Average pathology scores ± SEM are shown. 1MT group has no error bars due to the lack of variation in gross scores. *n*=3 per experimental group, scale bars = 500μm. B: Kinetics of infectious virus levels as measured by $TCID_{50}$ upon challenge of 1MT and control mice with Day 0's virus titer represents inoculum dose. Data is representative of three independent experiments (*n*=3 per experimental group).

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# CHAPTER 4

# IDO ACTIVITY DURING INFLUENZA VACCINATION MODIFIES THE MEMORY RESPONSE TO INFLUENZA CHALLENGE

## Introduction

Influenza A virus is a worldwide health threat causing morbidity and mortality [1]. In addition to seasonal strains, emerging strains of influenza continually pose a threat as exemplified by the recent 2009 H1N1 influenza pandemic (pH1N1) [2], and the 2013 H7N9 epidemic [3], producing illness in at-risk populations. Although it is largely a self-limiting disease, influenza causes substantial morbidity and has a significant impact on productivity in the society affecting public health measures, and may also affect agriculture, e.g. poultry industry. Influenza is a member of the *Orthomyxoviriade* family and has an inherently high mutation capacity linked to polymerase proof-reading. Influenza strains of high virulence such as the 1918 pandemic H1N1 and H5N1 avian influenza which caused substantial mortality in both humans and avian species may return periodically, thus there is a need for surveillance and the capacity for rapid vaccine development.

Vaccination can diminish influenza transmission and disease severity by driving potent B cell (antibody) and T cell mediated immunity. The B cell parameters which have been correlated with protection against influenza include hemagglutinin inhibition (HAI) [4], high serum IgG, and mucosal IgA titer [5, 6]. Protective T cell mediated immunity include a robust memory Th1 [7] and effector CD8+ T cell (CTL) response [8, 9]. T cells are particularly important to confer heterosubtypic immunity against a variety of seasonal and novel pandemic strains. Currently no vaccines exist which excel in all of these attributes. The standard influenza vaccines used as of

2013 are trivalent or quadrivalent [10], and are inactivated preparations administered intramuscularly (i.m.) which are designed to induce an antibody response against hemagglutinin (HA) [11]. Live attenuated influenza vaccines (LAIV), which are administered intranasally (i.n.), are currently licensed for use as a quadrivalent formulation (FluMist®) [12]. These vaccines are designed to induce an antibody response against the vaccine strains with the design of providing neutralization against closely related influenza strains upon challenge. These vaccines are generally successful in inducing anti-HA responses, but various problems face inactivated vaccines such as the lack of robust T cell response which confers heterosubtypic immunity by T cells [13, 14]. Lack of sufficient antibody mediated neutralization is also a problem, especially in children because higher HAI titer is needed to confer protective immunity compared to adults [15].

One possibility to counteract vaccine-associated problems may be to inhibit the immunomodulatory enzyme, indoleamine 2,3-dioxygenase (IDO). IDO is a rate-limiting enzyme in the kynurenine pathway where the combination of tryptophan (substrate) depletion and kynurenine production limits activation and expansion of effector T cells [16, 17] and Th1/Th17 cells favoring a Th2/Treg-type response [18, 19]. Thus, IDO inhibition during primary [20, 21] and memory responses to influenza virus (Chapter 3) result in increased Th1 and virus-specific CD8+ T cells. However, the outcome of including pharmacological IDO inhibitors during vaccination has not been examined.

This study addresses the hypothesis that inhibition of IDO during influenza vaccination will result in a more robust CD8+ and CD4+ T cell response and increased antibody titer.

## Results

*IDO inhibition during intranasal vaccination increases the CD8+ T cell response*

To test the effects of IDO inhibition during intranasal influenza vaccination, a widely used competitive IDO inhibitor, 1-methyl-tryptophan (1MT) [20-23] was included. A/Alaska/6/1977 (H3N2), a cold-adapted LAIV was i.n. administered with or without 1MT to emulate FluMist®. The effect of IDO inhibition on vaccination efficacy as measured by anti-HA antibodies was assessed. As expected, vaccination induced antibodies, and although the 1MT group had a higher serum IgG titer, the difference between treatment groups was negligible (Figure 4.1).

To evaluate a heterosubtypic influenza challenge, vaccinated mice were challenged with mouse-adapted A/California/04/2009 (pH1N1). Upon challenge, substantial morbidity (weight loss) was observed, and mice continued to lose weight out to day 7 (75% weight loss in both groups) (Figure 4.2 A). This corresponded to virus persisting through day 7 (Figure 4.2 B) and lack of protection is not surprising because anti-H3N2 antibodies generated by the vaccine were expected to have a minimal effect on the H1N1 challenge strain. Although statistically insignificant, the 1MT group had lower lung virus titers at days 3 and 7 pi.

Given the role of T cell immunity in heterosubtypic protection, vaccine induced memory CD4+ and CD8+ T cells were examined. At 7 days post challenge, CD8+ T cells were higher ($p$=0.05) in the airways (BAL) in the 1MT-vaccine group (Figure 4.3A). The CD8+ T cell phenotype was examined for effector ($CD44^{hi}$ $CD62L^{lo}$) T cells [24, 25]. The findings revealed that, although not significant ($p$=0.07), CD8+ effector cells at day 7 (Figure 4.3 B), and effector CD4+ T cells were significantly ($p<0.05$) higher in the mediastinal lymph node (MLN) at day 7 pi (data not shown). This suggests that IDO inhibition during i.n. LAIV increases the T cell response to challenge, although the incidence of morbidity and virus clearance is not significantly affected. Although some differences in T cells were observed, more profound differences were

expected, particularly with virus clearance, morbidity, CD4+ T cell response, and antibody production. The significant but small differences hinted that this may be due to an inadequate suppression of IDO by 1MT, possibly by the low delivery volume which was limited by i.n. administration. Thus, a subcutaneous (s.q.) mode of vaccine was used to increase the available volume of vaccine that can be safely administered.

*IDO inhibition during subcutaneous vaccination decreases morbidity and facilitates protection against lethal challenge*

Subcutaneous vaccination with CA/07/09 was performed subcutaneously as previously described [22]. The vaccine was administered using live virus to induce a maximal T cell response where IDO is hypothesized to exert its greatest effects. Similar to the findings for i.n. vaccine administration, no differences in IgG titers were observed between the groups (Figure 4.4 A). Virus-specific IgG1/IgG2a titers was also determined, but no differences were detected (data not shown); however, the anti-HA antibody titer in the 1MT group was significantly ($p<0.001$) higher compared to the control (Figure 4.4 B).

All mice vaccinated with 1MT survived a lethal challenge, whereas all mice in the control group succumbed to lethal infection (Figure 4.5 A), correlated to the mice to clear virus faster and is likely linked to the higher HAI titer. By day 7, only 1 out of 3 mice in the 1MT vaccinated group had infectious virus in the lung, and the titer of the one mouse which did have virus was 1000-fold lower than the average of the control group's virus titer (Figure 4.5 B). The T cell population was also analyzed to determine if this had any role in the difference in mortality, morbidity, and virus clearance, but no detectable differences in any CD4+ and CD8+ T cell parameters were evident (data not shown). Thus, the survival of 1MT group against the pH1N1 is likely due to the high titer HAI.

*Subcutaneous vaccination with a novel IDO inhibitor is associated with increased hemagglutinin inhibition antibody titers*

7-azatryptophan (7AT), an IDO substrate analogue like 1MT [26] inhibits IDO activity *in vitro* (Figure 4.6A). Murine lung epithelial cells (MLE-15) were stimulated with IFNγ to induce IDO expression and activity [27, 28] and cultured in the presence of medium only, 1MT, or 7AT at various concentrations. At the lowest concentration (47 μM), 7AT exhibited significant ($p$<0.001) inhibition of IDO activity as evident by the low IDO product [kynurenine] [29] compared to the control. MLE-15 cells also exhibited low cell death in the presence of 7AT compared to medium-only control for up to 375μM (Figure 4.6 B). This suggested that 7AT is a good candidate for an *in vivo* IDO inhibitor with low toxicity.

The X31-PR8 influenza model, which is used to study heterosubtypic immunity [30-32], was used to evaluate 7AT efficacy. Following s.q. vaccination of mice with X31, weight loss was measured to evaluate one aspect of potential *in vivo* toxicity. The weight loss post-vaccination was equivalent between the control and 7AT group, suggesting that 7AT does not cause substantial morbidity upon use in a vaccine (Figure 4.6 C). 7AT's IDO inhibitory *in vivo* activity in response to the vaccine revealed that it significantly ($p$<0.04) lowered the concentration of kynurenine in the serum 2 days after vaccination (Figure 4.6 D). This suggests that 7AT is bioactive and inhibits IDO at least 2 days after vaccination and possibly longer.

First, the effect of 7AT on the IgG1/IgG2a response was examined (Figure 4.7 A and B, respectively). At day 21 post-vaccination the 7AT group had higher antibody titers for both IgG isotypes, however the differences were not significant (p<0.05). The HAI titers were also evaluated at day 21 post vaccination, where the 7AT vaccine group had significantly ($p$<0.001) higher titer by 2-fold (Figure 4.7 C), mimicking 1MT-inclusion (Figure 4.4). Upon challenge with PR8 at 28 days post vaccination, weight loss was equivalent between the groups. The lack of protection afforded by the vaccine against PR8 was surprising (Figure 4.8 A), although the virus

titer was decreasing by day 7 in both groups with a marked decrease in virus titer at day 5 in the 7AT group (Figure 4.8 B). To determine if the pathology in response to PR8 was different, lung sections were assessed for differences by a pathologist but no significant differences were noted (data not shown). At day 7, the % of CD4+ T cells expressing IFNγ (Th1) was significantly higher ($p$=0.05) in the 7AT group (Figure 4.8 C).

## Discussion

This study examined the immunological outcomes linked to IDO inhibition during influenza vaccination. IDO inhibition using 1MT during i.n. LAIV resulted in increased CTL and Th1-type responses in the airways and lymph node following influenza virus challenge. Inhibition of IDO during s.q. vaccination with 1MT resulted in increased HAI titers, and also enhanced pulmonary virus clearance and survival against lethal challenge. 7AT administered during s.q. vaccination also increased HAI titer and Th1-type response. The use of different inhibitors in various contexts which mimic conventional vaccination routes (i.n., s.q.) suggests different administration routes can be used for IDO inhibition.

The increase in CTL in relation to IDO inhibition was consistent with a previous study which boosted CTL immunity by IDO inhibition during influenza vaccines [22]. This change was attributed to the relationship between IDO and Natural Killer T cell mediated CD40L ligation to induce a robust Th1-type and CTL response [33]. This could be one way that the CTL response can be boosted and is corroborated by the findings showing an increased Th1-type response following 7AT (Figure 4.8C). The increase in HAI titer due to these IDO inhibitors in the vaccine may also be explained by this, as CD40L ligation by CD4+ T cells also facilitate isotype switching and antibody production by B cells [34]. Since HAI titer is one way to measure vaccine efficacy [35] as it is a correlate of protection [4], 7AT and 1MT may be a possible candidate for boosting HAI titer to confer better protection especially for children [15].

Future studies should focus on understanding the mechanisms behind the changes observed, particularly at the point of antigen presentation and the role of IDO in influenza antigen recognition by Pattern Recognition Receptors (PRRs). IDO is expressed in response to stimulation by some TLRs [16, 36] and TLR stimulation is critical for driving optimal immune responses to influenza vaccines [37] and cross-presentation by plasmacytoid dendritic cells (pDC) [38] for protection against inflenza challenge [39]. IDO inhibitors may persist long enough to also affect where the antigen presenting cells such as IDO+ pDC interact with Tregs which are activated by IDO and inhibit activation and expansion of effector T cells [19, 40, 41]. Interrupting the feedback loop of Treg-pDC mediated suppression of effector T cell expansion may shift the immune response from a tolerogenic towards a more antigenic response.

IDO attenuates the generation of central and effector memory CD8+ T cells [42]. This suggests that IDO inhibition may be useful in enhancing the memory response for individuals experiencing influenza antigen for the first time, and for increasing the efficacy of those receiving vaccines annually to enhance the generation of secondary and subsequent memory response.

1MT is a good IDO inhibitor candidate as its safety is documented [43] and it is in use in phase III clinical trials for cancer treatment due to its success in serving to boost the effects of tumor vaccines [44, 45]. However, the use of other safe and effective IDO inhibitors should be considered. The different immunological outcomes linked to different routes of vaccination (i.n. and s.q.) led to interesting findings. The differences observed could be due to the different types of APCs present at sites of vaccination (Langerhans versus alveolar macrophage, respectively).

The development of vaccination models that will allow for more extensive analyses of the effects of IDO inhibition on vaccines is needed. There is some information now on the role of IDO in the primary and memory immune response [21, 46] but it will be a challenge to figure out how these established immunological principles translate to a vaccination model to understand the immunology behind constructing a more effective influenza vaccine.

## Materials and Methods

*Influenza*

Influenza strains California/07/2009 (CA/07/09), A/Alaska/6/1977, A/Puerto Rico/8/1934 (PR8), and X31, a recombinant virus with the HA and NA (H3N2) derived from A/Hong Kong/1/68 with the internal proteins from Puerto Rico/8/1934 were propagated in 9 day old embryonated chicken eggs. Allantoic fluids were extracted and stored at -80˚C until use. Mouse adapted influenza A/California/04/2009 (pH1N1; a kind gift from Daniel Perez) was grown *in vitro* by infecting MDCK cells in MEM (HyClone, Rockford, IL) supplemented with L-Glutamine and 1 µg/mL TPCK-Trypsin (Worthington, Lakewood, NJ) at a multiplicity of infection (MOI) of 0.01. 3 days after infection, cell culture supernatant was collected by centrifugation and stored at -80°C.

*Mice, Vaccines, and Challenge*

8-10 week old female, C57BL/6 mice (National Cancer Institute, Bethesda, MD) were anesthetized with 2,2,2-tribromoethanol (Avertin) and [47] vaccinated intranasally with 150µL of PBS with 5000 plaque forming units (PFU) of A/Alaska/6/1977. For subcutaneous vaccinations, mice were subcutaneously injected at their interscapular site with 0.2mL with 2000 PFU of CA/07/09 or $10^6$ PFU of X31 in PBS. Vaccine formulation contained either PBS with virus only (control) or IDO inhibitors 1MT (Sigma-Aldrich) or 7AT (a kind gift from Robert Phillips). The intranasal vaccine contained 50mg/mL of 1MT. The subcutaneous vaccine with CA/07/09 contained 10mg/mL of 1MT and the vaccine with X31 contained 10mg/mL of 7AT. Administering 0.2mL of 10mg/mL the IDO inhibitor is equal to 100mg/kg for a 20g mouse. The solution of 1MT/7AT were balanced to isotonic salt concentration (300 mEq/L) and neutral pH (7.2) using NaOH/HCl before adding influenza.

After vaccination, mice were tail bled (days 2, 21, or 28 as indicated in Figure Legends) for blood collection (maximum of 0.5mL/mouse) in CapiJect® Micro collection tubes with a clot

activator (Terumo). Serum was prepared by centrifugation according to the manufacturer's results and stored at -20°C until testing the serum for antibodies and analytes.

28 days post-vaccination, mice were anaesthetized with Avertin and intranasally challenged with lethal doses of pH1N1 or PR8. For BAL and whole lung collection, mice were euthanized with an overdose of Avertin. At various times post challenge and vaccination, mice were weighed on a scale to assess morbidity. All mouse work was approved by the Institutional Animal Care and Use Committee of the University of Georgia.

*Serum Influenza-Specific IgG Measurement*

Serum IgG against vaccine influenza strain was measured using indirect ELISA as described [35]. Briefly, 96 well plates (EIA/RIA high binding) were coated with 0.1mL of influenza vaccine strains A/Alaska, CA/07/09, or X31 at 100 pfu/mL overnight at 4°C. Plates were then wash with KPL Wash Solution (KPL) and overlaid with serum which was pre-diluted in blocking buffer (5% nonfat dry milk and 0.5% Bovine Serum Albumin [Gibco] in KPL wash solution) for 1 hour at room temperature. After washing with KPL Wash Solution, a secondary antibody (goat anti-mouse) was added IgG was added at 1µg/mL for one hour at room temperature. Color was developed using pNPP phosphatase substrate if the secondary antibody was conjugated to alkaline phosphatase (KPL) and the reaction was stopped by adding an equal volume of 5% EDTA and read at 405nm. If the secondary antibody was conjugated to horseradish peroxidase, 1-Step Ultra-TMB ELISA (Thermo) was added, followed by stopping the reaction by adding an equal volume of 2M $H_2SO_4$ and read 450nm using a microplate reader (TECAN).

*Hemagglutination Inhibition*

HAI was performed as described [35]. Briefly, heat-inactivated (56°C for 30 minutes) serum was mixed with an equal volume of 0.5% chicken erythrocytes (cRBC) in PBS to remove non-specific

agglutinating activity by the serum for one hour at room temperature. After incubation, erythrocytes were removed by centrifugation and the serum was then serially diluted 2-fold in a 96-well round bottom plate (Corning) in PBS in triplicates. Each well was co-incubated with 4 HAU of influenza vaccine strains 37°C for one hour except for negative control wells in which the serum was incubated with PBS only, not virus. Finally, the virus-serum solution was mixed with an equal volume of 0.5% cRBC in a V-bottom 96-well plate (Corning) and incubated at room temperature for one hour. Hemagglutination pattern was scored and the titer was determined by the average of the triplicates' titers.

*Determination of Serum Kynurenine Concentration*

Kynurenine from serum was determined as described [48, 49]. Briefly, serum was mixed with 30% trichloroacetic acid (Sigma-Aldrich) to precipitate protein. The mixture was vortexed and centrifuged at 1,500XG at 4°C for 10 minutes The supernatant was mixed with an equal volume of Ehrlich's Reagent (2% p-dimethyl-benzaldehyde in glacial acetic acid) (Sigma-Aldrich) in a 96-well plate (Costar). The samples were read at 490 nm to detect kynurenine. A standard curve was developed from a set of serial dilution of purified kynurenine (Sigma-Aldrich) to calculate the exact concentration (μg/mL) of kynurenine for each experimental sample.

*Cell preparation and flow cytometry*

Leukocytes from the airways of influenza challenged mice were collected by bronchoalveolar lavage (BAL). Briefly, 1.0 mL of PBS (HyClone) was instilled in the trachea using an 18-Gauge catheter followed by two PBS washes in the same volume. Mediastinal lymph nodes (MLN) were removed and single cell suspensions prepared in Hank's Buffered Saline Solution (HBSS, HyClone, Rockford, IL) following passage through 100μm cell strainers (BD Biosciences, San Jose, CA). Cell numbers from the BAL and MLN samples were enumerated using a Z2 Coulter

Counter (Beckman-Coulter, Brea, CA) after lysing erythrocytes using Zapo-Globin II (Beckman-Coulter) according to manufacturer's instructions.

To analyze cells by immunophenotyping, $10^6$ BAL or MLN cells were added to wells of round bottom 96-well plates (Corning, Lowell, MA), washed, and resuspended in FACS Buffer [Dulbecco's PBS (HyClone) with 0.09% (w/v) $NaN_3$ (Sigma-Aldrich), 2mM EDTA (Fisher Scientific, Pittsburgh, PA), and 1% (w/v) Bovine Serum Albumin (Sigma-Aldrich)] as previously described [50]. Plated cells were treated with 1μg/mL of FcBlock (BD Biosciences) to inhibit non-specific binding of antibodies. The cells were washed and stained at room temperature for one hour for CD8α, CD4, CD62L, and CD44 (BD Biosciences).

Surface staining was followed by intracellular IFN-γ cytokine staining [51]. Briefly, the cells were fixed and permeabilized with fixation/permeabilization buffer and washed with permeabilization buffer (BD Biosciences) and stained against IFNγ in permeabilization buffer for 30 minutes at 4°C. The cells were then washed with permeabilization buffer, and analyzed by flow cytometry using a BD LSR-II (BD Biosciences). At least 50,000 events were recorded following gating on CD4+ or CD8+ T cells. Data was captured using BD FACSDiva (BD Biosciences) and analyzed using FlowJo (Tree Star).

*Histopathology*

After X31 vaccinated mice was challenged with PR8, their lungs were perfused and fixed in cold 10% (w/v) neutral buffered formalin at days 3, 5, and 7 post challenge (Fisher). The lungs were treated with paraffin and sectioned as previously described [52]. Briefly, 5μm sections of lungs were cut and stained with Hematoxylin and Eosin (H&E). Histopathology of lung sections was evaluated in a single blind manner by a board certified pathologist in the College of Veterinary Medicine at the University of Georgia.

*Determining Influenza Viral Titer by $TCID_{50}$*

Infectious virus titer was measured by $TCID_{50}$ as previously described [53]. Briefly, following challenge with H1N1 or pH1N1, mice were euthanized and their lungs excised and placed in 1mL of PBS with 100 μg/mL of Streptomycin, 100 IU/mL of Penicillin, and 250ng/mL of Amphotericin B (Mediatech, Irving, TX). After homogenization with a tissue lyser (Eppendorf, Hamburg, Germany), the homogenate was centrifuged and the supernatant stored at -80˚C. MDCK cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, HyClone) with 5% (v/v) heat-inactivated Fetal Bovine Serum (FBS) (HyClone) at 37˚C in 96 well plates. Serial dilutions of virus in 100μL of Minimum Eagle's Medium (MEM, HyClone) containing 100 μg/mL of Streptomycin, 100 IU/mL of Penicillin, and 250ng/mL of amphotericin B (Mediatech), and 1μg/mL of TPCK-Trypsin (Worthington, Lakewood, NJ) was added to each well. The plates were incubated for 72 hours at 37°C and mixed with equal volume of 0.5% cRBC in PBS and incubated at room temperature for one hour. Hemagglutination was scored according to standard $TCID_{50}$ methods as described [53].

*Statistics*

All data points were assessed for statistical significance between means of 1MT or 7AT compared to the control ($p$ value $\leq 0.05$) using one or two-tailed Student's $t$-test or One-Way ANOVA followed by Tukey's post-hoc test as appropriate. Figure Legends state which test was used for each set of data. $p$-values are listed when significant. All statistical analyses were performed using Graph Pad Prism software (Graph Pad Software, San Diego, CA) and graphs displayed with Microsoft Excel (Microsoft) or Graph Pad Prism.

## Figures

**Figure 4.1**

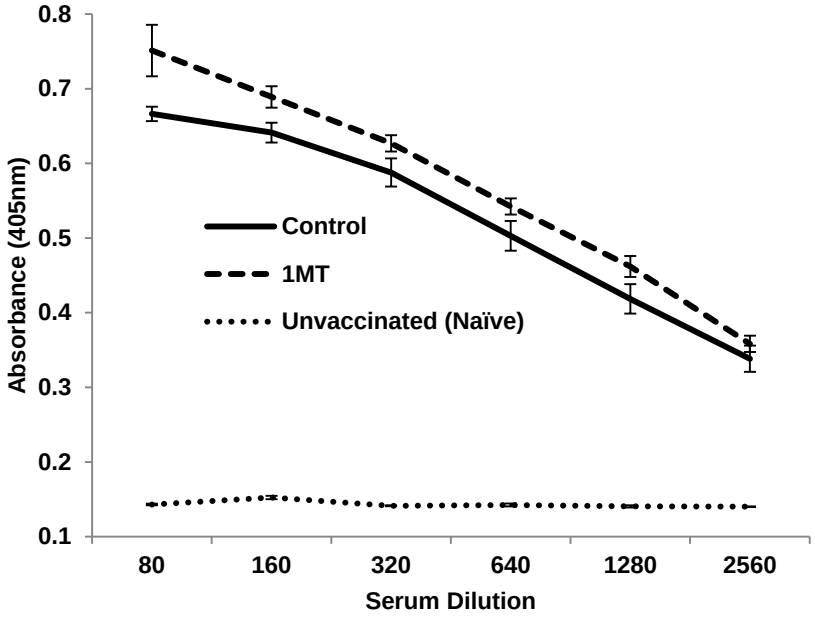


**Figure 4.1: 1MT inclusion in intranasal LAIV does not affect virus specific IgG titer.** C57BL/6 mice were vaccinated i.n. with A/Alaska/6/1977 with or without 1MT in the vaccine. 28 days later, serum from the blood of mice was collected. Serum IgG against A/Alaska/6/1977 was assessed by indirect ELISA compared to unvaccinated mice (Naïve). Absorbance at 405nm for each serum dilution is displayed from 1:80 to 1:2560 dilutions. Average values of all groups are presented ± SEM. *n*=10/time point for control and 1MT group. *n*=3/time point for unvaccinated group.

**Figure 4.2**

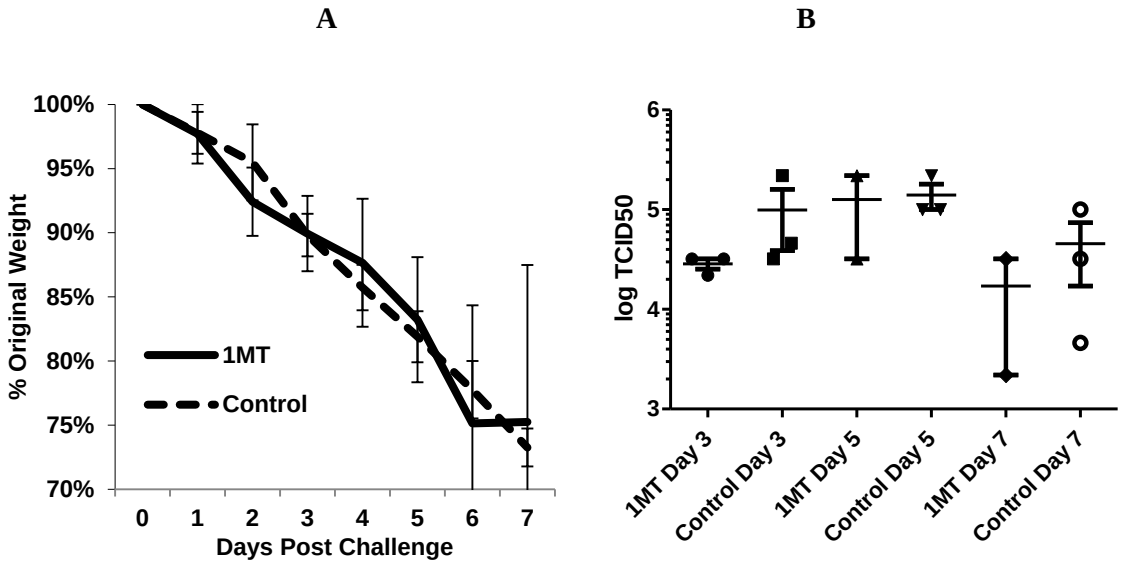


**Figure 4.2: 1MT inclusion in intranasal LAIV does not affect weight change or virus clearance upon challenge.** C57BL/6 mice were vaccinated i.n. with cold-adapted A/Alaska/6/1977 with or without 1MT in the vaccine. 28 days later, mice were challenged with 5x$LD_{50}$ pH1N1. A: Weight loss of mice relative to the pre-challenge weight was tracked for individual mice every 24 hours. Average values of both groups are presented ± SEM. B: Lung homogenate from challenged mice were assessed for infectious virus titer by $TCID_{50}$. Average values of both groups are presented with horizontal bars with individual titers at each time point/group ± SEM on a $\log_{10}$ scale. *n*=3/time point-group. Statistical significance was assessed using one-tailed student's *t*-test.

**Figure 4.3**

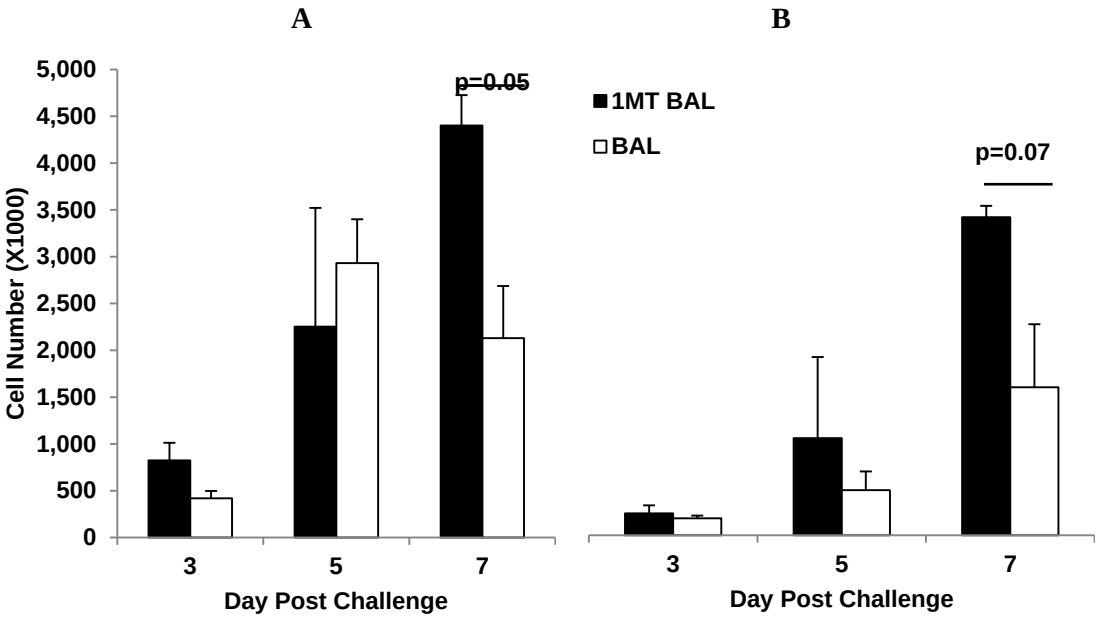


**Figure 4.3: 1MT inclusion in intranasal LAIV increases CD8+ T cell response upon challenge.** C57BL/6 mice were vaccinated i.n. with LAIV with or without 1MT in the vaccine. 28 days later, mice were challenged with 5x$LD_{50}$ pH1N1. At days 3, 5, 7 post challenge, cells in airways were collected by BAL and analyzed by FACS for CD8 expression (panel A) and effector phenotype ($CD44^{hi}$ $CD62L^{lo}$) (panel B). All figures contain the average value per time point/group ± SEM. Statistical significance was calculated using one-tailed student's *t*-test. *n*=3/time point-group.

**Figure 4.4**

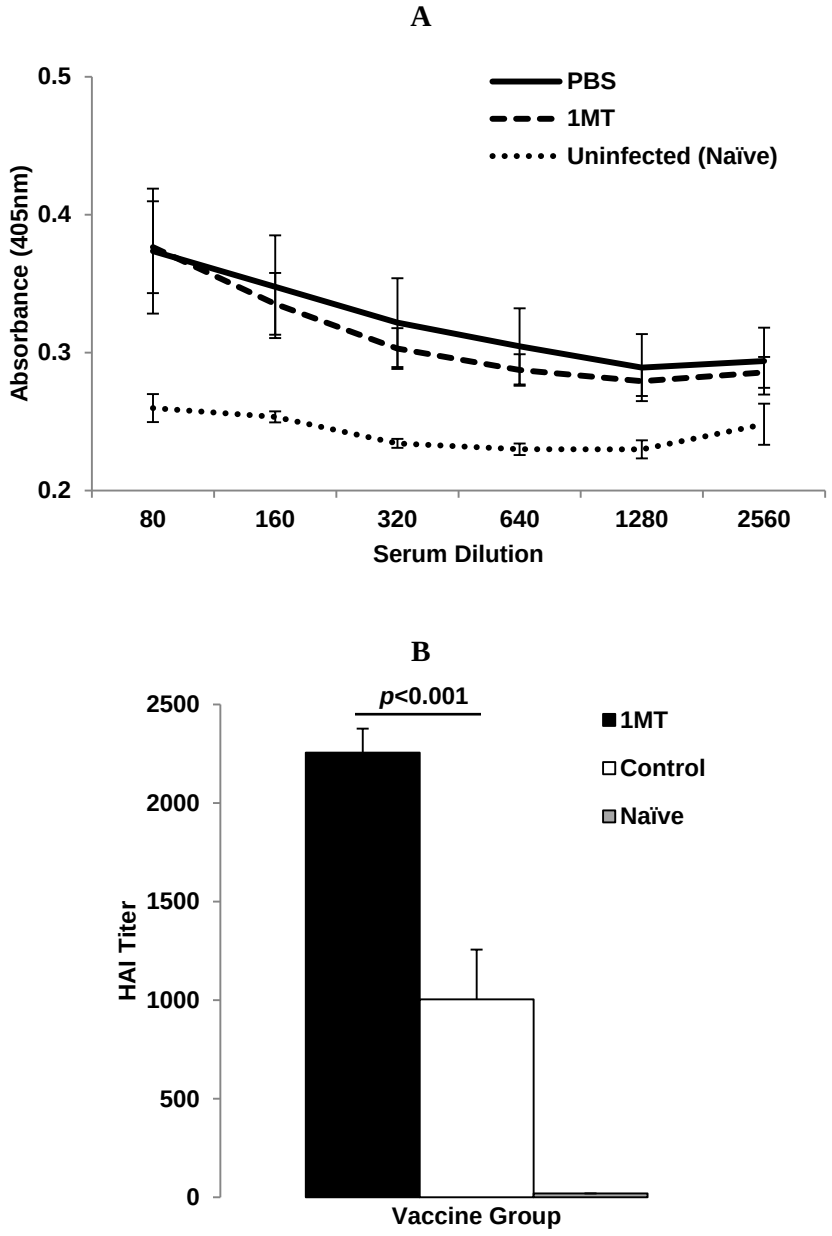


**Figure 4.4: 1MT inclusion in subcutaneous influenza vaccine increases HAI titer.** C57BL/6 mice were vaccinated subcutaneously with CA/07/09 with or without 1MT in the vaccine. 28 days later, serum from the blood of mice was collected. A: Serum IgG against CA/07/09 was assessed by indirect ELISA compared to unvaccinated mice (Naïve). Absorbance at 405nm (alkaline phosphatase) for each serum dilution is displayed from 1:80 to 1:2560 dilutions. B:

Antibodies against CA/07/09 HA was determined by HAI. Titers represent the highest point where HA inhibition dropped and agglutination appeared.

All figures contain the average value per time point/group ± SEM. Significance was calculated using One-Way ANOVA between experimental groups for each dilution followed by Tukey's post-hoc test. $n$=10/time point for control and 1MT group. $n$=3/time point for unvaccinated (naïve) group.

**Figure 4.5**

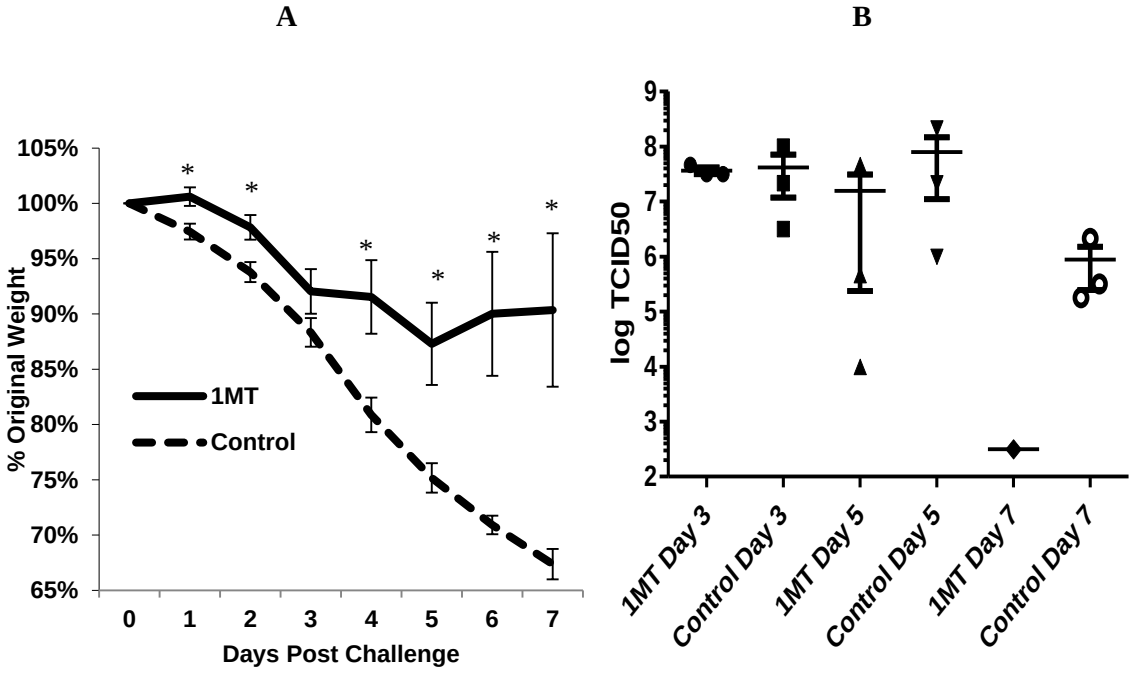


**Figure 4.5: 1MT inclusion in subcutaneous influenza vaccine reduced morbidity, mortality, and accelerated virus clearance.** C57BL/6 mice were vaccinated subcutaneously with CA/07/09 with or without 1MT in the vaccine. 28 days later, mice were challenged with 10x$LD_{50}$ pH1N1. A: Weight loss of mice relative to the pre-challenge weight was tracked for individual mice every 24 hours. Asterisk indicates *p*-value is <0.001 using two-tailed student's *t*-test. Average values of both groups are presented ± SEM. B: Lung homogenate from challenged mice were assessed for infectious virus titer by $TCID_{50}$. Average values of both groups are presented with horizontal bars with individual titers at each time point/group ± SEM on a $\log_{10}$ scale. *n*=3/time point-group. 2 out of 3 mice in 1MT Day 7 group had no detectable titer (not displayed).

**Figure 4.6**

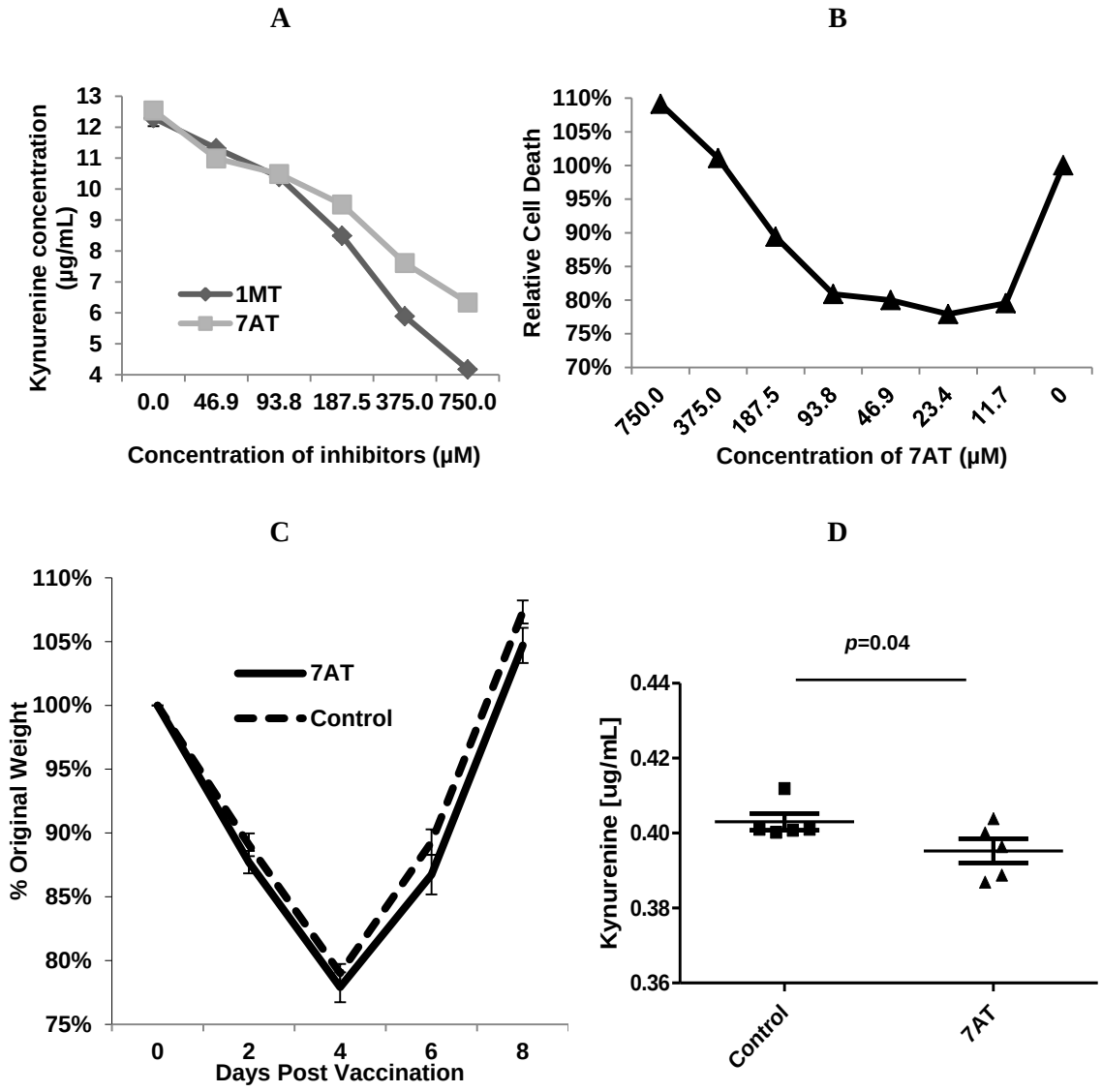
A
Kynurenine concentration (µg/mL)
13
12
11
10
9
8
7
6
5
4
1MT
7AT
0.0
46.9
93.8
187.5
375.0
750.0
Concentration of inhibitors (µM)
B
Relative Cell Death
110%
105%
100%
95%
90%
85%
80%
75%
70%
750.0
375.0
187.5
93.8
46.9
23.4
11.7
0
Concentration of 7AT (µM)
C
% Original Weight
110%
105%
100%
95%
90%
85%
80%
75%
7AT
Control
0
2
4
6
8
Days Post Vaccination
D
p=0.04
Kynurenine [ug/mL]
0.44
0.42
0.40
0.38
0.36
Control
7AT

**Figure 4.6: 7AT exhibits IDO inhibition activity *in vitro* and *in vivo* with minimal toxicity in subcutaneous influenza vaccine.** A: The IDO inhibitory activity of 1MT and 7AT *in vitro* against IFNγ stimulated MLE-15 cells as measured by kynurenine concentration in the supernatant compared to no inhibitors (0 μm). *n*=4/time point-group. B: Relative cell death caused by 7AT *in vitro* against MLE-15 cells compared to medium only control (0μM) at 48 hours post incubation. C57BL/6 mice were vaccinated subcutaneously with X31 with or without 7AT in the vaccine. C: Weight loss of mice relative to the pre-vaccination weight was tracked for individual mice every 48 hours. D: Immediately before and 2 days post-vaccination, serum was collected from mice to determine kynurenine levels. *n*=5/time point-group. All figures contain the average value per time point/group ± SEM. Statistical significance was assessed using one-tailed student's *t*-test.

**Figure 4.7**

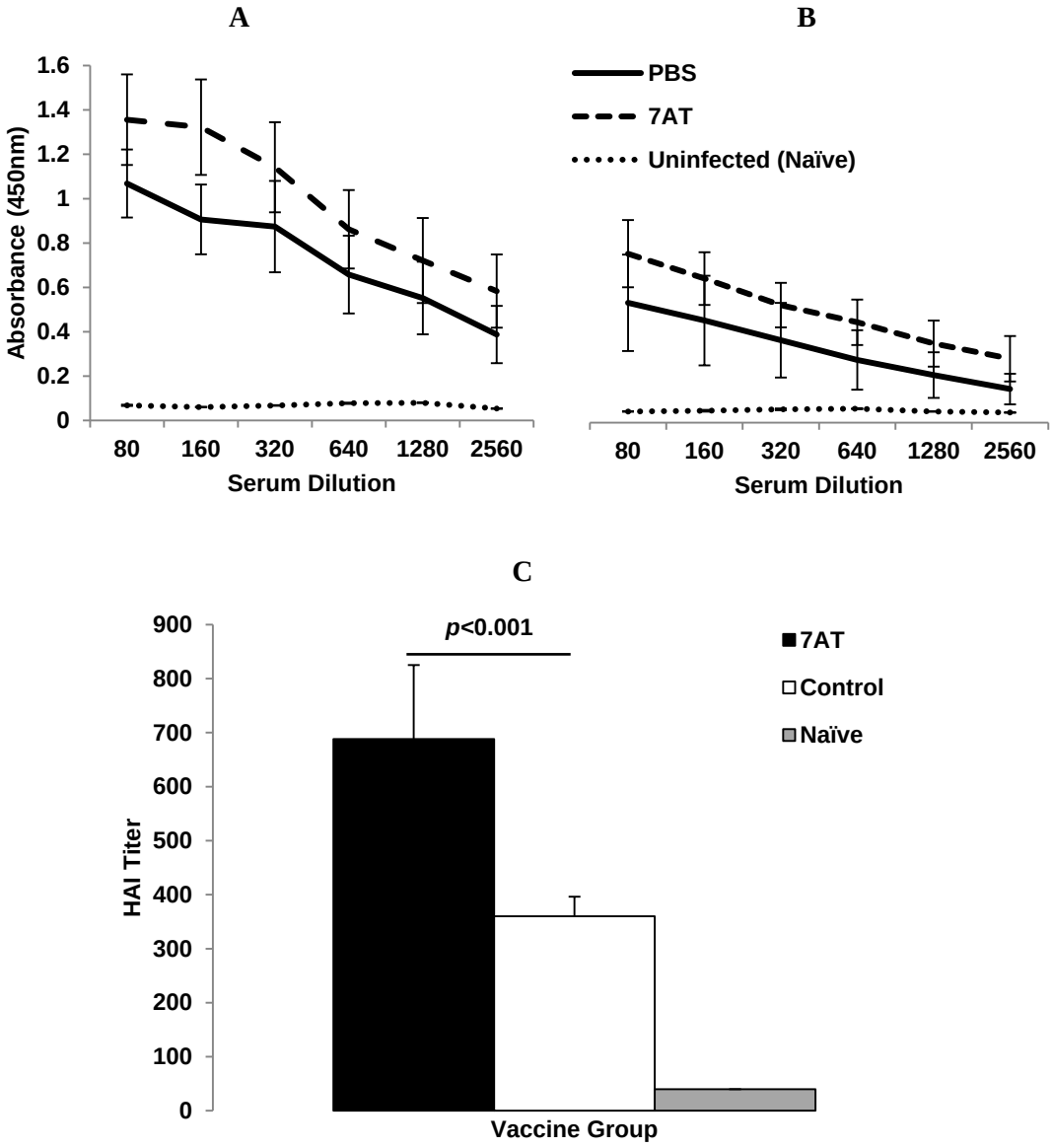


**Figure 4.7: 7AT inclusion in subcutaneous influenza vaccine does not significantly increase virus specific IgG1/IgG2a titer.** C57BL/6 mice were vaccinated subcutaneously with X31 with or without 7AT in the vaccine. 21 days later, serum from the blood of mice was collected. Serum IgG1 (panel A) and IgG2a (panel B) specific for X31 was measured by indirect ELISA compared to unvaccinated mice (Naïve). Absorbance at 450nm for each serum dilution is displayed from 1:80 to 1:2560 dilutions. C: Serum neutralizing antibodies against X31 HA was assessed using

HA Inhibition assay at day 21 post-vaccination. Average values of all groups are presented ± SEM. Significance was calculated using One-Way ANOVA between experimental groups for each dilution followed by Tukey's post-hoc-test. $n$=10/time point for control and 1MT group. $n$=3/time point for unvaccinated group. Data is representative of 2 independent experiments.

**Figure 4.8**

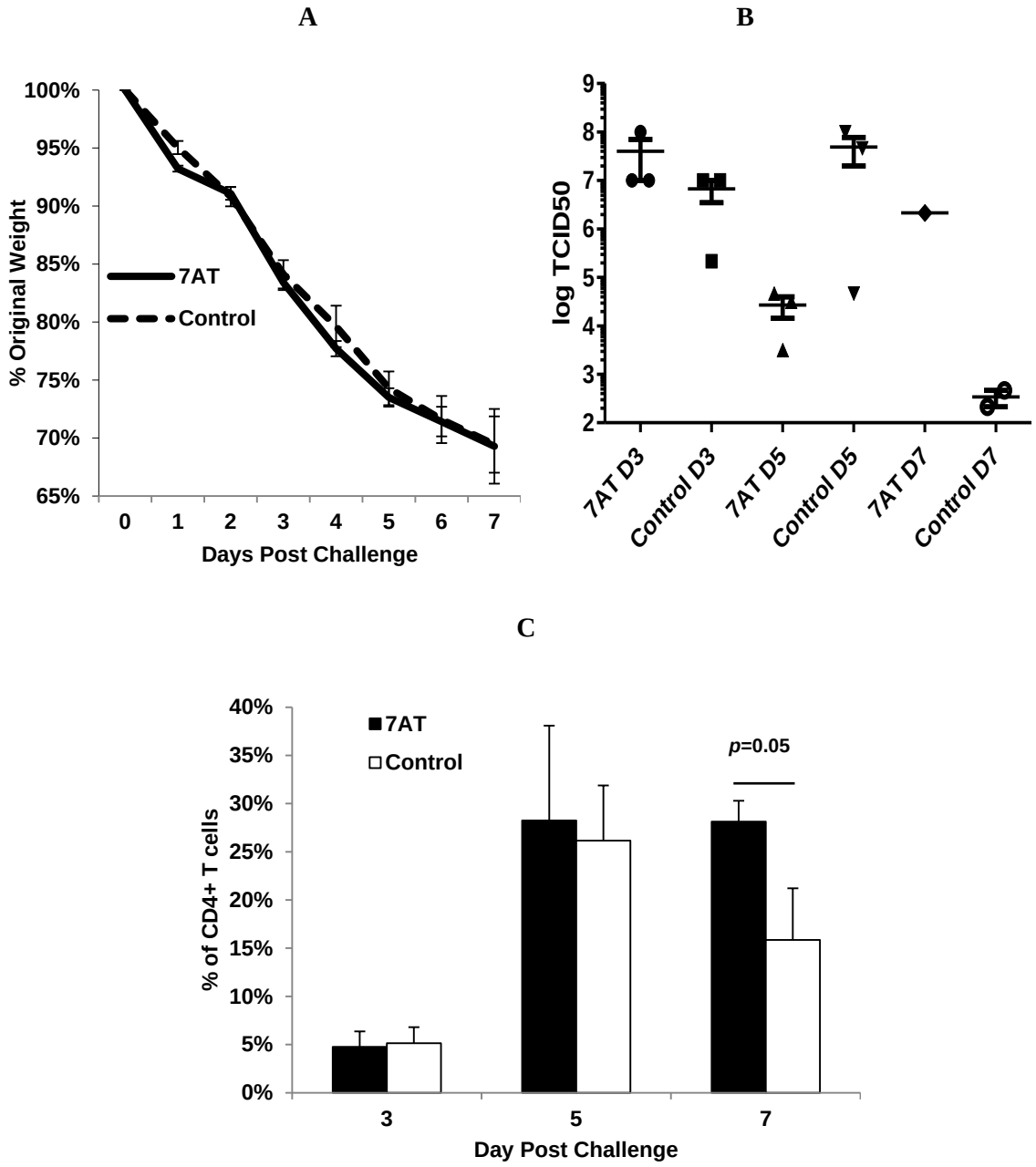
A
B
100%
95%
90%
85%
80%
75%
70%
65%
% Original Weight
7AT
Control
0
1
2
3
4
5
6
7
Days Post Challenge
9
8
7
6
5
4
3
2
log TCID50
7AT D3
Control D3
7AT D5
Control D5
7AT D7
Control D7
C
40%
35%
30%
25%
20%
15%
10%
5%
0%
% of CD4+ T cells
7AT
Control
p=0.05
3
5
7
Day Post Challenge

**Figure 4.8: 7AT inclusion in subcutaneous influenza vaccine does not affect morbidity, mortality, or virus clearance.** C57BL/6 mice were vaccinated subcutaneously with X31 with or without 7AT in the vaccine. 28 days later, mice were challenged with 10x$LD_{50}$ PR8. A: Weight loss of mice relative to the pre-challenge weight was tracked for individual mice every 24 hours. Average values of both groups are presented ± SEM. B: Lung homogenate from challenged mice were assessed for infectious virus titer by $TCID_{50}$. Average values of both groups are presented with horizontal bars with individual titers at each time point/group ± SEM on a $\log_{10}$ scale. *n*=3/time point-group. *n*=1 for 7AT Day 7. C: The % of CD4+ T cells expressing IFNγ (Th1) was tracked from day 3 to 7 post challenge between the groups.

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# CHAPTER 5

## SUBSISTING H1N1 INFLUENZA MEMORY RESPONSES ARE INSUFFICIENT TO PROTECT FROM PANDEMIC H1N1 INFLUENZA CHALLENGE IN C57BL/6 MICE[2]

[2] Sage, L. K., Fox, J. M., Tompkins, S. M., and Tripp, R. A. 2013. *Journal of General Virology* 94:1701-1711

## Abstract

The 2009 swine-origin pandemic H1N1 (pH1N1) influenza virus transmitted and caused disease in many individuals immune to pre-2009 H1N1 influenza virus. While extensive studies on antibody-mediated pH1N1 cross-reactivity have been described, few studies have focused on influenza-specific memory T cells. To address this, the immune response in pre-2009 H1N1 influenza-immune mice was evaluated after pH1N1 challenge and disease pathogenesis was determined. The results show that despite homology shared between pre-2009 H1N1 and pH1N1 strains, the effector memory T cell response to pre-2009 H1N1 was generally ineffective, a finding that correlated with lung virus persistence. Additionally, pH1N1 challenge generated T cells reactive to new pH1N1 epitopes. These studies highlight the importance of vaccinating against immunodominant T cell epitopes to provide for a more effective strategy to control influenza virus through heterosubtypic immunity.


## Introduction

Influenza remains a significant health and economic burden despite the availability of vaccines and therapeutics. As a zoonosis, control is challenging and novel strains often arise some of which have had the ability to productively infect humans [1], such as the emergence of the highly pathogenic H5N1 strain of avian influenza in 2004-2005 [2]. More recently, the 2009 swine-origin H1N1 influenza was transmitted from swine to humans (pH1N1) resulting in a pandemic.

Antibodies generated as a result of influenza infection or vaccination typically are protective against homotypic infections, but often fail to effectively cross-react with novel strains possessing distinct subtypes of hemagglutinin (HA) and neuraminidase (NA) [3]. Low levels of cross-reactivity with novel strains may exacerbate disease and enhance virus replication by a mechanism attributed to Original Antigenic Sin [4]. Interestingly, some elderly individuals with

antibodies against pre-2009 H1N1 strains, including the 1918 H1N1 virus, did not effectively cross-react with pH1N1 virus, yet did not develop substantial disease [5-7]. This result may be attributed to cross-protection mediated by T cells to conserved internal viral proteins, e.g. nucleoprotein (NP) which is known to confer heterosubtypic immunity [8, 9].

While antibody responses against pH1N1 have been studied with regard to cross-reactivity [10], vaccination [11, 12], and pre-existing immunity [13, 14], few studies have examined the contribution of pre-existing memory T cells to the immune response against pH1N1 in a mouse model. While the relatively conserved nature of T cell epitopes likely confers a level of heterosubtypic immunity, it is possible that T cell immunodominance to pH1N1 is different than pre-2009 H1N1 influenza. To evaluate the memory T cell response in this study, mice were primed with a H3N2 strain having HA and NA from A/Hong Kong/1/68 with the internal proteins from A/Puerto Rico/8/34 (X31). The T cell response and subsequent pathology were evaluated following challenge with pH1N1 (A/California/4/2009) or a pre-2009 H1N1 influenza (A/Puerto Rico/9/1934). Mice challenged with pH1N1 had inadequate heterosubtypic T cell responses compared to homotypic T cell responses, and adoptive transfer studies of H3N2-specific memory T cells confirmed the presence of overlapping primary and memory T cell responses to pH1N1 challenge. Subtle differences (1 amino acid) in NP and PA is known to affect MHC Class I restricted immunodominant epitopes [15] and may be sufficient to modify the memory T cell response against pre-2009 influenza and pH1N1 strains. This study shows how the 2009 pandemic H1N1 influenza may evade pre-existing H1N1 influenza memory T cells, and provides important insight into mechanisms that could contribute to immunity, pathology, and provides future consideration for vaccination strategies against influenza.

## Results

*CD8+ T cell epitopes are conserved between pH1N1 and pre-pandemic H1N1 influenza*

To determine whether heterosubtypic immunity might participate in H1N1 cross-protection, CD8+ T cell epitope homology between X31 (H3N2; priming strain) and pH1N1 (challenge strain) were compared to PR8, the prototypical H1N1 heterosubtypic challenge strain [16-18]. The reassortant X31 virus (PR8 X A/Aichi/68, (H3N2) differs from PR8 by expression of H3 and N2 providing an ideal model for comparing memory T cell responses [19, 20]. Amino acid comparison of immunodominant CD8+ T cell epitopes of influenza in Table 1 revealed the highest degree of immunodominance to be nucleoprotein ($NP_{366}$) > acid polymerase ($PA_{224}$) > basic polymerase 1 ($PB1_{703}$) in descending order [21, 22]. CD8+ T cells which recognize these epitopes constitute the majority of the CD8+ T cell response to influenza [23, 24]. pH1N1 has a high degree of homology to PR8/X31 (90-95%). The sixth amino acid of $NP_{366}$ (M371V) is a TCR contact residue [25, 26], and has been shown to affect immunity between PR8 and pH1N1 [27]. This suggests that while there is a high degree of homology, single amino acid differences in NP and PA epitopes may affect the expansion of heterosubtypic cross-reactive memory CD8+ T cells recognizing the same antigen [28].

*Delayed viral clearance following challenge with pH1N1*

Influenza has been known to mutate key amino acid residues with respect to neutralizing antibody epitopes [29, 30], thus suggesting the possibility that the amino acid differences in NP and PA may facilitate pH1N1 evasion of NP- and PA-specific memory CD8+ T cells. To address this, X31 primed mice were challenged with a lethal dose (10x $LD_{50}$) of PR8 (H1N1) or pH1N1. Lung virus titer was determined at days 1, 3, 5, 7, and 9 days post-challenge (Fig. 1). By day 3 post challenge, a 10-fold higher pH1N1 lung viral titer was evident compared to H1N1 challenged mice. H1N1 challenged mice had reduced lung viral titers through day 3, and the virus

was cleared at day 5 post challenge ($<10^2$ $TCID_{50}$/limit of detection). In contrast, lung viral titers in pH1N1 challenged mice remained significantly higher at both days 3 ($p$=0.022) and day 5 ($p$=0.032) post-challenge. Notably, detectable lung viral titers persisted in pH1N1 challenged mice until day 7 post-challenge, i.e. 4 days longer than H1N1 challenged mice. Despite greater lung viral burden in H1N1 challenged mice, none of the pH1N1 challenge mice succumbed to the lethal challenge providing evidence of an effective memory response, albeit with delayed kinetics compared to the response to H1N1 challenge.

*The kinetics of the memory T cell response is delayed in pH1N1 challenged mice*

In the absence of pre-existing neutralizing antibodies, antigen-specific T cells are requisite for viral clearance [31-33]. To address differences in the kinetics of viral clearance between H1N1 and pH1N1 challenged mice, the kinetics of memory T cell reactivation was determined based on the influx of effector T cells, defined as $CD62L^{lo}$ $CD44^{hi}$ [34-36] in the lung airways. At day 5 post-challenge, the majority (85%) of the CD8+ T cells in the BAL from H1N1 challenged mice were $CD62L^{lo}$ $CD44^{hi}$ effector cells, whereas for pH1N1 challenged mice, a lower proportion (55%) of $CD62L^{lo}$ $CD44^{hi}$ effector cells were in the BAL (Fig. 5.2(a), left). At day 9 post-challenge, the frequency of $CD62L^{lo}$ $CD44^{hi}$ effector CD8+ T cells in the BAL of pH1N1 challenged mice was similar to that in H1N1-challenged mice. The frequency of $CD62L^{lo}$ $CD44^{hi}$ effector CD8+ T cells in the MLN was also lower (by approximately 30%) in pH1N1 challenged mice and remained so through the time-course (Figure 5.2(a), right). It is notable that the peak response times of these T cell subpopulations correlated with the time of virus clearance (Fig. 1; day 5 and 9 post-challenge, respectively).

$CD62L^{lo}$ $CD44^{hi}$ CD4+ effector T cells in the BAL were similar in kinetics and frequency regardless of challenge virus strain (Figure 5.2 (b)). However, there were a slightly lower proportion of effector CD4+ T cells isolated from the MLN from day 5 through day 9 post-

pH1N1 challenges. This finding may suggest that CD4+ T cells are recognizing more conserved MHC class II epitopes between H1N1 and pH1N1; this hypothesis could explain why X31-primed mice are protected from a lethal pH1N1 challenge which is consistent with a previous finding showing MHC class II (CD4+ HLA) restricted epitopes were more conserved compared to MHC class I in another pH1N1 strain [37]. The high percentage (>80%) of effector CD4+ T cells may contribute to an anti-viral response, perhaps through the elaboration of cytokines, until there is sufficient CD8+ T cell to clear infected cells,

As the level of memory CD8+ T cells determined by effector phenotype (Fig. 5.2) is inversely proportional to lung virus titer for H1N1 and pH1N1 challenged mice (Fig. 5.1), it is possible that the T cells have different cytotoxic capacities toward H1N1 and pH1N1 infected cells. Thus, the level of memory CD8+ T cell cytotoxicity generated against H1N1 or pH1N1 was evaluated. X31 primed mice were challenged with a lethal dose of H1N1 or pH1N1 and evaluated at day 5 post-challenge, i.e. the peak CTL response, using secondary *in vitro* restimulation and expansion. After *in vitro* expansion, CTL cytolysis was assessed by flow cytometry, however there were no detectable differences in cytotoxicity generated in response to pH1N1 or H1N1 challenge (data not shown). Thus, the intrinsic killing ability of CD8+ T cells does not seem to be affected.

*Virus levels persist and are associated with pathology in pH1N1 challenged mice.*

Histopathology of the lungs and airways following influenza infection results from a combination of events involving immune cells and virus replication [reviewed in [38]], thus the level and tempo of lung pathology was determined. Accordingly, lung histopathology was evaluated in X31-immune mice challenged with PR8 or pH1N1 at days 5 and 9 post-challenge which are the peak T cell response days for H1N1 and pH1N1 challenged mice, respectively. At day 5, mice challenged with PR8 or pH1N1 showed similar levels and types of inflammatory

cells and exudates in the alveoli, but pH1N1 challenged mice had slightly more extensive necrosis of bronchioles (Fig. 5.3(a)). Where inflammation was present, the infiltrates consisted of mainly lymphocytes with small number of macrophages, consistent with the high effector T cell response (Fig. 5.2).

At day 9, pH1N1 challenged mice continued to exhibit pathological signs, but with markedly higher levels of neutrophils in the alveoli compared to the PR8 challenged mice. At day 9, PR8 challenged mice showed attenuated pathology with minimal necrosis and inflammation, whereas pH1N1 challenged mice still had necrosis and inflammatory exudates consistent with the delayed virus clearance (Fig. 5.1). Although no substantial differences were evident by gross histopathology scores (scale of 0-3) between the challenge groups, pH1N1 challenge was associated with a consistently higher average score (Fig. 5.3(b), left panel), and evaluation of specific pathological parameters (necrosis, alveolar exudates) revealed differences at day 9 pi primarily lung exudate in the alveolar lumen (Fig. 5.3 (a), (b)).

The presence of viral nucleoprotein (NP) antigen was evaluated by immunohistochemistry (IHC) (Fig. 5.3(c)). IHC of lung sections revealed little detectable NP antigen at day 5 post-H1N1 challenge in the bronchioles (B) and alveoli (A), but in pH1N1 challenged mice, terminal bronchiole (TB) and surrounding parenchyma showed bronchiolar epithelial cells with NP staining (solid arrows). The surrounding parenchyma displayed inflammatory infiltrate composed of macrophages, neutrophils and lymphocytes. Scattered inflammatory cells (dashed arrows) also exhibit strong nuclear staining. By day 9, NP was mostly undetectable, although there was slight inflammation around a terminal bronchiole (circled) with staining detectable. NP antigen was undetectable at day 9 post-pH1N1 challenge, a feature consistent with the virus titer findings.

*pH1N1 challenge induces a primary and cross-reactive memory T cell response*

To evaluate the cross-reactive memory T cell response, a pilot adoptive transfer study was performed to distinguish whether the T cells responding against pH1N1 were from a memory pool, or recently activated primary T cell response. Donor mice (CD90.1) were primed with X31 and their memory T cells transferred to naïve congenic mice (CD90.2), and challenged with pH1N1 or PBS. At day 9 post-challenge, flow cytometry revealed that donor CD90.1+ T cells (both CD4+ and virus-specific CD8+) were present at higher levels in PR8 challenged mice compared to pH1N1 (data not shown). As this study was not statistically powered, the cross-reactivity of memory T cells was evaluated by ELISPOT. The resting (Fig. 5.4(a)) and effector (Fig. 5.4(b)) memory CD8+ T cell response to pH1N1 immunodominant peptides (Table 1) was evaluated. Treatment with PMA/ionomycin treatment induced a large number of spot forming units (SFU) compared to unstimulated cells as expected. The resting CD8+ T cell memory response to PR8/H1N1 NP was significantly ($p$=0.01) higher compared to pH1N1 NP. The resting memory cell responses were similar between the PR8/H1N1 and pH1N1 PA. The difference in response to NP could be due to a Met to Val mutation occurring in the contact residue which may have a more profound effect on the cellular response to the peptides. It is noteworthy that some cross-reactive CD8+ T cells were able to respond to pH1N1 NP, which likely represents the effector CD8+ T cell fraction which responded to pH1N1 in Figure 2.

At day 5 post-challenge with PR8 or pH1N1, the level of IFNγ expression in effector memory CD8+ T cell in response to the same viral peptides was determined (Fig. 5.4(b)). As predicted, PMA/ionomycin treatment induced higher SFU numbers compared to unstimulated cells. Although not significant, SFU numbers for the controls was higher in H1N1 challenged mice, an effect that may be related to CD4+ and CD8+ T cell bystander activation. Thus, this does not represent strictly virus-specific CD8+ T cell responses. pH1N1 challenged mice had a higher SFU response ($p$=0.008) to pH1N1 NP peptide compared to PR8/H1N1 NP, a finding

consistent with the hypothesis that a low frequency of X31 memory cells are responding to pH1N1 peptides, and that a novel set of T cells is responding to pH1N1 peptides. It is interesting to note that the response to PR8/H1N1 NP is comparable at day 5 (Fig.4(b)) even though the NP response was higher in resting memory cells ($p$=0.01; Fig. 5.4(a)). In addition, the PR8/H1N1 challenge group mounted a more robust response ($p$=0.009) compared to PR8/H1N1 PA, although there was no difference in the resting memory T cells response. This could be due to skewing of the memory response towards PA and PB1 in secondary responses over NP [39]. Thus, subtle differences which may not be evident in a resting memory T cell subpopulation can become exaggerated in effector memory T cell responses. This may also explain why there is little difference between the challenge groups to PR8/H1N1 NP. Finally, stimulation with PB1 did not show any significant differences as expected because it is identical between both viruses.

## Discussion

This study shows that mice with T cell immunity to pre-2009 H1N1 mount a different response upon challenge with the pH1N1 compared to homotypic challenge with H1N1 (PR8). Interestingly, X31-immune mice mount a memory T cell response against pH1N1, as well as a primary response to pH1N1, resulting in delayed virus clearance and corresponding to exacerbated lung histopathology compared to PR8 challenge.

One explanation may be that PR8 challenged mice have a higher frequency of cross-reactive memory T cells compared to pH1N1 challenged mice. This explanation is supported by the findings in this study where after challenge, a higher proportion of memory T cells recognize cross-reactive immunodominant epitopes and are able expand and traffic to the airways to exert their effector functions evident by the higher number and proportion of effector ($CD44^{hi}$ $CD62L^{lo}$) T cells. These findings are corroborated by results from adoptive transfer studies of memory T cells from X31-immune mice being present at higher levels in PR8-challenged mice compared to

pH1N1-challenged mice. The presence of cross-reactive memory T cells responding to pH1N1 was supported by the survival of mice following lethal challenge, since X31-primed mice were challenged with an equal lethal dose of PR8 or pH1N1. Substantial differences in the kinetics of the T cell response and in CTL cytotoxicity were expected, but no detectable differences in the ability of memory CD8+ T cells generated in response to PR8 and pH1N1 challenge were observed. This suggests that the intrinsic CTL cytotoxicity is comparable in response to pH1N1 and PR8 infected cells, and is consistent with previous studies that have dissected the difference in T cell clonotypes [40, 41] and TCR usage [42], and whose findings revealed very small differences in T cells responding to the different viruses and their epitopes. Future studies will investigate the properties of memory CD8+ T cells in the response to pH1N1 and homotypic and heterosubtypic responses to influenza vaccination and challenge.

The difference in the level of CD4+ effector T cells were less pronounced between PR8 and pH1N1 challenged mice. One possibility is that a higher proportion of the memory CD4+ T cells are able to recognize conserved pH1N1 MHC Class II restricted epitopes. It is likely that effector CD4+ T cells contribute to protection against the lethal influenza challenge in this model as had been previously for other influenza studies [31, 32].

The primary T cell response following pH1N1 challenge may be explained by several mechanisms including by the level of viral antigen in the lungs. Established memory CD8+ T cells have been shown to suppress clonal expansion of naïve CD8+ T cells specific for identical and similar epitopes from the same pathogen [43]. This threshold may have been sufficient so that memory CD8+ T cell activation did not occur, allowing APCs to present antigen to naïve T cells in pH1N1 challenged mice. This is supported by virus titer ($TCID_{50}$) and antigen clearance (IHC) findings where virus antigen persisted at least 4 days longer compared to the H1N1 challenge.

It would have been optimal to compare the immune response in X31 primed mice to naïve mice challenged with pH1N1 as a positive control for a primary response, but naïve mice

do not survive beyond day 7 following lethal challenge (data not shown). Accordingly, a lower dose of pH1N1 challenge using 1 x $LD_{50}$ (70 pfu) was investigated, but yielded very low lung virus titers after intranasal infection preventing comparison (data not shown). However, the results presented here provide evidence that small differences in T cell response can be attributed to differences in the sequence between PR8 and pH1N1. While the overall homology is high (90%), the difference in the immunodominant epitopes as shown in Table 1 (H-2$D^b$ $NP_{366}$ and H-2$D^b$ $PA_{224}$) affects the CD8+ T cell response when challenged by pH1N1. The ELISPOT results (Figure 5.4) highlight this method as a useful indicator of T cell cross-reactivity for examining immunodominant epitopes when comparing the T cell response to closely related viruses, as IFNγ expression in response to immunodominant viral peptides showed some remarkably different responses. While the ELISPOT may serve as a useful indicator of cross-reactivity, it does not indicate to what extent the CD8+ T cells are cross-reactive, i.e. TCR avidity to MHC-peptide. This is important as different avidity induces different responses including cytokine secretion [44], subtle changes in immunodominant epitopes in pH1N1 can affect the host response and lead to increased immune evasion as has been observed regarding protection against H5N1 avian influenza [45, 46].

Although antibodies have a significant role neutralizing influenza, studies evaluating pre-2009 influenza antibody cross-reactivity against pH1N1 have concluded that is generally no cross-reactive antibodies, however there may be cross-protective antibodies [10, 47] such as antibodies against NP [48-50] and M1 [20]. As these proteins are not cell surface expressed, T cells dominate the response against these proteins. To our knowledge, this is the first study to evaluate the role of pre-2009 memory T cell responses against these pandemic influenza proteins. The study we presented here emphasize the need for generating cross-protective memory T cells following vaccination, and the need for considering pre-existing T cell responses when designing vaccination strategies against influenza.

## Materials and Methods

*RNA Isolation and Sequencing*

Viral RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA). The NP, PA, and PB1 gene segments for direct sequencing were amplified using the One-step RT-PCR kit (Qiagen) according to manufacturer's instructions. Purified cycle sequencing products were loaded onto an ABI 3130XL genetic analyzer (Applied Biosystems, Carlsbad, CA) and separated by capillary electrophoresis through a 80cm capillary array. Resulting sequence traces were trimmed and assembled using Sequencher software (Genecodes, Ann Arbor, MI). Nucleotide coding sequences were translated into the corresponding amino acid sequences using the online ExPASy Proteomics Server (http://www.expasy.ch/) provided by the Swiss Institute of Bioinformatics. Amino acid sequences of X31 and H1N1 were then aligned with pH1N1 sequence using the National Center for Biotechnology Information's Influenza Virus Sequence Database: http://www.ncbi.nlm.nih.gov/sites/genome

*Influenza Viruses and Infection of Mice*

Influenza strains H1N1 (A/Puerto Rico/8/1934) and X31, a recombinant virus with the HA and NA (H3N2) derived from A/Hong Kong/1/68 with the internal proteins (e.g., NP, PA, PB1) from Puerto Rico/8/1934 were propagated in 9 day old embryonated chicken eggs as previously described (39). Allantoic fluids were extracted and stored at -80˚C until use. Mouse adapted influenza A/California/04/2009 (pH1N1; a kind gift from Daniel Perez) was grown *in vitro* by infecting MDCK cells in MEM (HyClone, Rockford, IL) supplemented with L-Glutamine and 1 µg/mL TPCK-Trypsin (Worthington, Lakewood, NJ) at a multiplicity of infection (MOI) of 0.01. 3 days after infection, cell culture supernatant was collected by centrifugation and stored at -80°C.

For infections, 8-10 week old female C57BL/6 (National Cancer Institute, Bethesda, MD) mice were anaesthetized with 2,2,2-tribromoethanol (Avertin) [51]. Subsequently, mice were intranasally instilled with 1000 plaque forming units (PFU) of X31 diluted in 50μL of PBS. Thirty-six days after priming with X31, mice were challenged in the same manner with 10 x $LD_{50}$ of either H1N1 (1000 pfu) or pH1N1 (700 pfu). All mouse work was approved by the Institutional Animal Care and Use Committee of the University of Georgia.

*Cell preparation and flow cytometry*

At days 5 and 9 after H1N1 or pH1N1 challenge, mice were euthanized and bronchoalveolar leukocytes (BAL) collected by instillation of 1.0 mL of PBS (HyClone) in the trachea using an 18-Gauge catheter followed by two PBS washes in the same volume. Mediastinal lymph nodes (MLN) were removed and single cell suspensions prepared in Hank's Buffered Saline Solution (HBSS, HyClone, Rockford, IL) following passage through 100μm cell strainers (BD Biosciences, San Jose, CA). Cell numbers from the BAL and MLN samples were enumerated using a Z2 Coulter Counter (Beckman-Coulter, Brea, CA) after lysing erythrocytes using Zapo-Globin II (Beckman-Coulter) according to manufacturer's instructions.

To analyze cells by immunophenotyping, $10^6$ BAL or MLN cells were added to wells of round bottom 96-well plates (Corning, Lowell, MA), washed, and resuspended in FACS Buffer [Dulbecco's PBS (HyClone) with 0.09% (w/v) $NaN_3$ (Sigma-Aldrich), 2mM EDTA (Fisher Scientific, Pittsburgh, PA), and 1% (w/v) Bovine Serum Albumin (Sigma-Aldrich)] as previously described [52]. Plated cells were treated with 1μg/mL of FcBlock (BD Biosciences) to inhibit non-specific binding of antibodies. The cells were washed and stained at room temperature for one hour for CD8α, CD4, CD62L, and CD44 (BD Biosciences) in combination with MHC Class I Tetramers (H-2D/$K^b$) loaded with the appropriate immune dominant peptides: $NP_{366-374}$: ASNENMETM (H-2$D^b$), $PA_{224-233}$: SSLENFRAYV (H-2$D^b$), $PB1_{703-711}$: SSYRRPVGI (H-2$K^b$).

All tetramers were provided by the National Institute of Health (NIH) Tetramer Facility (Emory University, Atlanta, GA).

Surface staining was followed by intracellular IFN-γ cytokine staining [44]. Briefly, the cells were fixed and permeabilized with fixation/permeabilization buffer and washed with permeabilization buffer (eBioscience, San Diego, CA) and stained against IFNγ in permeabilization buffer for 30 minutes at 4°C. The cells were then washed with permeabilization buffer, and analyzed by flow cytometry using a BD LSR-II (BD Biosciences). At least 50,000 events were recorded following gating on CD4+ or CD8+ T cells. Data was captured and analyzed using BD FACSDiva (BD Biosciences).

*Determining Influenza Viral Titer by $TCID_{50}$*

Infectious virus titer was measured by $TCID_{50}$ as previously described [27]. Briefly, following challenge with H1N1 or pH1N1, mice were euthanized and their lungs excised and placed in 1mL of PBS with 100 μg/mL of Streptomycin, 100 IU/mL of Penicillin, and 250ng/mL of Amphotericin B (Mediatech, Irving, TX). After homogenization with a tissue lyser (Eppendorf, Hamburg, Germany), the homogenate was centrifuged and the supernatant stored at -80˚C. MDCK cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, HyClone) with 5% (v/v) heat-inactivated Fetal Bovine Serum (FBS) (HyClone) at 37˚C in 96 well plates. Serial dilutions of virus in 100μL of Minimum Eagle's Medium (MEM, HyClone) containing 100 μg/mL of Streptomycin, 100 IU/mL of Penicillin, and 250ng/mL of amphotericin B (Mediatech), and 1μg/mL of TPCK-Trypsin (Worthington, Lakewood, NJ) was added to each well. The plates were incubated for 72 hours at 37°C and mixed with equal volume of 0.5% (v/v) chicken erythrocytes in PBS and incubated at room temperature for one hour. Hemagglutination was scored according to standard $TCID_{50}$ methods as described and normalized to weights of the extracted lung [27].

*In Vitro Restimulation and CTL Killing Assay*

Mice primed with X31 and challenged with H1N1 or pH1N1 were used to obtain memory T cells which were expanded as previously described with minor modifications [53]. Briefly, memory T cells were isolated from the spleens and MLN at 5 day post-PR8 challenge of X31 immune mice and stimulated *in vitro* with naïve syngeneic splenocytes co-incubated with 100 hemagglutinin units (HAU) of X31 for 12h at 37C followed by mitotic inactivation using Mitomycin C (Sigma-Aldrich) [54]. The secondary *in vitro* restimulation was maintained for 6 days at 37C in complete RPMI (RPMI 1640 with 10% FBS, antibiotics, 50μM β-mercaptoethanol, and 10U/mL of recombinant mouse IL-2 [BD Biosciences]). After stimulation, the cell cultures were washed by centrifugation and the T cells co-incubated at indicated effector-to-target ratios with syngenic MC57G target cells infected with 100HAU of PR8 for 12h at 37°C. The target cells were stained with PKH67 (Sigma-Aldrich) according to manufacturer's instructions. The CTL and target cells were added to 96 well V-bottom plates (Corning) and gently centrifuged (200xg for 1 minute) to maximize cell contact and incubated at 37°C for 4 hours. Cell cytotoxicity was analyzed by flow cytometry, i.e. after co-culture for 4 hours, MC57G (PKH67+) were gated and assessed for apoptosis as defined by binding of APC-Annexin V (early apoptosis) or double positive for 7AAD and Annexin V (late apoptosis), but not 7AAD alone (necrosis) [55].

*Histopathology and Immunohistochemistry (IHC)*

Lungs from mice challenged with pH1N1 or H1N1 were perfused and fixed in cold 10% (w/v) neutral buffered formalin (Fisher). The lungs were treated with paraffin and sectioned as previously described [56]. Briefly, 5μm sections of lungs were cut and stained with Hematoxylin and Eosin (H&E). Expression of influenza antigen was also examined by immunohistochemistry following deparaffinizing and rehydrating the same tissues. For this analysis, sections were

blocked with 1% bovine serum albumin in PBS and treated with Proteinase K (Dako, Carpentaria, CA) to minimize non-specific staining and expose epitopes. Subsequently, sections were incubated with 1 µg/mL goat anti-influenza A H1N1 antibody (Meridian Life Science, Inc., Soca, ME) diluted in Dako antibody diluent (Dako) for 1 hour at 25˚C, washed with PBS, and then incubated with a biotinylated anti-goat rabbit antibody (Dako) added at a concentration of 5 µg/mL in Dako antibody diluent for 10 minutes at room temperature. Finally, strepavidin/horseradish peroxidase (HRP) complex (Dako) was added according to manufacturer's instructions for 10 minutes with color development by HRP substrate diaminobenzidine (DAB) addition for 10 minutes and assessed by microscopy. Histopathology and IHC of lungs were evaluated in a single blind manner by a certified, independent pathologist in the College of Veterinary Medicine at the University of Georgia.

*Adoptive transfer of congenic memory cells*

A congenic mice model was used to assess the source of T cells (primary or memory) responding to pH1N1 or H1N1 challenge by adapting methods previously described [57, 58]. Briefly, CD90.1$^{+}$ C57BL/6 (donor) mice were primed via intranasal infection of $10^{3}$ pfu of X31 and rested for 28 days. Mice were sacrificed to extract single cells from mediastinal lymph node, mesenteric lymph nodes, and spleen. CD3+ T cells were enriched by negative selection using AutoMACS (Miltenyi Biotec, Auburn, CA) magnetic cell separation kit according to manufacturer's instructions. Post-enrichment analysis by flow cytometry confirmed that the cells were > 98% CD3+. Memory cells were sorted from CD3+ T cells by isolating CD44$^{hi}$ cells [21] using a cell sorter BD FACS Aria II (BD Biosciences). Post-sort analysis revealed that the sorted cells were >90% memory CD3+ T cells. The cells were washed and resuspended in PBS for intraperitoneal injection to CD90.2$^{+}$ congenic C57BL/6 mice ($10^{5}$ memory T cells/recipient). Mice were then allowed to rest for one day after the adoptive transfer and then challenged with 0.1 x $LD_{50}$ either

H1N1 or pH1N1. At day 9 post challenge, mice were sacrificed and the cells from MLN, spleen, and BAL were immunostained and analyzed by flow cytometry for: CD90.1, CD90.2, CD8+, IFNγ, CD44, CD62L, expression and the CD8+ T cells' epitope specificity by MHC Class I tetramers. Only 1 of 3 mice survived until day 9, so *n*=1 for the H1N1 challenge.

*ELISPOT*

ELISPOT was used to assess the frequency of IFNγ expressing memory CD8+ T cells responding to pH1N1 and H1N1 immunodominant peptides as previously described [23]. Briefly, multi-screen ELISPOT plates (Millipore, Billerica, MA) was coated with 10μg/mL of anti-mouse IFNγ antibody (capture antibody; BD Biosciences) overnight at 4°C. At day 5 post challenge, mice were euthanized and serial dilutions of single cell suspensions of splenocytes and MLN were extracted and plated onto the antibody coated plates ($5x10^5$ to $1.25x10^5$ cells/well) and cultured in complete RPMI. The splenocytes were stimulated with 10μM of H1N1 or pH1N1 derived NP, PA, and PB1 derived peptides (Table 1), in addition to an irrelevant peptide ($GFP_{200-208}$ HYLSTQSAL), no peptide (cells only), or phorbol myristate acetate and ionomycin at 25 and 1.25ng/mL, respectively, for maximum IFNγ induction. All peptides were custom manufactured by New England Peptides (Gardner, MA). 2 days after culture, the plates were washed away with PBS to remove cells. After 3 washes, biotinylated anti-IFNγ (detection antibody; BD Biosciences) resuspended in PBS with 0.5% (v/v) FBS was added and incubated at room temperature for 1 hour. The detection and capture antibodies recognize distinct epitopes and does not interfere with each other's binding to secreted IFNγ. After washing again, strepavidin-alkaline phosphatase (Thermo Scientific) was added at 1:500 dilution in PBS with 0.5% FBS for 30 minutes. Finally, the plates were washed 5 times with PBS and IFNγ spots were developed using One-Step BCIP/NBT kit (KPL, Gaithersburg, MD) according to manufacturer's instructions, and the number of spots between experimental groups was compared.

*Statistics*

All data points were assessed for statistical significance between means of 1MT treatment and control ($p$ value $\leq$ 0.05) using a one-tailed Student's $t$-test. Exact $p$-values are listed when significant. All statistical analyses were performed using Graph Pad Prism software (Graph Pad Software, San Diego, CA) and graphs displayed with Microsoft Excel (Microsoft).

## Tables and Figures

**Table 5.1**

| Virus | % Amino Acid Identity Relative to X31 | | | Residues of Immunodominant Epitopes | | |
|---|---|---|---|---|---|---|
| | NP | PA | PB1 | NP 366-374 | PA 224-233 | PB1 703-711 |
| A/Puerto Rico/8/1934 (PR8; H1N1) | 100% | 100% | 100% | ASNENMETM | SSLENFRAYV | SSYRRPVGI |
| A/California/4/2009 (pH1N1) | 91.3 % | 95.5 % | 95.2 % | ASNEN**V**ETM | **P**SLENFRAYV | SSYRRPVGI |

**Table 5.1: Amino acid comparison of immunodominant epitopes of influenza.** Amino acid sequences of influenza internal proteins NP, PA, and PB1 containing immunodominant epitopes, derived from genomic RNA sequence comparison between the priming strain X31 and challenge strains, H1N1 and pH1N1. % Amino acid identity is indicated. Where difference is present in the immunodominant epitopes, the residue is bolded and underlined for the pH1N1.

**Figure 5.1**

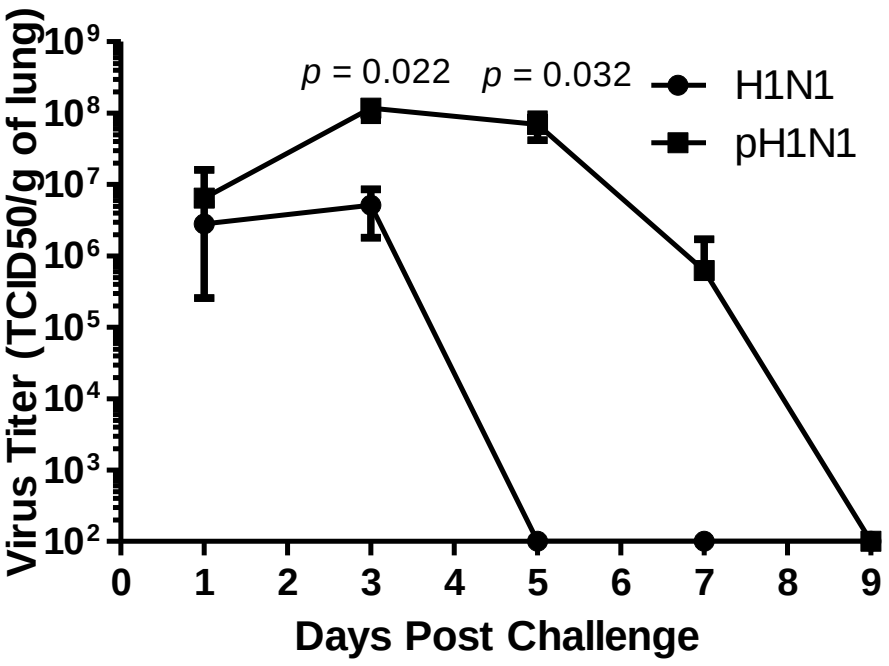



**Figure 5.1: pH1N1 persists longer than H1N1 upon challenge.** Mice were primed with X31. Twenty-eight days after whole lungs from either PR8 (H1N1, circle) or pH1N1 (square) challenged mice were homogenized and viral titers assessed by $TCID_{50}$ at days 1, 3, 5, 7, and 9 days post challenge. Exact *p* - values are indicated where $p<0.05$. Data is representative of three independent experiments.

**Figure 5.2**

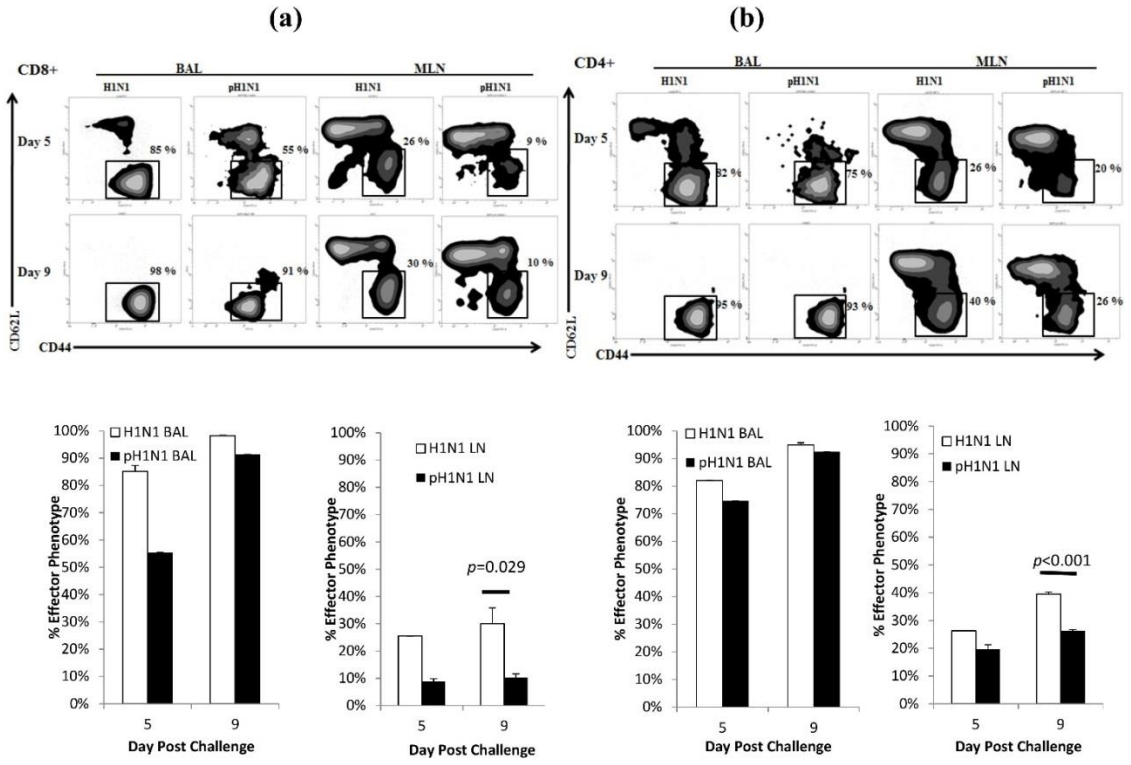



**Figure 5.2: Effector T cell responses are delayed in pH1N1 compared to PR8 challenge.** X31 primed mice were rested for 28 days and then challenged with PR8 or pH1N1. Representative contour plots of effector ($CD44^{hi}$ $CD62L^{lo}$) CD4+ and CD8+ T cells at days 5 and 9 post challenge, the proliferative peak of T cells in response to H1N1 and pH1N1 challenge, respectively. Gated plots of CD8+ (Panel A) and CD4+ (Panel B) T cells show CD62L CD44 expression from BAL (left) and MLN (right) for PR8 and pH1N1 challenge. Average percentage from three mice/experiments of CD4+ and CD8+ T cells expressing the effector phenotype are indicated above the gate. Bar graphs showing the average percentage +/- SEM are also included below each panel. Data is representative of two independent experiments.

**Figure 5.3 A**

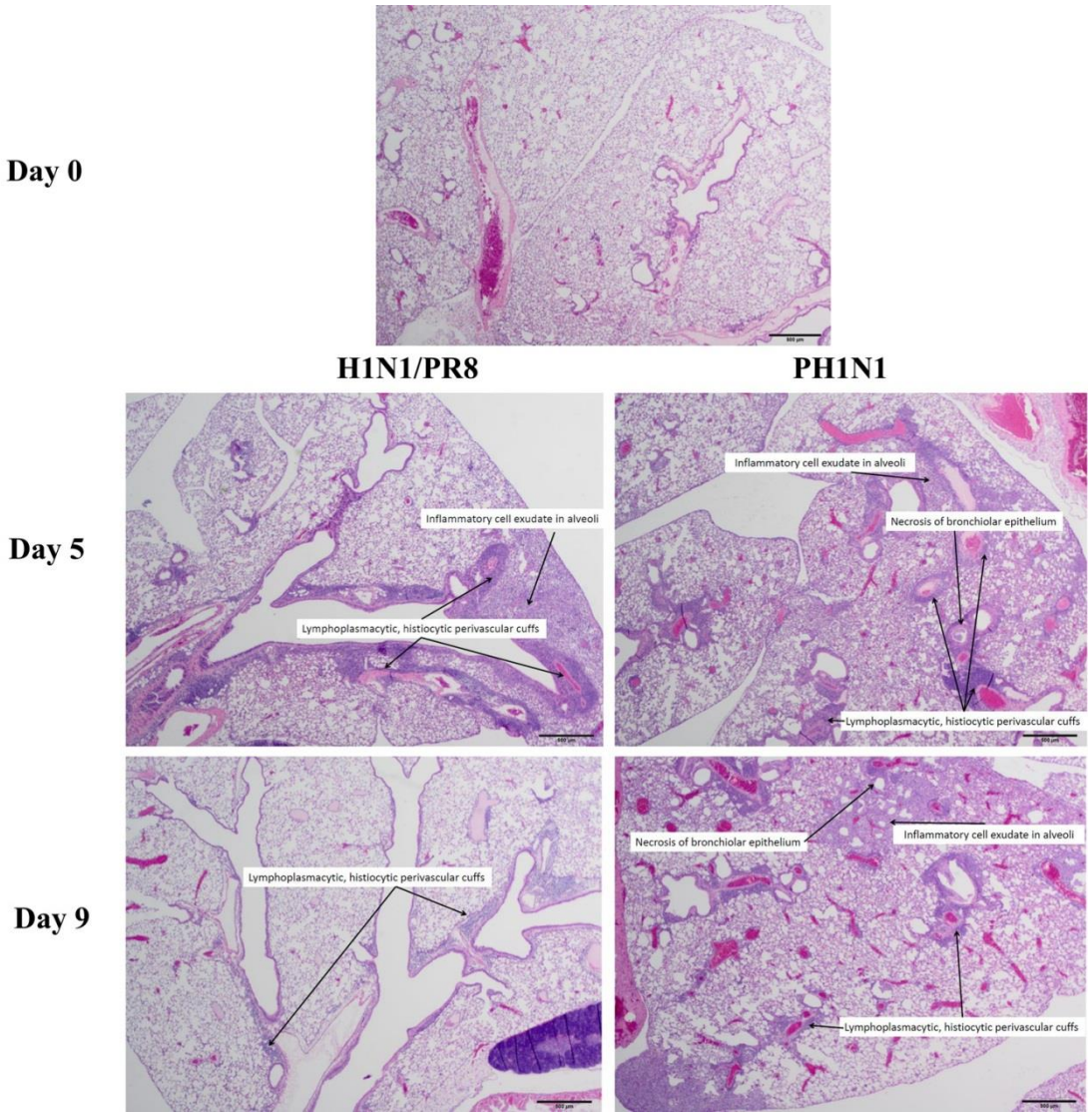

Day 0
H1N1/PR8
PH1N1
Day 5
Inflammatory cell exudate in alveoli
Lymphoplasmacytic, histiocytic perivascular cuffs
Inflammatory cell exudate in alveoli
Necrosis of bronchiolar epithelium
Lymphoplasmacytic, histiocytic perivascular cuffs
Day 9
Lymphoplasmacytic, histiocytic perivascular cuffs
Necrosis of bronchiolar epithelium
Inflammatory cell exudate in alveoli
Lymphoplasmacytic, histiocytic perivascular cuffs

**Figure 5.3 B**

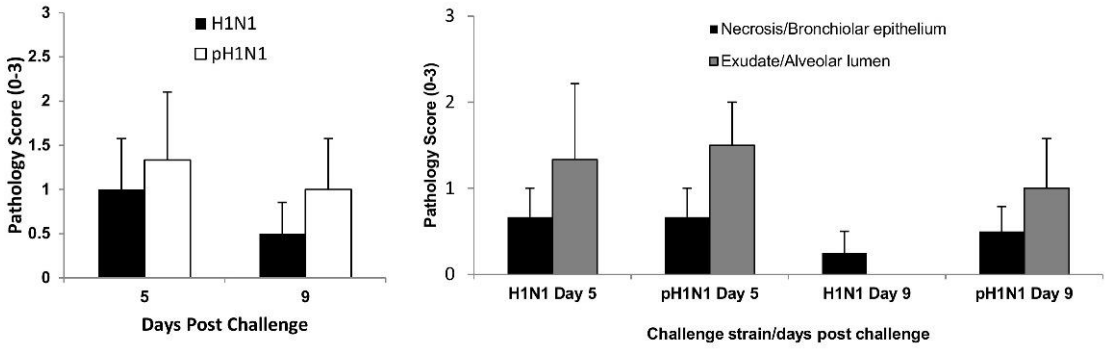



**Figure 5.3 C**

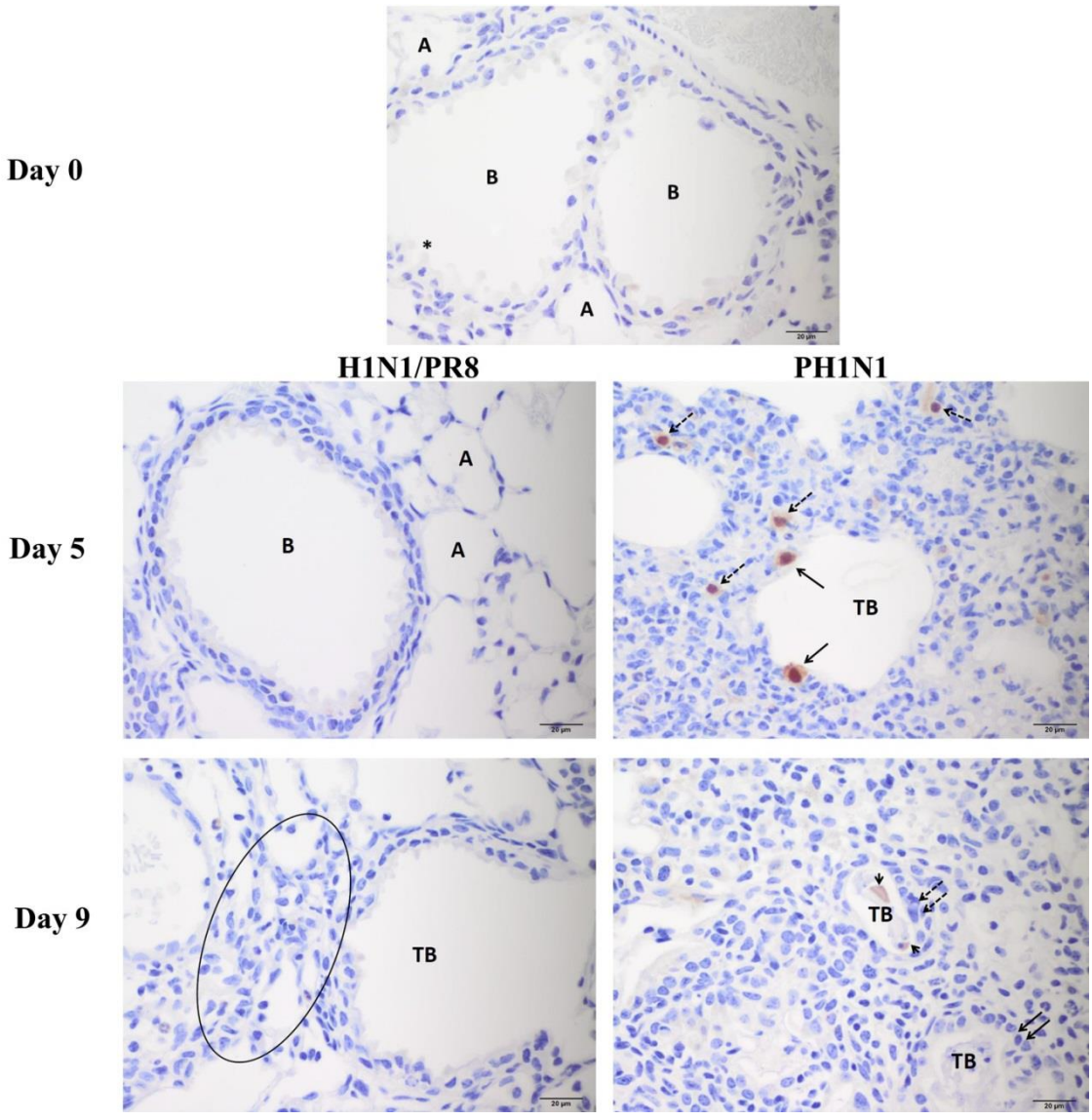
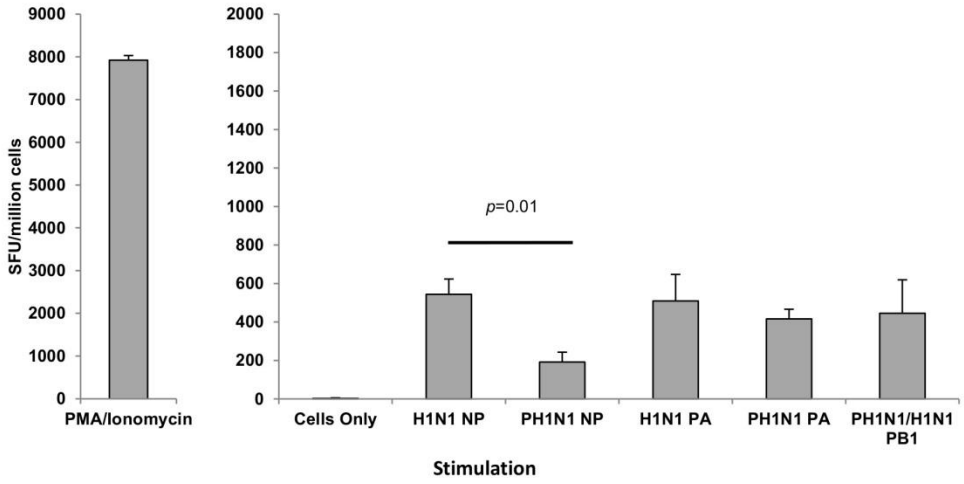


**Figure 5.3: Lung pathology is more severe and viral antigen persists in mice challenged with pH1N1 compared to PR8.** X31 primed mice were rested for 28 days and then challenged with PR8 or pH1N1. A: Whole lungs from challenged mice were fixed, sectioned, and evaluated for histopathology by H&E staining for various parameters which constitute pulmonary lesions. Panels are representative H&E stains of triplicate lung samples. Scale bars show the cross section length in each panel. Day 0 shows representative normal, uninfected tissue for both experimental groups. Specific pathological parameters are noted on each figure B: Bottom bar graphs indicates the comparative gross histopathological scores between challenge groups in addition to scores associated with specific pathological parameters (right) at day 5 and 9 post challenge. C: Representative immunohistochemistry images of influenza antigen (H1N1) on days 5 and 9 post challenge with PR8 or pH1N1 ($n$=3/group). Day 0 shows representative normal tissue lacking influenza antigen for both experimental groups. The images and scores are representative of two independent experiments.

**Figure 5.4**

**(a)**


SFU/million cells
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
PMA/Ionomycin
2000
1800
1600
1400
1200
1000
800
600
400
200
0
p=0.01
Cells Only
H1N1 NP
PH1N1 NP
H1N1 PA
PH1N1 PA
PH1N1/H1N1 PB1
Stimulation

**(b)**

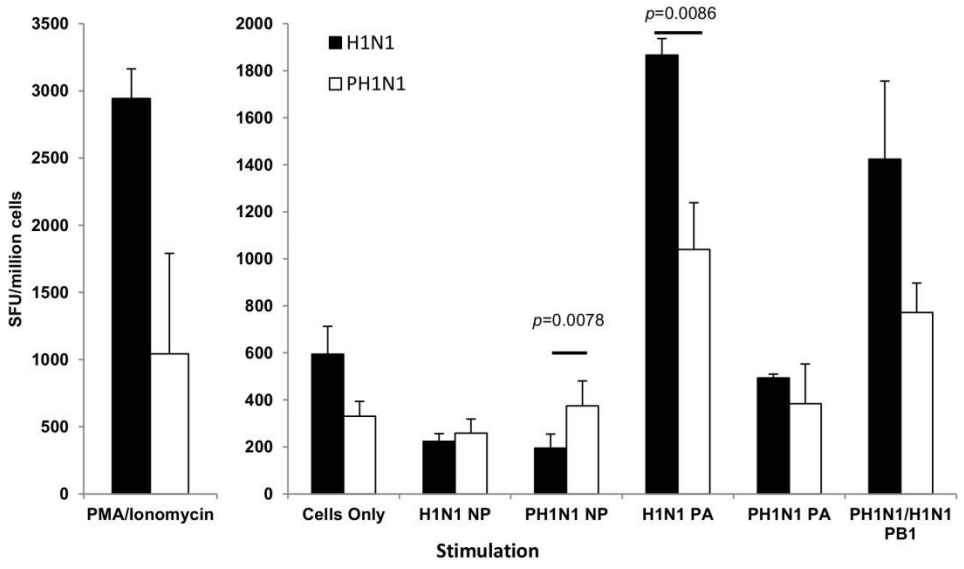

SFU/million cells
3500
3000
2500
2000
1500
1000
500
0
PMA/Ionomycin
2000
1800
1600
1400
1200
1000
800
600
400
200
0
H1N1
PH1N1
p=0.0086
p=0.0078
Cells Only
H1N1 NP
PH1N1 NP
H1N1 PA
PH1N1 PA
PH1N1/H1N1 PB1
Stimulation

**Figure 5.4: Cross-reactivity of memory and resting CD8+ T cells measured by ELISPOT against immunodominant epitopes.** Resting (Figure 4A) memory CD8+ T cell response from X31 primed mice were evaluated by measuring the number of IFNγ spot forming units (SFU) per million cells stimulated with immunodominant epitopes or media alone (cells only). Effector (4B) memory CD8+ T cells were evaluated by challenging X31 primed mice with PR8 (H1N1) or pH1N1. 5 days later, the spleen cells were evaluated for SFU in response to peptides. PMA/Ionomycin was used as positive control and is listed separately. The cells only control were stimulated with media only with no peptides. Statistical significance is shown by error bars representing SEM, and *p* values as indicated for the comparisons indicated. Data is representative of three independent experiments.

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# CHAPTER 6

# CONCLUSIONS

This dissertation has examined the immune response against influenza A virus infection in a mouse model, specifically the role of heterosubtypic immunity, and indoleamine 2,3-dioxygenase in the modulation of memory T cell response against influenza challenge. The following Specific Aims in the Introduction were addressed and the conclusions are as follows.

- **IDO inhibition during primary response to influenza enhances the memory T cell response**

  This study showed that IDO inhibition during primary immune response against influenza virus increased the antiviral memory T cell response, specifically the CD4+ Th1 response and virus-specific IFNγ+ CD8+ T cell response. The CD8+ T cells had broader epitope specificity, and CD8+ T cells which are specific for subdominant epitopes were overrepresented. Although virus clearance was not affected, pathological parameters correlated with accelerated tissue repair were increased. Finally, IDO inhibition decreased regulatory T cell CTLA-4 expression due to IDO inhibition was decreased in response to influenza virus challenge. This work demonstrates the role of IDO during memory T cell formation and serves to uncover the relationship between IDO, memory T cells, influenza virus, as well as the functional consequences of their interaction.

- **IDO inhibition during vaccination to influenza enhances the memory T cell response**

  This study shows promising results in IDO inhibition during conventional influenza vaccination modified various immunological parameters that are correlated with

protection. Although no one vaccine delivery method or IDO inhibitor displayed superior protective immunity in all aspects, IDO inhibition resulted in increased antibody response, T cell response, faster virus clearance, depending on the route of vaccination and IDO inhibitor. This work indicates that IDO inhibition in conventional influenza vaccines may be a solution to the problems with the current influenza vaccines.

- **2009 pandemic H1N1 influenza evades subsets of memory T cells mounted against pre-2009 H1N1 influenza**

  This study showed that the T cell immune response generated by effector memory T cells in response to pre-2009 H1N1 was generally ineffective against pH1N1. Consequently, the pH1N1 virus was able to persist in the lungs longer and cause exacerbated pathology compared to infection with pre-2009 H1N1 strains. This is likely caused by the lack of reactivity against novel pH1N1 epitopes, as evidenced by the generation of a second wave of T cells after the influx of memory T cells. This study highlights the importance of vaccinating against a broad range of immunodominant T cell epitopes in conferring a more effective heterosubtypic immunity to control influenza virus.

Collectively, these results emphasize that heterosubtypic immunity is critical and one of the ways to induce protection against a broad range of influenza viruses and that IDO is important for the regulation of formation of virus specific memory T cells to enhance heterosubtypic immunity and holds a potential for incorporation in vaccine development. Thus, the use of IDO inhibitors in vaccines may be a promising way to enhance the antiviral memory response against future influenza epidemics and pandemics which plague various avian and mammalian species.