LOCALIZATION OF VIRUS NEUTRALIZING I/LNJ H2-Oβ ALLELE USING CONFOCAL MICROSCOPY

By

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And approved by

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ABSTRACT OF THE THESIS

Localization of Virus Neutralizing I/LnJ H2-Oβ Allele Using Confocal Microscopy

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Class II MHC peptide presentation is a key process in the adaptive immune response and maintenance of self-tolerance. The loading of peptides on to the class II MHC molecule is facilitated by the non-classical MHC molecule H2-M. The actions of H2-M are inhibited when complexed with another non-classical MHC molecule, H2-O. A functionally null H2-O molecule has been identified in the I/LnJ strain of mice; this strain has the ability to clear persistent retroviruses that other strains (B6 and BALB/cJ) cannot. This study aims to localize the I/LnJ H2-O peptide in dendritic cells using confocal microscopy in order to determine if mis-localization of free I/LnJ H2-O contributes to the ability of I/LnJ mice to produce retrovirus neutralizing antibodies. Preliminary data suggests that free I/LnJ H2-O is localized to compartments other than the endosome and endoplasmic reticulum, which has been previously reported. Data suggests that free I/LnJ H2-O may localize to mitochondria, which introduces the potential for a novel H2-O function in these mice.

Acknowledgement

I would like to thank Dr. Lisa Denzin for taking a chance on a “non-conventional” masters student and allowing me to learn in her lab. This research would not have been possible without the assistance of Francesca Virdis, Louis Osorio, and the rest of the Denzin/Sant’Angelo lab. I also have to thank my students and my family for inspiring me to continue learning.

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Introduction

The adaptive immune response is dependent upon antigen presentation at the surface of antigen presenting cells, such as dendritic cells (DCs) and B cells. Stable peptides are presented by major histocompatibility complex proteins (MHC) at the cell membrane. MHC proteins, both class I and class II, are polygenic; these highly polymorphic genes are coded for by the Human Leukocyte Antigen (HLA) genes in humans and H-2 genes in mice. The high degree of polymorphism allows for the presentation of a diverse peptide repertoire, containing both antigenic and self-peptides.[2] MHC class I proteins on the surface of antigen presenting cells (APCs) present peptides to $CD8^+$ T cells, while MHC class II on APCs present peptides to $CD4^+$ T cells. The binding of APCs to these T cells allows for the initiation of the adaptive immune response, as well as the maintenance of self-tolerance.

The focus of this thesis is the MHC Class II protein (Figure 1A), which is a four-subunit transmembrane glycoprotein composed of two immunoglobulin domains ($\alpha 2$ and $\beta 2$) and two protein binding domains ($\alpha 1$ and $\beta 1$).[6] The protein binding domains create a binding groove capable of presenting a complex mix of self and non-self-peptides with shared motifs containing a negatively charged and hydrophobic amino acid. Each allelic variant of MHC Class II is capable of binding to a different collection of peptides, therefore organisms containing multiple alleles can present a broad peptide repertoire at the cell surface. This diverse binding ability allows for the activation of an immune response when confronted with a multitude of pathogens, as well as the prevention of an autoimmune response.

The process of MHC class II antigen presentation begins in the endoplasmic reticulum, where the α and β subunits (1 and 2) are assembled. This assembly is facilitated by the invariant chain (I chain); a non-polymorphic, chaperone protein not encoded in the H-2/HLA region. The I chain contains a di-leucine repeat at the N-terminus of the cytoplasmic domain, which directs the MHC class II molecule to endosomal compartments where they will be loaded with an antigenic peptide for presentation.[6] Another functional domain of the I chain is Class II-associated Invariant Chain Peptide (CLIP); CLIP fits into the MHC Class II peptide binding groove (Figure 1B) to prevent the premature binding of peptides while MHC is still in the endoplasmic reticulum and Golgi apparatus.[6]

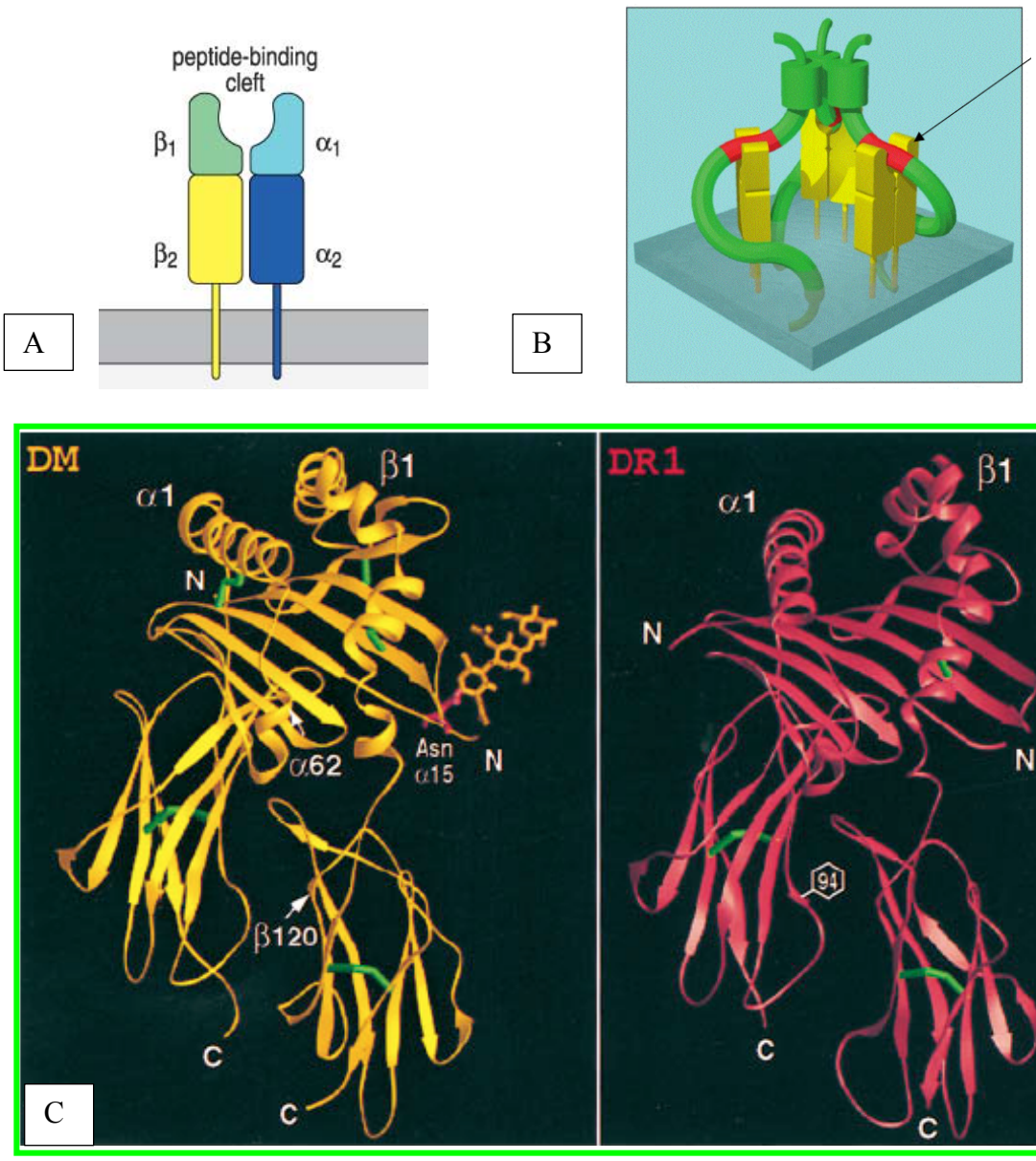

Figure 1: Structure of MHC Class II (DR1) and HLA-DM/H2-M Regulator

(A) Schematic diagram of MHC Class II showing four subunits and peptide binding cleft (labeled).[2]

(B) Schematic diagram of MHC Class II (yellow) bound to invariant chain (green) with CLIP domain (red-indicated by arrow) filling the peptide binding site.[1]

(C) Side by side comparison of human MHC Class II (pink) and HLA-DM (yellow) secondary structure.[3]

Upon arrival in endosomes and lysosomes, the majority of the invariant chain is degraded by resident proteases that are activated due to the acidic pH of these compartments. However, CLIP remains bound in the groove of the MHC class II molecule until the presence of highly stable antigenic or self-peptides triggers its removal by HLA-DM/H2-M (referred to as DM), a MHC Class II regulator. DM is structurally similar to MHC class II (Figure 1C) except for the lack of a peptide binding groove. DM edits antigenic peptides to create highly stable molecules, removing low affinity peptides from the binding groove of MHC, as well as stabilizing empty MHC class II (without CLIP).[7] The latter is facilitated by the induction of a rearrangement of the MHCII peptide groove when DM binds to the Class II/CLIP complex in close proximity to the $\alpha 1$ and $\beta 1$ subunits.[6] Once DM is bound, CLIP will be released. Then the binding of high-stability peptides, which enter the cell via endocytosis and are processed in endosomes, will enter the binding site and trigger the release of DM from MHC Class II. If the peptide that binds to MHC Class II is low affinity it will be edited by DM (Figure 2). The presence of high-stability, antigenic peptides at the cell surface allows for $CD4^+$ T cell activation and the initiation of the adaptive immune response.

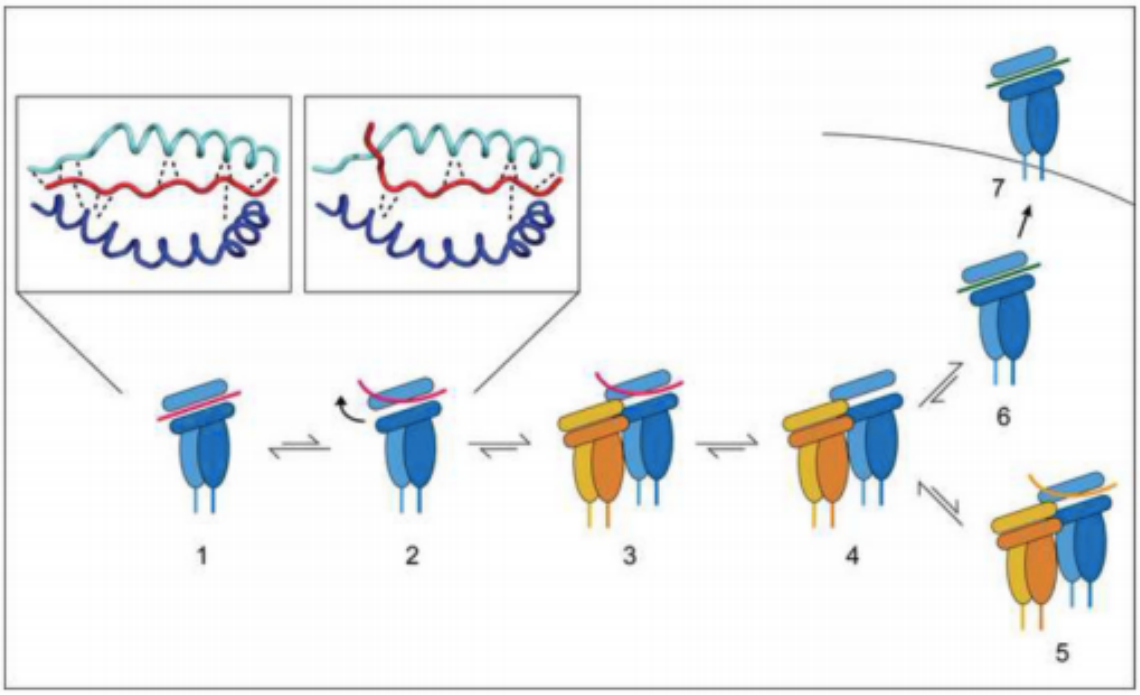

Figure 2: Interaction between MHC Class II and DM[5]

(1) MHC Class II (blue) with CLIP (pink) in binding groove arrives at endosome (2) Movement of CLIP allows for temporary exposure of the DM (yellow) binding site (3) DM binds to the peptide binding groove of MHC Class II and forces the release of CLIP (4) DM stabilizes the empty binding groove of MHC Class II (5) DM facilitates the editing of a low affinity peptide (6) MHC Class II is released from DM when it is bound to a highly stable peptide (7) MHC Class II presents a highly stable peptide at the cell surface of an APC. Ribbon diagrams show the binding of MHC Class II α1 and β1 subunits (teal and blue helices) to a peptide (red) and how the peptide moves to expose the DM binding site.

DM is regulated by another Class II-like molecule, HLA-DO/H2-O (referred to as DO). This highly conserved, substrate mimetic of MHC Class II is expressed in dendritic cells (DCs), B cells, and thymic epithelium; the degree of similarity between DO and MHC Class II led to its original classification as an allele of the Class II β chain.[5] Upon further study, however, it became clear that DO was a non-classical MHC protein that functioned in the regulation of DM.[5] This hypothesis was generated once DO was found to associate with DM in the endoplasmic reticulum and subsequently transported as a DO/DM complex to endosomes (Figure 3A). This is contrary to the process of DM-MHC Class II binding which occurs in the endosomes (Figure 3B).[8] The movement of DO to endosomes is dependent on the formation of a complex with DM; this is supported by the analysis of cells from DM-deficient mice where a minimal amount of DO protein is detected only in the endoplasmic reticulum.[1,8] The endoplasmic reticulum associated protein degradation (ERAD) machinery detects this unbound DO as misfolded and translocates it into the cytosol for degradation by the proteasome.[8] Similarly, when DO is released from DM at the endosome, the free DO is readily degraded in the lysosome or proteasome. However, primary mouse B cells have shown evidence of a relatively stable pool of free H2-O (DO) outside of the endoplasmic reticulum.[12] The function of this free H2-O has yet to be determined.

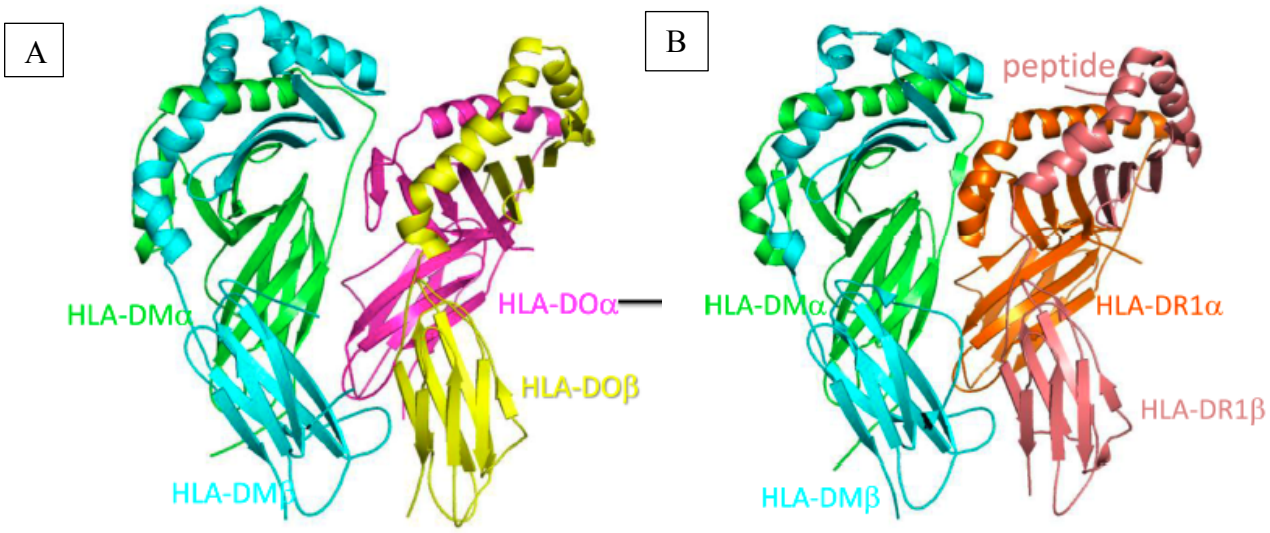

Figure 3: Structural Associations between DM, DO, and MHC Class II (DR1)[4]

(A) The interaction between HLA-DM and HLA-DO is represented in this image. There are many structural similarities between the two proteins, however HLA-DO shares greater similarity with MHC Class II (HLA-DR1) leading to its classification as a structural mimetic.

(B) The interaction between HLA-DM and MHC Class II (HLA-DR1) is represented in this image. The presence of DO in a cell can prevent this interaction and ultimately affect the immune response of the organism.

Studies have shown that when DM is bound to DO, DM is unavailable to bind to MHC Class II which alters peptide loading and editing. Because of this inhibition of DM activity by DO, the presence of MHC-CLIP is increased (this correlation is observed in humans, but less so in mice).[4] Conversely, DO-deficient cells show decreased levels of MHC-CLIP complexes in endosomes and greater MCH class II peptide presentation to T cells.[3] Similarly, biochemical studies have revealed that DO is down-regulated in activated dendritic cells (DC) and B cells compared to their resting counterparts.[4,12] This evidence implicates DO as an inhibitor of DM when no immunologic challenge is present, however comparative studies of the peptide repertoire of DO-expressing and DO-deficient cells have not been conducted.[4] In preliminary studies, presentation by DO-deficient antigen specific B cells (in mice) was found to be less than 2-fold better than that observed in DO-expressing B cells.[4] This relatively small positive change in peptide presentation, as well as epitope specific responses observed in certain experiments, support the possibility of multiple functions for DO in the immune response.[14]

Modulation to the immune response by DO has been observed even though the changes to peptide repertoire have not been conclusive. An *in vivo* B cell study showed that DO-deficient B cells outcompeted wild type B cells (expressing DO) for entrance into the germinal center.[4] Movement of B cells into the germinal center (location of B cell maturation in lymph nodes) and their subsequent hypermutation and proliferation triggers an immunological response. Therefore, it can be hypothesized that one function of DO is to prevent B cell activation (which relies on T cell assistance) when significant immunological challenge is absent. Similarly, non-obese diabetic (NOD) mice that were

engineered to express human DO in their dendritic cells (DCs) failed to develop type 1 diabetes (an autoimmune condition) and $DO^{-/-}$ mice show expression of antinuclear antibodies (associated with Lupus) later in life.[4] Therefore, it has been proposed that APCs expressing DO present a broader self-peptide repertoire because the inhibition of DM and the subsequent presentation of lower-affinity peptides that would normally not be presented. The presence of a diverse peptide population at the cellular surface of APCs allows for the death of self-reactive thymocytes and renders mature $CD4^+$ T cells functionally inactive. Both of these actions prevent autoimmunity and promotes self-tolerance. However, these ideas remain to be formally proven.

Recently, DO has been implicated in retroviral clearance in mice.[3] Persistent retroviruses plague both humans and mice and certain individuals are capable of producing neutralizing antibodies that clear the viruses. The abilities of these "elite controllers" has historically been associated with the MHC gene region, however until recently very little evidence existed to implicate the mechanisms that led to the development of neutralizing Abs. Recent progress has been made largely because of the discovery of the I/LnJ mouse model; this mouse has the ability to clear two persistent retroviruses unlike most other mouse strains, including B6 and BALB/cJ.[3] The ability of I/LnJ mice to clear these viruses via the production of neutralizing antibodies has been traced to a single recessive locus, which has been termed *virus infectivity controller 1* (*vic1*).[3]

Positional cloning was utilized to identify the critical region of the *vic1* locus. Within this region was the entire H2-Oβ (β chain of the DO protein) gene. Further analysis revealed four mutations that distinguish the I/LnJ H2-Oβ allele from the B6 and BALB/cJ allele (Figure 4); three of the amino acid substitutions were present in the immunoglobulin domain and the fourth was in the cytoplasmic tail.[3] Evidence for the role of H2-Oβ in viral resistance came from the comparison of I/LnJ to $Ob^{-/-}$, $Ob^{+/-}$, and $B6^{vic1/I}$ mice. $Ob^{-/-}$ mice express no H2-Oβ, $Ob^{+/-}$ mice express half the amount of H2-Oβ as a wild type mouse, and $B6^{vic1/I}$ mice express the I/LnJ Oβ allele while maintaining the remainder of the B6 genome. Mice that express only the I/LnJ Oβ allele (*I/LnJ* and $B6^{vic1/I}$) have been shown to produce viral neutralizing antibodies in a manner similar to cells not expressing Oβ ($Ob^{-/-}$). This supports the notion that H2-Oβ is responsible for viral resistance.[3]

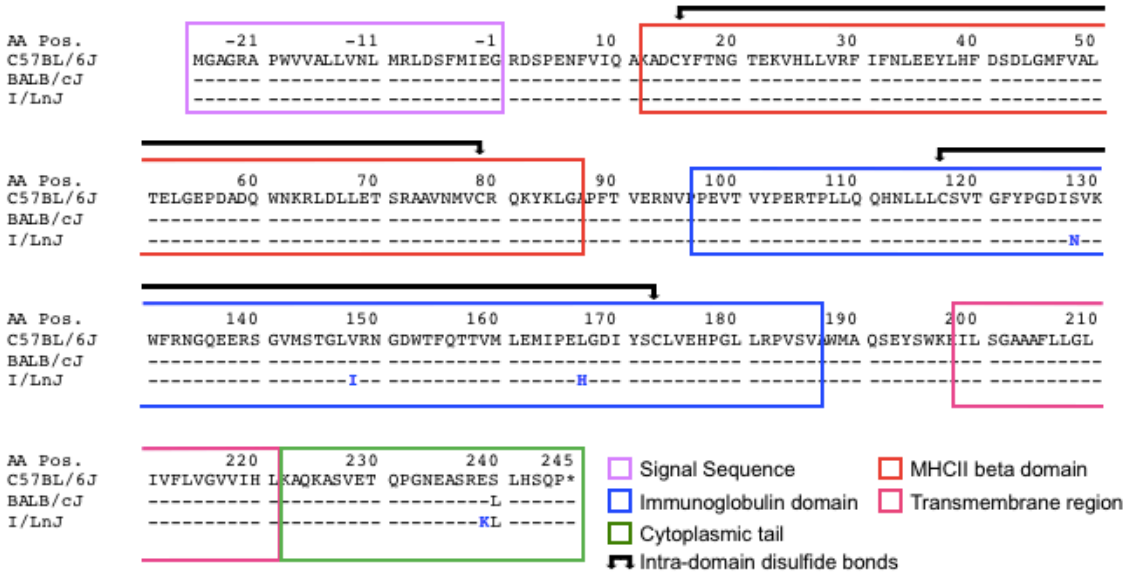

Figure 4: Amino acid alignment of B6 (C57BL/6J), BALB/cJ, and I/LnJ H2-Oβ[6]

The lavender box (negative amino acids) denotes the signal sequence that is cleaved. Three mutations have been identified in the immunoglobulin domain (blue box) and one in the cytoplasmic tail (green box). The MHC II beta domain (red) and transmembrane region (pink) are also included as well as locations of disulfide bonds.

The expression levels of H2-Oβ in I/LnJ mice, as well as the presence of H2-M/H2-O complexes, are unchanged when compared to virus-susceptible strains.[3] Because expression levels are unaffected in comparison to virus-susceptible mice (B6 and BALB/cJ) and the ability to bind to H2-M (DM) is not deterred, the mutations in I/LnJ Oβ likely render it a functionally null allele.[3] This finding, as well as previously collected data on the varied effect of H2-O on the presentation of specific peptide epitopes led us to hypothesize that I/LnJ DO is not only trafficked to the endosome, but is mis-localized to another cellular compartment where it takes on a novel function. The basis for this mis-localization hypothesis is the presence of an amino acid substitutions in the cytoplasmic tail of I/LnJ H2-Oβ (Figure 4). The cytoplasmic tail mediates adaptor protein binding, which influences subcellular localization. Mis-localization could allow for a novel H2-Oβ function that contributes to the ability of I/LnJ mice to neutralize retroviruses that other strains (B6 and BALB/cJ) cannot. The idea of a novel function for I/LnJ DO is also supported by the failure to detect autoimmunity in I/LnJ mice.[3] If the sole effect of the I/LnJ H2-Oβ allele was an increase in H2-M activity, one would expect a greater presentation of self-peptides and a higher incidence of autoimmune disease as is seen in *Ob*$^{-/-}$ mice.

To test this hypothesis, an antibody reagent specific for the I/LnJ H2-Oβ peptide was generated, purified and tested for specificity. This reagent was utilized with already available reagents to compare the subcellular localization of H2-O in virus-susceptible and virus-resistant strains of mice via confocal microscopy.

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Methodology:

Peptide Specific Sulfhydryl Bead Preparation:

The B6 (KAQKASVETQPGNEASR**ESL**HSQP) and I/LnJ (KAQKASVETQPGNEAS**KLL**HSQP) Oβ peptides containing terminal Cys residues to allow for conjugation were dissolved in DMSO at 10 mg/ml and 2 mg of peptide was diluted to a final concentration of 5 mg/ml in PBS containing 0.1ml of 0.5M TCEP (tris(2-carboxyethyl)phosphine hydrochloride**).** The peptide was then incubated at room temperature for 30 minutes. ThermoScientific SulfoLink Column beads were washed extensively with PBS. The activated peptides were then added to the washed beads (1mg peptide/ml of beads) and rotated for 15 minutes at room temperature followed by incubation without rotation for an additional 30 minutes. Beads were washed twice in wash solution (1.0M NaCl and 0.05% NaN_3) and twice in PBS. After washing, the beads were rotated for 15 minutes at room temperature in L-Cysteine buffer (15.8mg/2 mL PBS) to block any remaining reactive sites on the beads and allowed to stand for 30 minutes at room temperature. The resulting beads were washed three times in PBS and stored in 15mL conical tubes in PBS with sodium azide (1:1000) at 4°C until use.

Purification of I/LnJ H2-Oβ specific antibodies from polyclonal rabbit sera:

Rabbit polyclonal sera generated after injecting rabbits with the I/LnJ-Oβ/c peptide (C-KAQKASVETQPGNEASR**KLL**HSQP) conjugated to keyhole limpet hemocyanin (KLH) was depleted of reactivity to B6 Oβ by incubation (with rotating) with the B6 Oβ beads (10ml sera/4ml beads) at 4°C overnight. To ensure total elimination of the B6 Oβ specific antibody, each 10ml sample was incubated with the B6 Oβ beads six times. After verification of B6 Oβ elimination by Western blotting, the

I/LnJ Oβ-specific antibodies were collected by transferring the depleted sera to the I/LnJ H2- Oβ beads followed by rotation at 4°C overnight.

Elution of I/LnJ H2- Oβ specific antibodies from sulfhydryl beads:

To elute the I/LnJ Oβ specific antibodies from the beads, the beads were first washed in PBS four times and then transferred to a spin column. The beads were spun at 2000 rpm to remove excess PBS and three sequential elutions were performed with 2 ml 2.0M glycine HCl at pH 2.5. 3M Tris (pH 8.45) was present in the collection tube to neutralize the eluted antibody immediately. Antibody was detected in the flow through using spectroscopy (A_{280}). Beads were then washed twice with PBS and transferred back to a 15mL conical tube for storage in PBS with sodium azide (1:1000).

Visualization of Antibody Specificity:

Throughout the purification process, Western blotting was utilized to verify the depletion of B6 Oβ reactivity as well as the I/LnJ Oβ reactivity of the final product. Splenocytes from B6, BALB/cJ, *Ob*$^{-/-}$, and I/LnJ mice were lysed for 30 minutes at 4°C in lysis buffer (20mM Tris, pH 8.0, 130mM NaCl containing 1% Triton X-100 and protease inhibitor (Roche Life Science)). The lysates were spun at 4°C for 5 minutes at 14,000 rpm to pellet nuclei and 20 µl (~50,000 cells) of each splenocyte lysate were loaded onto Criterion Any kD SDS-PAGE gels (BioRad; item 567-1124) and run at 200V for 1 hour. The separated proteins were transferred from the gels to PVDF membrane (Millipore) at 100V for 35 minutes in a wet chamber. Blocked membranes were incubated in depleted rabbit sera (1:1000) followed by detection with HRP-conjugated donkey anti-rabbit (1:5000), as well as Mags.Ob1 (200ng) and Mags.Ob3 (500ng) followed by HRP-conjugated goat anti-Armenian hamster (1:5000). Blots were

developed with SuperSignal West Pico chemiluminescent peroxidase substrate (Pierce Biotechnology) followed by exposure to film.

Table 1: Antibodies utilized for Western Blot analysis

Antibody	Source	Identified
Donkey anti-Rabbit IgG (H+L)-Horseradish Peroxidase	Jackson ImmunoResearch	Cat#711-005-152; RRID: AB_2340585
Hamster Monoclonal Anti-Oβ cytoplasmic tail	Denzin Lab	Clone Mags.Ob1
Goat anti-Armenian Hamster IgG (H+L)-Horseradish Peroxidase	Jackson ImmunoResearch	Cat#127-025-160; RRID: AB_2338974
Hamster Monoclonal Anti-Oβ cytoplasmic tail	Denzin Lab	Clone Mags.Ob3

Mice:

H2-Ob$^{-/-}$ B6, *H2-Ma*$^{-/-}$, and B6.CD45.1 mice were bred and maintained at the animal facility of Rutgers University. I/LnJ and IxB6 (F1) mice were bred and maintained by colleagues at The University of Chicago.

Purification of dendritic cells:

Spleens were harvested from the indicated strains of mice and digested in 400U/mL collagenase D and 100 μg/mL DNase I for 30 minutes at 37°C. Cells were collected into conical tubes and spun at 2000 rpm for 5 minutes at 4°C. Red blood cells were water lysed and immediately diluted in MACS buffer (PBS supplemented with 0.5% BSA, 2mM EDTA, and 0.05% sodium azide). Cells were collected after spinning and resuspended in MACS buffer followed by filtration to remove debris from the sample. Dendritic cells were purified by incubating with CD11c Microbeads_mouse

(Miltenyi Biotech #130-052-001) followed by MACS separation. After separation, the cells were counted and diluted in HBSS++ at ~$8x10^6$ cells/mL.

Confocal Slide Production and Staining:

Purified dendritic cells were added to chambers drawn on a Fisherbrand Superfrost/Plus microscope slide (25x75x1.0) with a Pap Pen (~150,000cell/chamber). Cells were incubated for 60 minutes in a moist chamber at 37°C. Excess HSBB++ was carefully aspirated from each chamber post-incubation and the cells were fixed to the slide with 4% paraformaldehyde in PBS. The cells were washed with PBS two times prior to permeabilization in PBS containing 10% calf serum, 0.05% saponin, 10mM glycine, and 10mM HEPES, pH 7.4. To optimize the staining procedure, B6, *Ob*$^{-/-}$, and *Ma*$^{-/-}$ cells were stained with Mags.Ob3-A647 (1:500) and 2C3A-A488 (1:1000) followed by goat-anti-Armenian hamster-A647 (1:2000) for 1 hour at room temperature. To observe localization of I/LnJ Oβ using the affinity purified antibody, B6, *Ob*$^{-/-}$, *Ma*$^{-/-}$, I/LnJ, and F1 cells were stained with the purified I/LnJ H2- Oβ antibody (1:1000), and 2C3A-A488 (1:1000) followed by goat-anti-rabbit-A594 (1:3000) for 1 hour at room temperature. I/LnJ H2-Oβ antibody utilized for imaging studies was one time depleted on the I/LnJ column; cross-reactivity was avoided by pre-treating slides with Mags.Ob3-A647 (1:500) for 30 minutes at room temperature. Prior to treatment with secondary antibody all slides were incubated in 20% natural goat sera (NGS). The staining of slides was preserved with one drop of Anti-fade gold with DAPI prior to the addition of a coverslip.

Table 2: Antibodies utilized for Confocal Imaging

Antibody	Source	Identified
Alexa 647 Mouse MHCII/H2-O beta	Denzin Lab	Mags.Ob3
Alexa 488 Mouse MHCII H2-M	Denzin Lab	2C3A
Alexa Fluor 647 AffiniPure Goat Anti-Armenian Hamster IgG (H+L)	Jackson ImmunoResearch	Cat#127-605-160
Alexa Fluor 594 AffiniPure Goat Anti-Rabbit IgG	Jackson ImmunoResearch	

Confocal Imaging:

Slides were imaged using 63xW lens of confocal microscope (Zeiss, LSM700) and Zen Imaging Software.

Results:

We hypothesized that the virus neutralizing capability of I/LnJ mice might be due to a mis-localization of H2-O, resulting in a novel function that complements the altered viral peptide presentation by MHC Class II. To test this hypothesis, reagents were needed to distinguish between the I/LnJ Oβ cytoplasmic tail peptide and the B6 and BALB/cJ peptide. Appropriate Ab reagents specific for the targeting of the B6 peptide (Mags.Ob3), as well as H2-M (2C3A) have been previously purified by the Denzin lab, but no Abs were available that specifically recognize I/LnJ Oβ. The difference between the cytoplasmic tails of I/LnJ, BALB/cJ, and B6 mice is only 1 or 2 amino acids, respectively, which makes specificity of the reagent key for the future success of the study. I/LnJ Oβ has a substituted lysine followed by a substituted leucine where B6 Oβ has a glutamic acid followed by serine (Figure 5). Therefore, the generation of an appropriate reagent was necessary to determine the localization of Oβ in B6 versus I/LnJ cells by confocal microscopy.

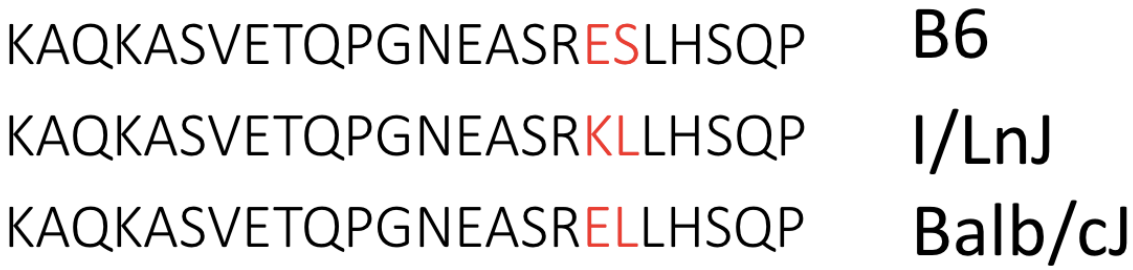


Figure 5: Comparison of B6, I/LnJ, and BALB/cJ Oβ cytoplasmic tail

Generation of I/LnJ Oβ specific antibody reagent

To create an I/LnJ Oβ specific antibody reagent, a polyclonal rabbit serum was generated by injection of rabbits with the I/LnJ-Oβ/c peptide conjugated to keyhole limpet hemocyanin (KLH). The resulting polyclonal serum was systematically depleted of B6 reactivity using sulfhydryl beads to which the B6 Oβ cytoplasmic tail was covalently linked. In order to completely eliminate B6 Oβ reactivity, 6 successive depletions were preformed, however minimal reactivity was reached by depletion 4 which was verified using Western blotting (Figure 6A). The remaining I/LnJ Oβ tail-specific Abs were collected by incubation of the depleted sera with beads conjugated with the I/LnJ Oβ/c peptide. The specific antibodies eluted from the I/LnJ Oβ/c peptide beads were capable of binding to the I/LnJ Oβ cytoplasmic tail with greater specificity than the B6 Oβ cytoplasmic tail (Figure 6B). Western blotting with mAbs Ob1 and Ob3 was utilized to verify the presence of the B6 cytoplasmic Oβ tail in the cellular lysates. As had been previously determined the Ob3 antibody bound preferentially to the B6 cytoplasmic tail peptide, while Ob1 detected both the B6 and BALB/cJ Oβ allele. The apparent lower molecular weight of the I/LnJ H2-Oβ is due to differential SDS binding when compared to the B6 and BALB/cJ peptide.[3]

The anti-I/LnJ Oβ antibody eluted from the I/LnJ cytoplasmic tail beads succeeded at distinguishing between the B6 and I/LnJ Oβ cytoplasmic tail but retained reactivity to the BALB/cJ cytoplasmic Oβ tail because of sequence similarity. The I/LnJ Oβ cytoplasmic tail differs from the BALB/cJ cytoplasmic tail by the substitution of a single lysine while the B6 Oβ cytoplasmic tail differs by both a lysine and leucine

(Figure 5). After the success seen via Western blots, the purified I/LnJ Oβ tail specific antibody was tested by confocal microscopy (data not shown), however extensive depletion of the sera led to poor antibody recovery and insufficient Oβ signal and thus, I was not able to use this reagent for my confocal studies.

Therefore, we next asked if affinity purification of the polyclonal rabbit sera directly on the I/LnJ Oβ cytoplasmic tail beads would provide a reagent that was preferentially specific for the I/LnJ Oβ tail. To test this idea, the polyclonal sera was incubated on the I/LnJ Oβ tail beads overnight, followed by extensive washing and elution of the I/LnJ Oβ tail specific Abs. Specificity of the new affinity purified reagent was once again verified by Western blotting of splenocyte lysates from B6, I/LnJ, BALB/cJ and *Ob*$^{-/-}$ mice (Figure 7). Although reactivity to the B6 Oβ cytoplasmic tail remained, the reactivity to I/LnJ Oβ was much greater and significantly amplified compared to the depleted sera (Figure 6B). To limit the binding of the purified I/LnJ Oβ tail Ab to B6 Oβ peptide, pre-treatment with the Mags.Ob3 Abs was tested. This was proven to limit the non-specific binding of the I/LnJ sera to B6 Oβ by Western blotting (Figure 7). Therefore, this affinity purified reagent along with pre-treatment were used for future confocal studies.

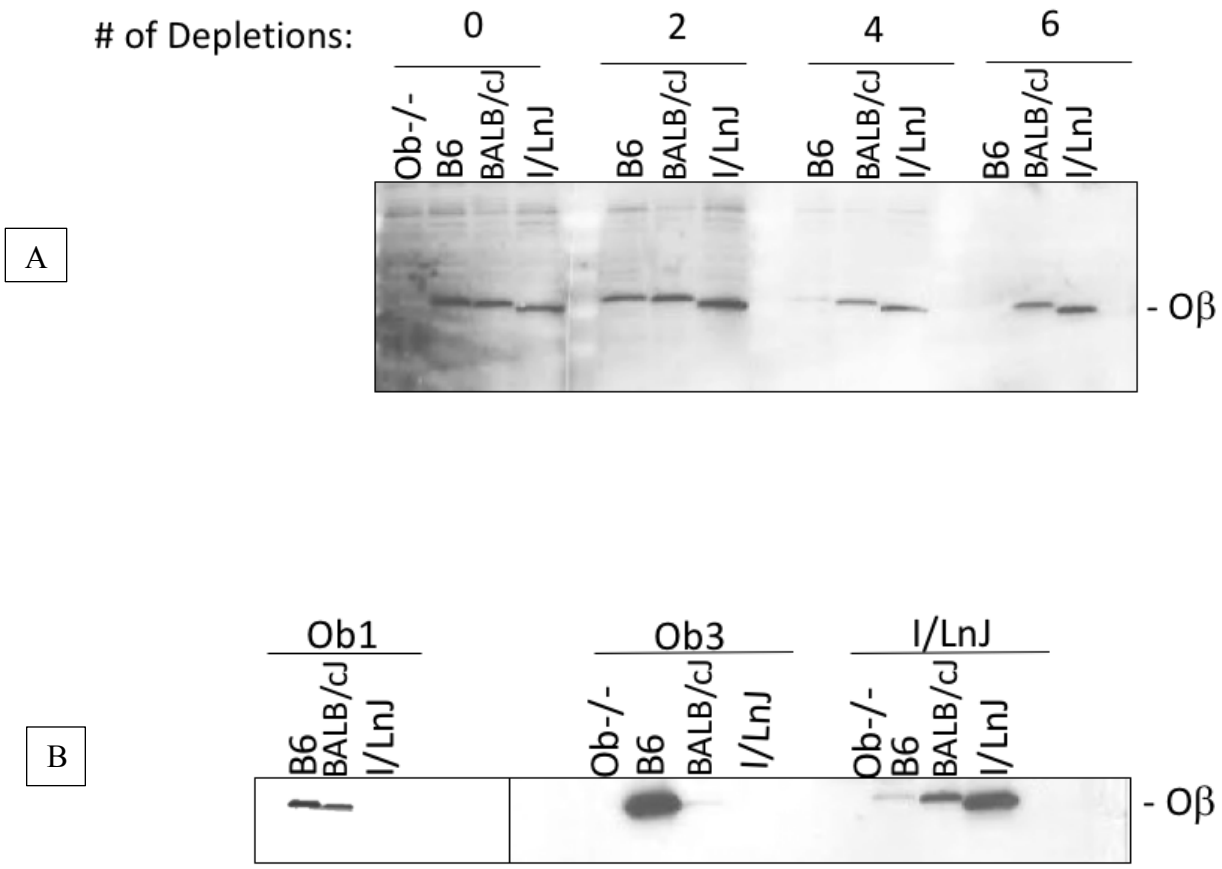


Figure 6: Verification of I/LnJ Oβ reactivity of depleted rabbit sera by Western blot analysis

(A) B cell lysates from indicated mouse strains were probed with rabbit serum after each successive depletion on the B6 Oβ sulfhydryl beads. There is a clear decrease in B6 Oβ reactivity from the starting sera (0) to that resulting after the final depletion (6). Purified $Ob^{-/-}$ were used as negative controls.

(B) B cell lysates from indicated mouse strains were probed with Oβ cytoplasmic tail antibodies specific for B6/BALB/cJ (0b1), B6 (Ob3), and I/LnJ (eluted from I/LnJ column). Purified $Ob^{-/-}$ were used as negative controls. The purified I/LnJ Oβ sera bonded specifically to the I/LnJ cytoplasmic Oβ tail with minimal B6 cross reactivity.

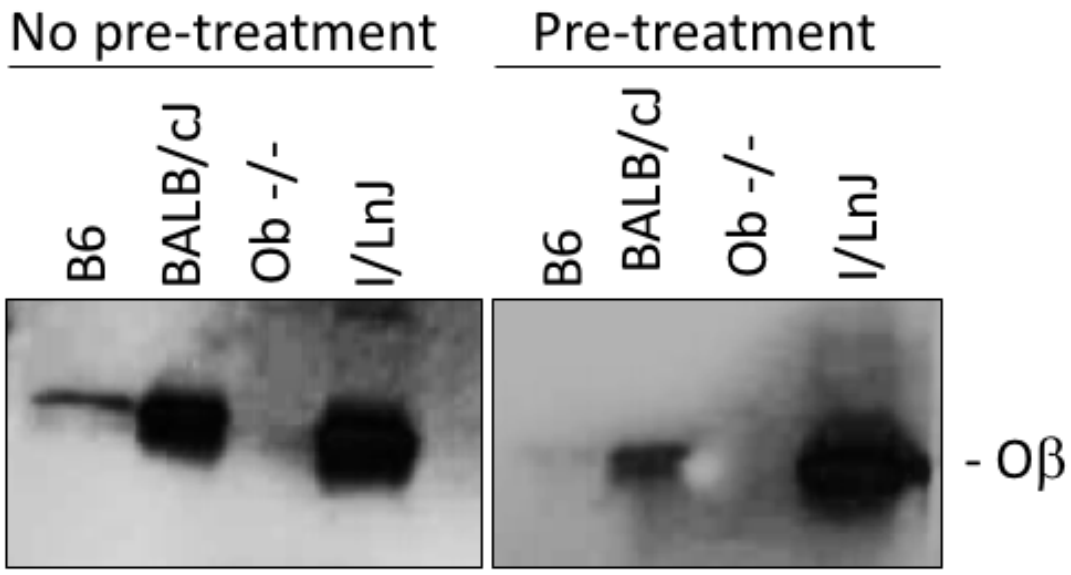


Figure 7: Verification of I/LnJ Oβ reactivity in affinity purified rabbit sera by Western blot analysis.

B cell lysates from indicated mouse strains were probed with rabbit serum after affinity purification on I/LnJ Oβ cytoplasmic tail sulfhydryl beads. The gel on the right was pre-treated with Mags.Ob3 (1:500) prior to incubation with the I/LnJ Oβ sera. Cross-reactivity with the B6 Oβ peptide was greatly reduced after pre-treatment. Purified $Ob^{-/-}$ were used as negative controls.

Co-localization of B6 H2-O peptide and H2-M in Dendritic Cells

It has been well established that H2-O forms a complex with H2-M in the endoplasmic reticulum and the complex is passed through the Golgi apparatus and into endosomes as a H2-M/H2-O complex.[4] In most H2-O expressing cells, H2-O which is not complexed with H2-M remains in the endoplasmic reticulum and is targeted for degradation.[12] Thus, H2-O requires H2-M for transport from the ER to endosomal compartments. Prior to testing splenocytes with the affinity purified I/LnJ Oβ cytoplasmic tail antibody, it was necessary to visualize the B6 Oβ cytoplasmic tail peptide and H2-M using available reagents. Mags.Ob1 (Ob1) is a monoclonal antibody that bonds with greater affinity to the B6 Oβ cytoplasmic tail than the I/LnJ Oβ cytoplasmic tail (see Figure 6); 2C3A is a monoclonal antibody that is specific for the H2-M heterodimer. These Abs were conjugated to A647 and A488, respectively allowing them to be used for confocal without secondary antibodies. However, the relatively low energy of the A647 fluorophore led to the need for secondary amplification (by incubating the cells with Goat-anti-hamster-A647).

In B6 DCs co-localization of H2-O and H2-M was observed after staining with the Mags.Ob1 and 2C3A antibodies (Figure 8A). All H2-O that is visualized seems to be co-localized with H2-M, there was no evidence of free H2-O congregation in another cellular compartment. However, co-localization was not observed in the I/LnJ DCs because the Mags.Ob1 fails to bind to the cytoplasmic tail of I/LnJ Oβ (Figure 8B). Minimal H2-O expression is seen in $Ma^{-/-}$ (cells deficient in the *H2-Ma*) since H2-O does not leave the ER in the absence of H2-M and is degraded by the ERAD machinery. The residual protein detected is therefore likely ER localized (Figure 8C).[8] As expected, $Ob^{-/-}$

DCs fail to express H2-O but show regular expression of H2-M in what appears to be endosomes (Figure 8D). The results of these experiments showed that established antibodies can be utilized to specifically localize the B6 H2-O protein and H2-M protein via confocal microscopy.

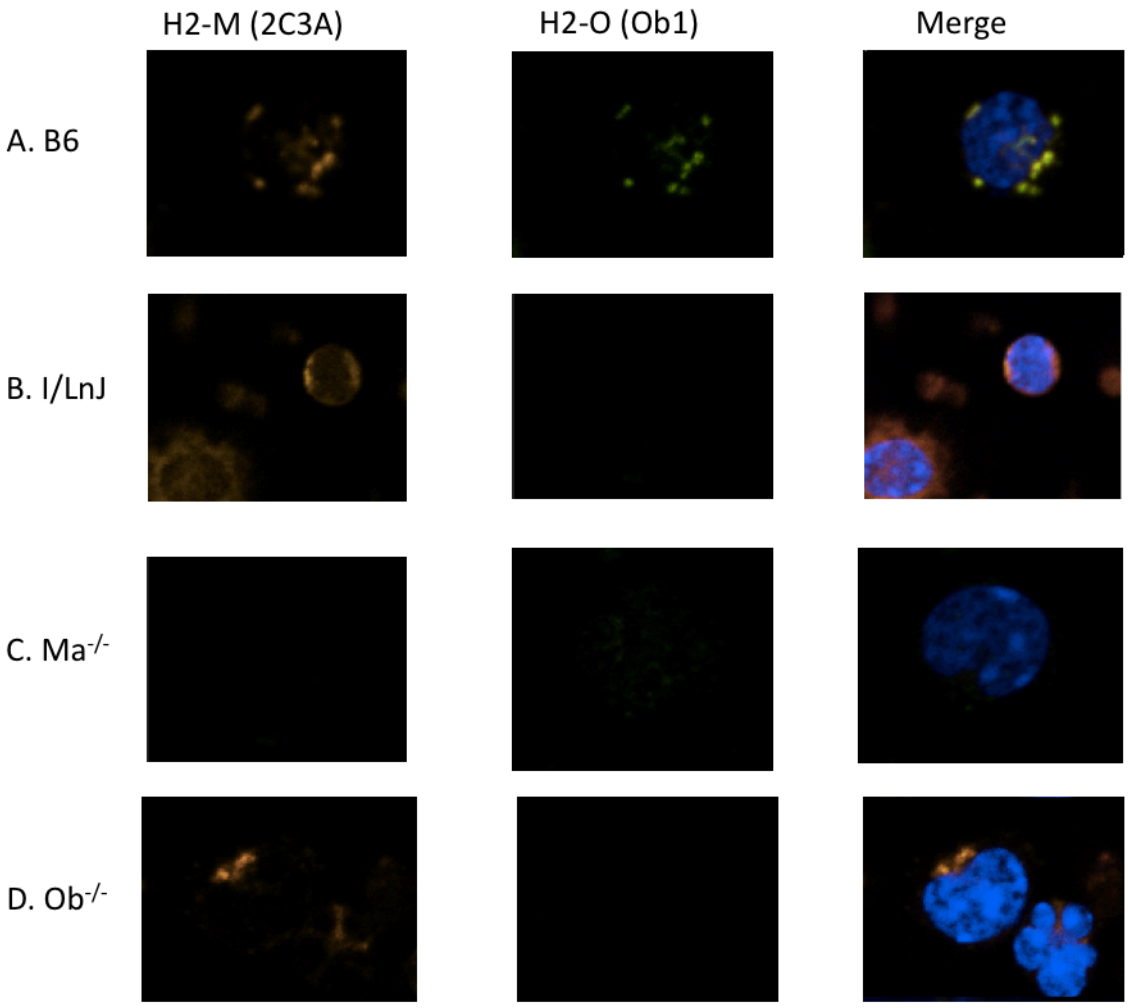


Figure 8: Co-localization of B6 H2-O and H2-M in DCs via Confocal Microscopy Immunofluorescence staining analyzed by confocal imaging of DCs of noted strains co-stained for H2-O (Ob1) and H2-M (2C3A). Ob1 detected B6 Oβ cytoplasmic tail, but not I/LnJ Oβ cytoplasmic tail. The H2-O visualized in B6 cells overlapped with the detected H2-M. *Ma*$^{-/-}$ and *Ob*$^{-/-}$ served as negative controls for 2C3A and Ob1, respectively. These images are representative of at least 2 similar experiments.

Co-localization of I/LnJ H2-O and H2-M in Dendritic Cells

In order to compare the subcellular localization of the B6 and I/LnJ H2-O peptide, the affinity purified I/LnJ Oβ cytoplasmic tail antibody was used to stain B6, I/LnJ, *Ob*$^{-/-}$, and *Ma*$^{-/-}$ dendritic cells (Figure 9). As expected, the I/LnJ Oβ cytoplasmic tail antibody detected the I/LnJ peptide in only the I/LnJ dendritic cells, but not B6 dendritic cells. Slides were pre-treated with the B6-Oβ tail-specific mAb Mags.Ob3 to block binding of the I/LnJ Ab reagent to the B6 Oβ peptide as had been previously supported using Western blots (Figure 7). This allowed for minimal cross reactivity with the B6 peptide, as well as minimal non-specific binding (Figures 9A and B). Images revealed that there was a similar degree of co-localization between I/LnJ H2-O and H2-M as that seen in B6 cells (Figure 8A). This had been previously supported by the Denzin lab using co-immunoprecipitation studies.[3] However, a significant amount of H2-O was detected in outside endosomes suggesting that H2-O might localize to compartments other than endosomes and endoplasmic reticulum in I/LnJ mice (Figure 9B and Supplemental Figure S1). This was not detected in either the B6 or *Ma*$^{-/-}$ DCs. This raises the question of where the free H2-O is located within the cell. As would be expected, the I/LnJ H2-O peptide was not detected in *Ob*$^{-/-}$ and *Ma*$^{-/-}$.

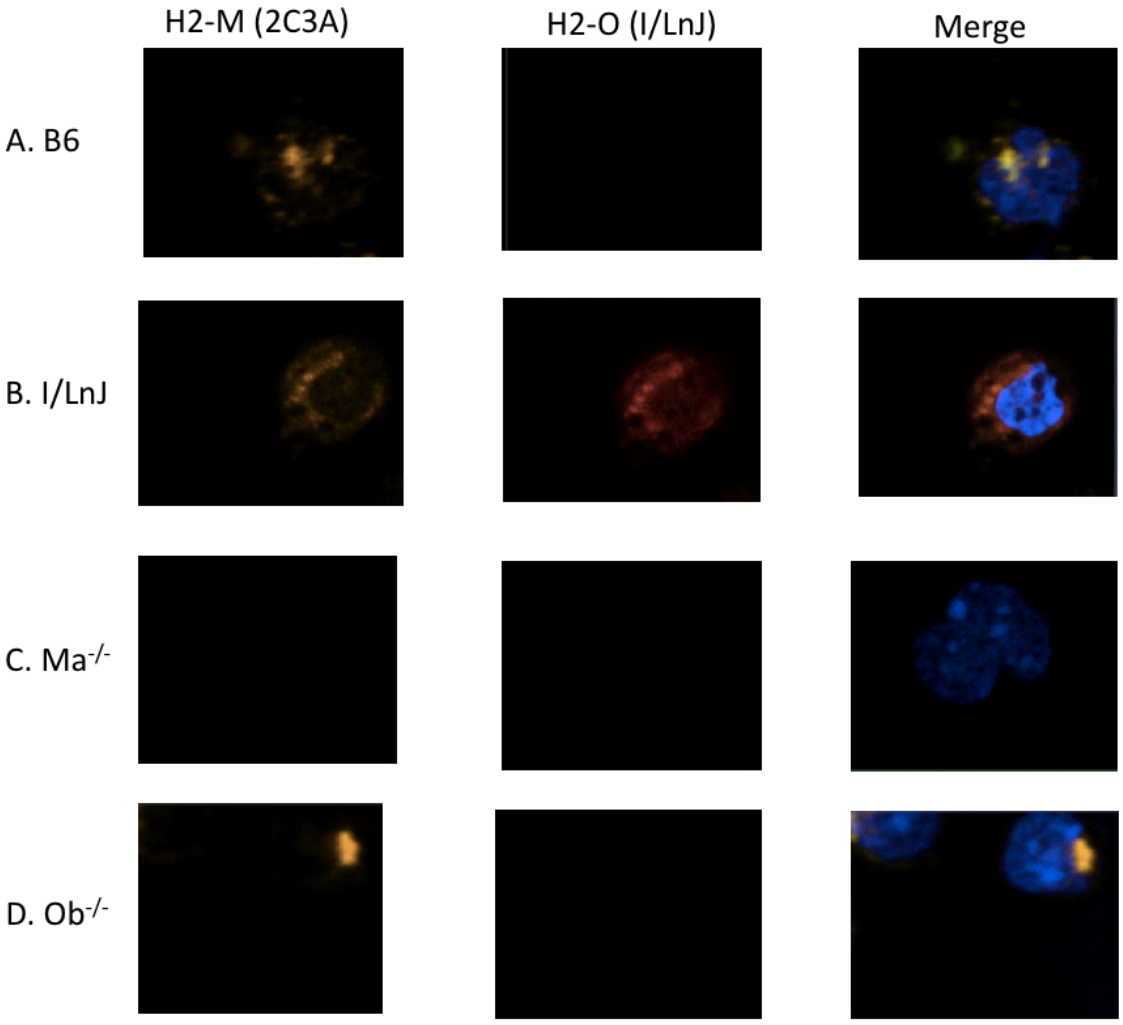


Figure 9: Co-localization of I/LnJ H2-O peptide and H2-M in DCs via Confocal Microscopy

Immunofluorescence staining analyzed by confocal imaging of DCs of noted strains co-stained for H2-O (I/LnJ) and H2-M (2C3A). The I/LnJ Oβ antibody detected I/LnJ Oβ cytoplasmic tail, but not B6 Oβ cytoplasmic tail. H2-O was detected with H2-M and in endosomes and in an unknown subcellular compartment in I/LnJ cells. *Ma*$^{-/-}$ and *Ob*$^{-/-}$ served as negative controls for 2C3A and I/LnJ, respectively. These images are representative of at least 2 similar experiments.

Visualization of I/LnJ and B6 H2-O in F1 mice

To verify the observations made in the I/LnJ dendritic cells, F1 (B6xI/LnJ) cells were stained with the I/LnJ and B6 (Ob1) Oβ cytoplasmic tail antibody and visualized using confocal microscopy. To generate F1 mice, our collaborator at the University of Chicago, Dr. Tatyana Golovkina, crossbred I/LnJ mice with B6 mice; these mice express both the B6 and I/LnJ *H2-Ob* cytoplasmic tail at equivalent levels.[3] It was initially predicted that the B6 Oβ peptide would be detected only with H2-M in endosomes, while the I/LnJ Oβ peptide would be seen both in endosomes and other cellular compartments. This hypothesis was based upon the observations made in I/LnJ and B6 dendritic cells (Figure 8A and 9B). Confocal microscopy revealed that all three proteins (B6 H2-O, I/LnJ H2-O, and H2-M) co-localize in endosomes and are not present elsewhere (Figure 10). It seems likely that the presence of the B6 H2-O peptide influences the localization of the I/LnJ peptide.

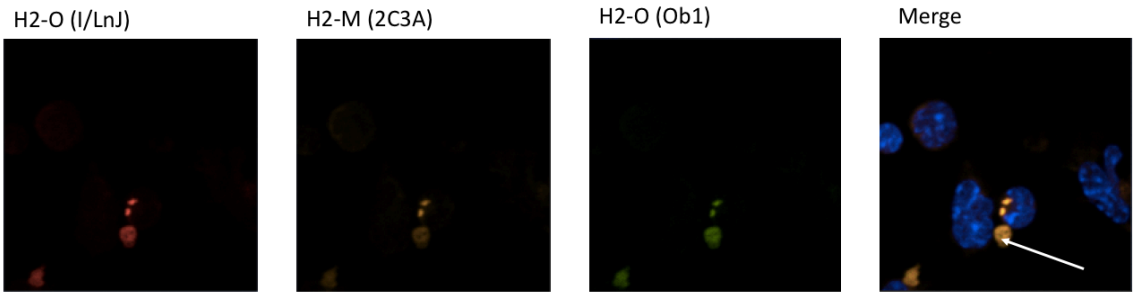


Figure 10: Co-localization of I/LnJ H2-O, B6 H2-O and H2-M in F1 Dendritic Cells Immunofluorescence staining analyzed by confocal imaging of DCs of F1 mice co-stained for I/LnJ H2-O (I/LnJ), B6 H2-O (Ob1) and H2-M (2C3A). Both H2-O peptides and H2-M appear to co-localize in endosomes. Compartment indicated by an arrow in the merged image is likely an antibody aggregate and does not indicate protein localization.

Discussion:

The hypothesis for this thesis was that the ability of I/LnJ mice to neutralize persistent retroviruses was partially due to mis-localization of H2-O into cellular compartments other than the endosomes. This hypothesis has been initially supported by confocal microscopy. Dendritic cells of virus-susceptible mice (B6) show expression of H2-O in endosomes with H2-M (Figure 8A). This pattern of H2-O/H2-M co-localization has been well established.[3,4,7] Conversely, dendritic cells expressing solely the I/LnJ *H2-Ob* peptide show evidence of endosome localization as well as the presence of H2-O in other cellular compartments (Figure 9B). This can be seen in the more diffuse staining pattern that is not seen in virus-susceptible I/LnJ cells (Figure 9B and Supplemental Figure S1). Although free H2-O has been detected in B6 cells, it is not found in compartments other than the endosome. The function of this free H2-O has yet to be elucidated but might be key to understanding why the I/LnJ peptide is functionally null.

When the B6 H2-O peptide was co-expressed with the I/LnJ peptide (F1 cells), staining outside of endosomes was not detected (Figure 10). This observation correlates with the inability for F1 to clear persistent retroviruses in a manner similar to I/LnJ mice.[8] This introduces the possibility that B6 H2-O can influence the localization of I/LnJ H2-O. Further experimentation will be required to elucidate the mechanism at work.

A failure of some H2-O/H2-M complexes to localize to the endosome of I/LnJ mice may contribute to the ability to create virus neutralizing antibodies. The lack of functional H2-O that does complex with H2-M and arrive at the endosome also contributes to the virus neutralizing capability by altering MHC class II peptide loading.[3] Therefore, the quantity of peptide repertoire being presented at the I/LnJ cell surface by

class II MHC is likely different than that seen in virus-susceptible mice (B6 and BALB/cJ). This altered peptide presentation and the function served by the mis-localized H2-O in an unconfirmed cellular compartment will allow the I/LnJ mice to respond to persistent retroviruses in a manner similar to that of *Ob*$^{-/-}$.[3]

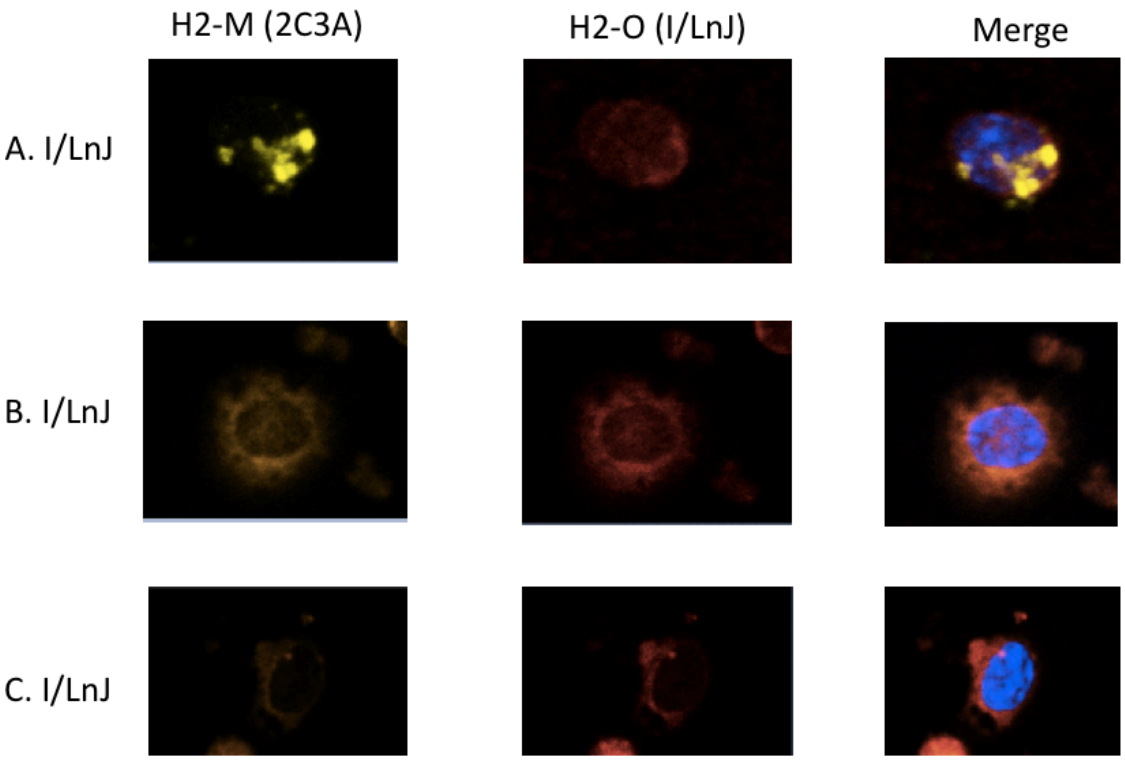
These results raise the question of where I/LnJ H2-O localizes. Initial observations of the staining pattern suggest the mitochondria. Further support for this hypothesis can be found in preliminary mass spectrometry studies of H2-O and H2-M associated proteins. H2-O/H2-M complexes have been found to associate with both mitochondrial and ER proteins in B6 and I/LnJ mice. The association with mitochondrial proteins is stronger in I/LnJ mice than virus-sensitive B6 mice. Because one key mutation in the I/LnJ allele occurs in the cytoplasmic tail, it is likely that adaptor proteins bond with differential affinity and potentially traffic the I/LnJ H2-O protein to a different cellular compartment. Future studies will analyze the possibility of that cellular compartment being the mitochondria.

Mitochondrial proteins that were found to associate with H2-0/H2-M include: GRIM-19, Glycerol-2-Phosphate Dehydrogenase 2, and ATPase Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} Transporting 3 (LKD and TG, unpublished data). The up-regulated association of I/LnJ H2-O/H2-M (compared to B6 H2-O/H2-M) with GRIM-19 is of particular interest because it is a subunit of NADH dehydrogenase (mitochondrial inner membrane complex) that has been found to inhibit signal transducer and activator of transcription 3 (STAT3).[10] STAT3 is a transcription factor that has recently been associated with B cell germinal center maintenance in lupus mouse models.[11] This presents an alternative mechanism to explain the virus neutralizing

phenotype observed in I/LnJ mice. If mis-localized H2-O is associated with GRIM-19 in the mitochondria of I/LnJ mice it could prevent the inhibition of STAT3, thus allowing for germinal center maintenance of virus neutralizing B cells and subsequent clearance of persistent retroviruses.

The well-established connection between the endoplasmic reticulum and mitochondria provides further support for this hypothesis and adds validity to future research efforts. Over 50 years ago, the physical interaction between these two organelles was observed; contact sites and the proteins localized there have been implicated in overlapping functions including mitochondrial fission, calcium transfer, autophagy, and inflammation.[9]

In order to begin testing this hypothesis, localization of the I/LnJ H2-O in the mitochondria must be confirmed. This can be done utilizing confocal microscopy and an antibody specific for a mitochondrial protein, such as MAVS (mitochondrial antiviral-signaling protein). Future studies will also aim to quantify the amount of co-localization of I/LnJ H2-O and B6 H2-O with H2-M. This will potentially support the claim that a significant amount of H2-O is found outside of the endoplasmic reticulum and endosome in I/LnJ DCs, supporting the legitimacy of a novel H2-O function in association with GRIM-19. Cell fractionation studies will also be utilized to confirm the presence of H2-O in the mitochondria. The process of mitochondrial fractionation has been well established and can be useful in confirming the qualitative results of confocal microscopy.[9] The data reported in this thesis and that which will be collected in the future could potentially define a novel function for H2-O in the immune response and reveal a therapeutic target, either GRIM-19 or H2-O, for persistent retroviruses.



Supplemental Figure S1: Visualization of mis-localized I/LnJ H2-O peptide in I/LnJ dendritic cells.

Immunofluorescence staining analyzed by confocal imaging of DCs of noted strains co-stained for H2-O (I/LnJ) and H2-M (2C3A). H2-O was detected with H2-M and in endosomes and in an unknown subcellular compartment in I/LnJ cells

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