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DELETION OF ANTIBODY ENCODED
TOLEROGENIC SIGNALS TO IMPROVE A
DENDRITIC CELL TARGETED VACCINE DELIVERY
PLATFORM SYSTEM

BY

DANIELLE AGUIRRE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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IN
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2014

MASTER OF SCIENCE THESIS
OF
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2014

ABSTRACT

Antibodies specific for DEC205, a dendritic cell (DC) endocytic receptor that traffics to the antigen presentation pathway, have been shown to be excellent tools for raising robust, sustained immune responses to co-delivered vaccine antigens; however, strong immune responses are only elicited with the aid of non-specific dendritic cell maturation factors, without which a tolerogenic immune response is induced. We hypothesize that regulatory T cell epitopes (Tregitopes) located in the α DEC205 sequence promotes tolerance, requiring the use of non-specific immuno-stimulators to promote pro-inflammatory immune responses. This hypothesis is based on previous research performed by De Groot *et al.* 2008 who characterized a set of natural regulatory T cell epitopes derived from human immunoglobulins (IgG) that were found to induce tolerance by stimulating regulatory T cells (CD4⁺CD25⁺FoxP3⁺).

We believe that α DEC205 can be rendered less tolerogenic by modifying its regulatory T cell epitope content and improve its capacity to induce inflammatory responses without the aid of non-specific maturation factors to activate the immune system.

In this work, the α DEC205 sequence was computationally screened for putative HLA-Class II-restricted, regulatory T cell epitopes as targets for elimination by mutation. Mutations affecting key amino acid sites, relevant to peptide-HLA-DRB1*0401 binding, were carried out to reduce epitope binding affinity to the HLA-DRB1*0401 allele. Sequence modifications confirmed to

disrupt peptide-HLA binding were incorporated into an array of α DEC205:OVA-ORG (original sequence) variants (mutant sequences) via site directed mutagenesis. Protein was produced by CHO-S cell transient transfection. Purified variant α DEC205:OVA-ORG recombinant proteins were utilized in DR4 in vivo immunizations and functional assays to observe T cell activation and proliferative immune responses.

The α DEC205:OVA variants (HC54-MOD1, VH77-MOD1 & VH77-MOD2) were shown to target and bind to dendritic cells as effectively as the non-modified α DEC205:OVA antibody. Splenocytes were re-stimulated with ovalbumin in the T cell proliferation assay and with OVA Class I₍₂₅₇₋₂₆₄₎ and OVA Class II₍₃₂₃₋₃₃₉₎ peptides in the ELISpot assay to measure the memory responses. Both α DEC205:OVA-VH77-MOD1 and α DEC205:OVA-VH77-MOD2 showed statistically significant differences in CD8⁺ and CD4⁺ T cell proliferation in comparison to splenocytes previously immunized with the α DEC205:OVA-ORG antibody condition, respectively. However, a lack of statistically significant IFN- γ cytokine production was observed for all variant antibody immunization conditions. Further analyses are required to determine the true significance observed from the α DEC205:OVA-VH77-MOD1 data set as evidenced by the possible outliers, which may be skewing the results. The α DEC205:OVA-HC54-MOD1 sequence failed to generate elevated levels of T cell proliferation and IFN- γ secretion responses.

It was concluded that the α DEC205:OVA antibodies (VH77-MOD1 and VH77-MOD2) induced statistically significant elevated T cell proliferative

responses in comparison to the baseline immune response levels of the non-modified α DEC205:OVA-ORG antibody, suggesting that an improved vaccine delivery system is underway due to the epitope modifications at the VH77 Tregitope sequence, which potentially decreased the tolerogenicity of the α DEC-205:OVA antibody. In regards to the other variant antibodies, further modifications to the Tregitope sequences in the α DEC205:OVA antibody's overall sequence may be necessary to reduce tolerogenicity further to begin to create a detolerized antibody capable of inducing a pro-inflammatory immune response without the aid of a non-specific immuno-stimulator.

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CHAPTER 1

INTRODUCTION

Since their discovery in 1973, dendritic cells (DCs) have proven to be specialized immune cells and potent stimulators of primary immune responses (Hart, 1997, Janeway et al. 2001, Granucci et al. 2005, Lipscomb et al. 2002). Initial interest peaked when these bone marrow-derived cells were found to be not only in mice, but also in most human lymphoid (spleen, thymus, and lymph nodes) and non-lymphoid tissue (Hart, 1997, Lipscomb et al. 2002). Information regarding DCs has only continued to grow, leading to the knowledge that DCs play a central role in the immune system by controlling both immune tolerance and immunity (Palucka et al. 2002, Granucci et al. 2005, Ueno et al. 2007, Steinman et al. 2006). Because of these qualities, researchers are considering DCs as viable candidates to contribute to the improvement of targeted vaccine delivery platform systems (Palucka et al. 2002, Hart, 1997, Tacke et al. 2007).

Myeloid lineage DCs originate from bone marrow and are a part of a specific sub-population of cells (professional antigen presenting cells) (APCs), which comprise both Macrophages and B cells (Chatterjee et al. 2012, Hart, 1997, Nagl et al. 1997, Steinman et al. 2006). A primary function of DCs is to alert the immune system to any invading organism they may potentially

encounter (Janeway et al. 2001). Thus, APCs' antigen uptake process is an essential and critical function of the immune system (Steinman et al. 2006, Hart, 1997, Lipscomb et al. 2002). These cells efficiently process antigen, and present it in peptide-form in context of up-regulated MHC complexes (Hart, 1997, Steinman et al. 2006, Steinman et al. 2000, Janeway et al. 2001). Antigen-uptake facilitates directed antigen-specific T cell mediated immune responses, leading to the initiation of an adaptive immune response, a more specific and targeted immune response (Steinman et al. 2000, Hart, 1997, Janeway et al. 2001, Male et al. 2006, Wilson et al. 2004, Tacke et al. 2007, Bonifaz et al. 2002).

In order for DCs to perform this task, immature DCs circulate along the tissue periphery, efficiently and continuously sampling their environment for antigen in a variety of manners: phagocytosis of particles, fluid phase macropinocytosis, receptor-mediated endocytosis, or direct contact with apoptotic or infected cells (Nagl et al. 1997, Steinman et al. 2006, Hart, 1997). Following the antigenic encounter, peripheral-immature DCs process and present the antigen in peptide-form on DCs surfaces. Immature DCs express a variety of CLRs that are down regulated after maturation (Tel et al. 2011).

Immature DCs then travel from the tissue periphery via the lymphatic system to the nearest draining-lymph node, a secondary lymphoid organ, only after receiving appropriate direct-pathogenic signals or environmental inflammatory stimuli, causing the up-regulation of cell surface-chemokine receptors and subsequent DC migration (Hart, 1997, Nagl et al. 1997). Within

lymph nodes, the site of antigen localization, naive T cells are exposed to antigenic peptides by mature DCs. Mature DCs then acquire the ability to activate and facilitate naive T cell polarization; a hallmark of mature DCs, resulting in an antigen-specific T cell mediated immune response (Steinman et al. 2000, Hart, 1997, Tel et al. 2011).

Since DCs can play a role in both innate and adaptive immunity, their uniqueness as nature's adjuvant has attracted the attention of the scientific community (Steinman et al. 2000, Steinman et al. 2007, Nagl et al. 1997, Masten et al. 2006). As such, researchers have begun taking advantage of their functional roles within the immune system as vaccine targets for therapeutic antibodies (Chames et al. 2009, Dimitrov et al. 2010). Because of the roles DCs play and their natural propensity for receptor-mediated endocytosis, it only seems fitting for therapeutic antibodies to be viewed as a viable option for therapy against the human immuno-deficiency virus (HIV), cancer, and other autoimmune diseases; researchers have even considered it to be a viable delivery vehicle in cases of cancer (Tacke et al. 2007, Dimitrov et al. 2010, Steinman et al. 2006, Steinman et al. 2000, Kreutz et al. 2011). These therapeutic targeting antibodies have demonstrated moderate success in past clinical trials (Tacke et al. 2007, Steinman et al. 2006).

A monoclonal antibody (mAb) that has proven to be effective in raising robust and sustained immune responses is the α DEC-205 antibody (Boscardin et al. 2006). In past *in vitro* research, the α DEC-205 antibody has proven a capable delivery vehicle of tumor antigen that showed to elicit broad range

antigen specificity (Tsuji et al. 2010). Due to its targeting abilities, the α DEC-205 antibody elicits sustained immune responses to co-delivered vaccine antigens (Bonifaz et al. 2002, Bonifaz et al. 2004, Bozzaco et al. 2007, Tacken et al. 2007, Jiang et al. 1995, Mahnke et al. 2000, Boscardin et al. 2006). During immunizations, this antibody, when co-delivered with antigen, targets the DEC-205 endocytic receptor that internalizes the antibody-antigen complex (Bonifaz et al. 2002, Bozzaco et al. 2007, Mahnke et al. 2000). DCs display the processed antibody-antigenic peptides in the Major Histocompatibility Complex (MHC) molecule's binding groove on the cell's surface for presentation to specific CD4⁺ T cells (Steinman et al. 2000, Koren et al. 2007). When CD4⁺ T cells bind to the peptide-MHC complex, this forms the immunological synapse, a key interaction between DCs and T cells that initiate the adaptive immune response (Janeway et al. 2001, Male et al. 2006). As the adaptive immune response is called into action, two types of immune responses can be induced: either a tolerogenic or an effector-pro-inflammatory immune response (Janeway et al. 2001, Male et al. 2006).

In previous BALB/C-C57BL/6 mice immunizations involving α DEC-205:OVA antibody absent of co-stimulatory molecules, a tolerogenic immune response was observed in both *in vitro* and *ex vivo* cultures (Bonifaz et al. 2002). Further studies confirmed that the tolerogenic immune response was not induced due to the attached vaccine antigen, ovalbumin. The results showed that even when the α DEC-205 antibody was coupled with various antigens, the tolerogenic immune response remained induced because a co-

stimulatory molecule was not present during immunizations (Bonifaz et al. 2002, Bonifaz et al. 2004, Tacke et al. 2007, Matos et al. 2013). This suggested that the antibody itself, rather than the various attached antigens, induced the tolerogenic immune response. Strong inflammatory T cell responses were only generated with co-administration of a maturation factor (α CD40) or adjuvant (Steinman et al. 2000, Bonifaz et al. 2002, Matos et al. 2013)

The tolerogenicity finding is well supported by De Groot et al. (2008) who demonstrated that the presence of T-regulatory cell epitope sequences (Tregitopes) within antibody sequences activates regulatory T cells (Tregs) (modulators of the immune system that prevent it from reacting against self-antigen by readily inducing a tolerogenic immune state) (Josefowicz et al. 2012, Cousens et al. 2013). De Groot and colleagues hypothesized that various natural T regulatory cell epitopes that are highly conserved amongst human and mouse Immunoglobulin G (IgG) cause the tolerogenic effect (De Groot et al. 2008, De Groot et al. 2013). When those particular sequences were presented, an increase in regulatory T cell expansion ($CD4^+CD25^+FoxP3^+$) and activation was detected in studies involving Tregitope stimulation of peripheral blood mononuclear cells (PBMCs) (De Groot et al. 2008, De Groot et al. 2013). It is these Tregitopes within α -DEC-205's antibody sequence that are thought to promote tolerance and require the use of a non-specific immuno-stimulator. If the Tregitopes can be located and modified, it is then possible that the α DEC-205:OVA antibody may be

rendered less tolerogenic, thereby improving its capacity to induce a pro-inflammatory immune response without non-specific activation of the immune system. This is a novel approach that has not been performed prior.

The purpose of this study is to locate and modify tolerogenic Tregitopes within the α DEC-205 sequence, to prevent them from being presented on MHC molecules and subsequently induce a Treg suppressive immune response. Criteria pre-determined by in silico algorithmic tools identifies MHC-II restricted, putative T cell epitope sequences and generates suggested amino acid modifications to the targeted T cell epitopes in order to reduce binding potential to the MHC-II molecules. Suggested modifications are then incorporated into full-length variant α DEC-205:OVA-ORG (original) antibodies. These variant α DEC-205:OVA antibodies are then tested in an array of memory response-immunogenicity assays to determine if the variant antibodies can induce greater levels of pro-inflammatory immune responses, rendering each antibody less tolerogenic due to the specific modifications applied to each antibody's specific Tregitope sequence. These modifications would lead to an improved DC-targeted vaccine platform system that could potentially safely and effectively deliver vaccine test antigens to target T cells by way of the DEC-205 endocytic receptor, found ubiquitously on DCs (Steinman et al. 2000, Bonifaz et al. 2002, Bonifaz et al. 2004, Tacke et al. 2007).

CHAPTER 2

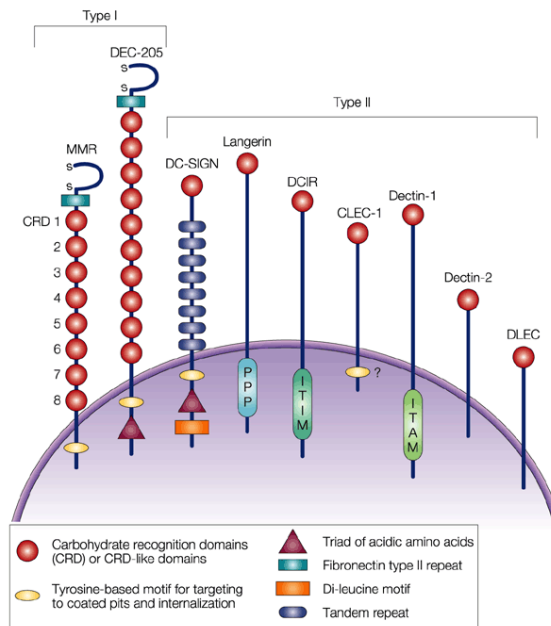
REVIEW OF LITERATURE

Dendritic cells and the Host Defense

A functional priority of immature DCs is to capture antigen, found while patrolling the periphery, and relay acquired processed antigenic peptides to naïve T cells (Steinman et al. 2000, Lipscomb et al. 2002, Hart, 1997, Janeway et al. 2001, Tacke et al. 2007, Wilson et al. 2004). Immature DCs require specialized receptors to recognize and capture antigen (Steinman et al. 2000, Lipscomb et al. 2002, Hart, 1997, Janeway et al. 2001, Wilson et al. 2004). During an infection, DCs are attracted to sites of inflammation via environmental stimuli, where the processes of engulfing invading microorganisms, foreign bodies, or apoptotic self-tissues via phagocytosis and endocytosis occur (van Vliet et al. 2007, van Vliet et al. 2008, Lipscomb et al. 2002). To defend the host and opsonize pathogens, DCs use pattern recognition receptors (PRR) that are highly evolutionarily conserved, germline-encoded receptors, expressed on DC cell surfaces (Kerrigan et al. 2011, Wilson et al. 2004, Pyz et al. 2006). These PRRs recognize characteristics specific to bacterial or viral components known as pathogen-associated molecular patterns that include lipopolysaccharides and peptidoglycans (PAMPs) (van Vliet et al. 2007, Pyz et al. 2006, Kerrigan et al. 2011, Granucci

et al. 2005). PRRs are present in high quantity on immature DCs and are most notably represented by Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (Pyz et al. 2006, van Vliet et al. 2007 den Dunnen et al. 2012, Wilson et al. 2004). PRR activation leads to intracellular signaling and triggers innate immunity, initiating a cascade of events that include secretion of cytokines and the up-regulation of cell surface receptors, which further define the process of DC maturation and T cell interaction (Granucci et al. 2005, Kerrigan et al. 2011, Steinman et al. 2001, Tacke et al. 2007, Hart, 1997, van Vliet et al. 2007, Wilson et al. 2004). Ultimately, this process results in a DC-tailored pathogen-specific adaptive immune response that leads to modulation of subsequent cellular and humoral immune responses (Ueno et al. 2007, Kreutz et al. 2012, Granucci et al. 2005, Kerrigan et al. 2011, Tacke et al. 2007, Geijtenbeek et al. 2009, Steinman et al. 2000, Wilson et al. 2004).

C-type Lectin Receptors



Nature Reviews | Immunology

Figure 1. A dendritic cell and its various C-type lectin receptors (CLRs). These receptors can be found on various subsets of DCs. C-type lectin receptors mainly bind to carbohydrate moieties specific to pathogens, such as peptidoglycan. This recognition process is mediated by the carbohydrate recognition domains and is Ca^{2+} dependent, a hallmark of classical CLRs. Image provided from Figdor et al. 2002.

Both TLRs and CLRs are crucial for DC recognition of pathogen and damaged self-tissue, as well as in “self-homeostasis” (Kerrigan et al. 2011, van Vliet et al. 2007, Steinman et al. 2000, Steinman et al. 2006, Van Kooyk et al. 2008). CLRs, in particular, represent a large superfamily of membrane-associated DC surface-protein receptors that share primary structural homology in the carbohydrate-recognition domains (CRD) (Varki et al. 2009, Kerrigan et al. 2011, van Vliet et al. 2007). Further CLR classification is decided by the consensus, primary-protein sequence, a 115-130 amino acid

length sequence, found within the folded lectin domain of the CRD that primarily determines carbohydrate-binding specificity (van Vliet et al. 2007, Varki et al. 2009, Hart, 1997, Wilson et al. 2004). Numerous pathogens express cell-surface carbohydrate structures that function as pathogen-specific “sugar-fingerprints” that the CLRs, mediated by the CRD, recognize in a calcium dependent manner, a hallmark of classical CLRs (den Dunnen et al. 2012, Pyz et al. 2006, Hart, 1997, Varki et al. 2009).

Immature DCs express a variety of CLRs; classic examples are the Mannose receptors (MR) (CD206), DEC-205 receptors (CD205), Dectin-1 and Dectin-2 receptors, DC-SIGN, Mincle, and asialoglycoprotein receptors (Figure 1) (Tacke et al. 2007, Tel et al. 2011, van Vliet et al. 2007, den Dunnen et al. 2012, Mahnke 2000, Idoyaga et al. 2008). All of these receptors have the capacity to capture glycosylated antigen and mediate interactions between specific pathogens and tailor immune responses (Van Kooyk et al. 2008, Tacke et al. 2007, Steinman et al. 2000, Tel et al. 2011, Wilson et al. 2004).

The Multi-lectin DEC-205 Receptor

Several CLRs have demonstrated the proclivity to not only capture specific antigen, triggering an innate immune response, but also facilitate efficient loading of antigen onto MHC Class I and Class II molecules (Rutella et al. 2006, Lipscomb et al. 2002, Hart, 1997, Tacke et al. 2007, van Vliet et al. 2007, den Dunnen et al. 2001, Idoyaga et al. 2008). Thus, CLRs have been explored as target receptors for targeted antigen delivery via antibodies

(Kreutz et al. 2012). One receptor in particular that shows such functional tendencies is the DEC-205 receptor (Tacke et al. 2007, van Vliet et al. 2007, Lahoud et al. 2012, Kreutz et al. 2012).

The DEC-205 receptor is a C type-1 multi-lectin endocytic receptor that is found ubiquitously on DC surfaces; however, they are also found on various immune cells such as macrophages, B cells, and T cells at varying levels of expression dependent on cell-state maturation (Shrimpton et al. 2009, Steinman et al. 2006, Bonifaz et al. 2004, Mahnke et al. 2000, Tacke et al. 2007, Lahoud et al. 2012, Caminischi et al. 2012, Kato, 2006). DEC-205 is a 205 kD integral membrane protein receptor, homologous to the macrophage mannose receptor, that has a cysteine-rich domain, a fibronectin type II domain, and 10 extracellular, contiguous, C-type lectin-like domains (Lahoud et al. 2012, Mahnke, 2000). The DEC-205 receptor binds to specific-pathogenic carbohydrate moieties in a calcium dependent manner, and takes part in the receptor-mediated endocytic process (Tel et al. 2011, van Vliet et al. 2007, Steinman et al 2006, Geijtenbeek et al. 2009). Within the DEC-205 receptor's distinct distal region of the cytosolic tail is an internalization sequence (requisite coated pit localization sequence) consisting of an acidic EDE triad, which facilitate receptor-mediated endocytosis and efficient recycling through late endosomal/lysosomal compartments (Lahoud et al. 2012 Jiang et al. 1995, Varki et al. 2009, Geijtenbeek et al. 2009, Steinman et al. 2000, Mahnke et al. 2000).

Immunotherapy - Targeted antibodies

There have been a number of treatment options such as surgery, chemotherapy, or pharmaceutical drugs over the years to try and treat most forms of autoimmune diseases, tumors, and cancers (Tacke et al. 2007, Steinman et al. 2000). However, within the past decade, immunotherapy has started to increase in popularity and become a viable option for disease treatment. In immunotherapy, a body's own immune system is used to help fight off infection and disease (Lipscomb et al. 2002, Tabrizi et al. 2006). For example, either immune system's cells are used to elicit a general immune response or mAbs are used to induce an antigen-specific immune response (Lipscomb et al. 2002, Tacke et al. 2007, Steinman et al. 2000, Tabrizi et al. 2006). Cellular-based immunotherapy clinical studies, for example, utilize autologous DCs, *ex vivo* cultured, which are stimulated and loaded with tumor-lysate antigen (Tacke et al. 2007). These DCs are then re-introduced to patients as a form of cellular vaccination against tumors (Tacke et al. 2007, Steinman et al. 2000).

A benefit to this type of vaccination is that cells are stimulated outside of the body, preventing non-specific systemic activation often associated with most vaccines and attributed to the harmful side effects of vaccines, ranging from soreness at the injection site to systemic distress (Stills et al. 2007, Tacke et al. 2007). However, there are limitations associated with cellular-based immunotherapy: availability of readily isolated DC subsets or precursors, isolation of a sufficient number of DCs, *in vitro* culture capacity of

isolated subsets, and DC distribution once administered to the patient (Tacke et al. 2007).

Those limitations and the need for an improved approach lead to the development of antibody targeted immunotherapy against autoimmune diseases, the human immunodeficiency virus (HIV), and cancers (Steinman et al. 2000, Tacke et al. 2007, Lahoud et al. 2012, Lipscomb et al. 2002, Rutella et al. 2006, Pyz et al. 2006, Bonifaz et al. 2004). Given the unique roles DCs play in innate and adaptive immunity and the various cell surface receptors found on DCs capable of endocytotic processes, strident efforts were made to harness and utilize DCs to develop a novel immuno-therapeutic vaccine strategy involving targeted delivery of antigens to resident DCs (Steinman et al. 2000, Tacke et al. 2007, Rutella et al. 2006, Tel et al. 2007, Boscardin et al. 2006). This approach became a viable option in patient care because mAbs' provide beneficial vaccine specificity, which directly target the choice-receptor, requiring less dosage of antigen, and as a result lessens the harmful impact of non-specific activation (Tacke, Steinman, Chan, 2010, Rutella et al. 2006, Pyz et al. 2006, Torchillin et al. 2003, Tabrizi et al. 2006).

Proof of principle-clinical trials reported that initial targeting of DCs by mAbs in vivo lead to favorable ex vivo immune responses once autologous DCs were re-stimulated with antigen (Tacke et al. 2007). Further studies reported the same results, that mAbs are capable of effectively targeting specific receptors for uptake of antigen and immunize against a specific pathogen, though in some cases immune responses were reported to be

limited in regards to various forms of cancer and tumors (Tacke et al. 2007, Lipscomb et al. 2002, Bonifaz et al. 2004, Steinman et al. 2000, Singh et al. 2012). Reports also indicated that targeting antigen to DCs in vivo not only showed cellular immune responses, but also a boost in humoral immune responses (Caminschi et al. 2012).

α DEC-205 Antibody

Past studies showed that the DEC-205 receptor is an effective endocytic receptor capable of internalizing targeted antigen with subsequent presentation of antigen on MHC molecules, on the DC surface, to CD4+ and CD8+ T cells (Bonifaz et al. 2002, Steinman et al. 2006, Tacke et al. 2007, Caminschi et al. 2012, Corbett et al. 2012, Boscardin et al. 2006, Kato, 2006). Previous studies have shown that antigen targeted to the DEC-205 receptor is two orders of magnitude more effective at inducing T cell proliferation and IFN- γ secretion rather than non-targeted antigens (Boscardin et al. 2006). Thus, a therapeutic antibody capable of targeting the DEC-205 receptor was selected to move forward with. α DEC-205:OVA (original) (ORG) is a chimeric mAb that has a rat variable region and a mouse constant region. It is a recombinant fusion protein with full-length ovalbumin whole protein attached to both ends of the α DEC-205 antibody's heavy chains. Ovalbumin is a known mouse immunogen that readily induces antigen-specific cellular and humoral mediated immune responses (Sun et al. 2010). Nevertheless, the α DEC-205 antibody is like any other IgG molecule containing both a F'ab (antigen binding

fragment) and Fc region (fragment crystallizable region) that consists of four polypeptide chains: two light chains and two heavy chains, each with their own constant and variable regions and connected by disulfide bonds (Janeway et al. 2001, Kumagai et al. 2001).

MHC Class II Processing Pathway

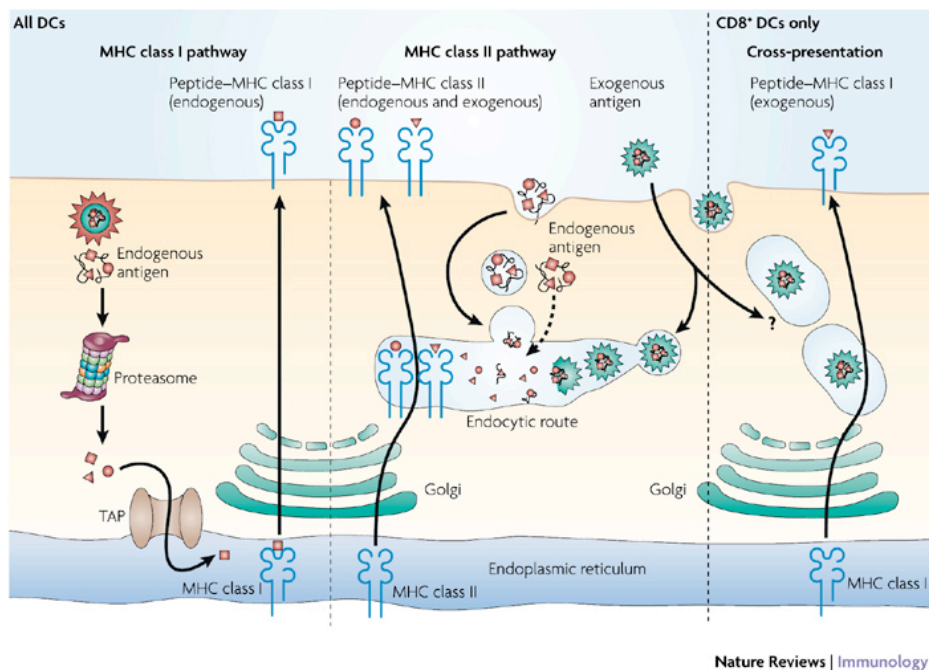


Figure 2. The Class I and Class II antigen-presentation pathways found in all dendritic cells. The MHC I molecule presents peptides that are derived from endogenous proteins that are degraded generally in the cytosol. The MHC II molecule presents peptides that are derived from exogenous protein and degraded by proteolytic enzymes within endosomal compartments. Image provided by Villadangos et al. 2007.

Previous research has shown the α DEC-205:OVA antibody to directly target the DEC-205 endocytic receptor, found ubiquitously on dendritic cells, where after receptor-mediated endocytosis occurs (Mahnke et al. 2000,

Steinman et al. 2006, Tacke et al. 2007, Nagl et al. 2002, Bonifaz et al. 2002). Following the receptor-ligand internalization, the antibody-antigen complex is loaded into a late endosome, which initiates the Major Histocompatibility Complex Class II (MHC II) processing pathway (Steinman et al. 2000, Bonifaz et al. 2004, Tacke et al. 2007, Holling et al. 2004, Mahnke et al. 2000). The DEC-205 receptor facilitates the targeting of late endosomes to load the receptor-ligand complex, due to the coated pit localization sequence in the cytosolic tail (Lahoud et al. 2012, Jiang et al. 1995). The late endosomes are specific to the MHC II pathway and eventually fuse with lysosomes containing internal proteases (Janeway et al. 2001, Male et al. 2006, Lahoud et al. 2012, Bonifaz et al. 2002, Steinman et al. 2007). The low pH of the vesicle enables the antibody-antigen complex to dissociate from the DEC-205 receptor and proteolytic processing begins, generating antibody and antigenic peptide fragments (Janeway et al. 2001, Mahnke et al. 2000, Tacke et al. 2007, Lahoud et al. 2012, Bonifaz et al. 2002, Steinman et al. 2006, Varki et al. 2009). Concurrently, MHC II formation initiates in the Endoplasmic Reticulum (ER) (Male et al. 2006, Janeway et al. 2001).

There are two types of MHC molecules, Class I (MHC I) and Class II (MHC II) (Janeway et al. 2001). Both are uniquely inherited proteins and are the ultimate molecules presenting the peptide fragments generated through the MHC processing pathway (Janeway et al. 2001). The type of antigen engulfed (viral, bacterial) and its location to the cell (extracellular, intracellular) determines which of the two MHC molecules is used and which pathway

(Class I or Class II) to initiate (Janeway et al. 2001, Male et al. 2006). The MHC I molecule generally deals with intracellular antigens such as virus infected cells, while MHC II molecules generally present peptides from extracellular proteins, such as from bacteria (Janeway et al. 2001, Steinman et al. 2000, Mahnke et al. 2000). Since an extracellular antibody-antigen protein is targeting the DC, an MHC II molecule will present the peptide fragments (Figure 2) (Janeway et al. 2001).

An MHC II molecule is a cell surface protein heterodimer with 2 homogenous polypeptide chains (α , β) consisting of 2 intracellular, trans-membrane, and extracellular domains (Mangalam et al. 2013). The 2 extracellular polymorphic domains ($\alpha 1$, $\beta 1$) result in the formation of the open-ended antigenic peptide-binding groove (Mangalam et al. 2013, Janeway et al. 2001). For the MHC II molecule, there are 3 pairs of α - and β -chain genes, called HLA-DR, -DP, and -DQ (Janeway et al. 2001, Male et al. 2006). With several possibilities of different genes for each MHC II molecule, an individual is capable of recognizing and presenting a broader range of antigenic peptides (Janeway et al. 2001). The MHC II in humans is referred to as Human Leukocyte Antigen (HLA) complex and H-2 in mice (Janeway et al. 2001).

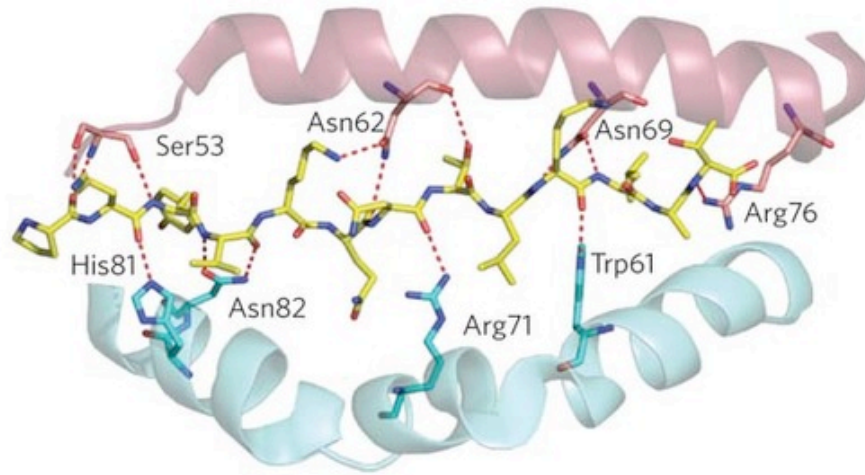


Figure 3. Peptide bound to an MHC II molecule. This is a top view of a MHC II molecule with a specific peptide antigen (Influenza HA peptide 3006-318) bound in the MHC II molecule's binding groove. Key peptides can be seen anchoring the peptide into the binding groove. This peptide-MHC complex is displayed on the dendritic cell surface for presentation to naïve T cells in the lymph nodes. Image from Neefjes and Ovaa, 2013.

During this MHC II processing pathway, while the MHC II forms in the ER, an invariant (Ii) chain also forms (Janeway et al. 2001, Male et al. 2006). The Ii is ultimately situated in the MHC II peptide-binding groove, and acts as a chaperone-peptide throughout the MHC II molecule's travel to the Golgi apparatus (Janeway et al. 2001). The Ii chain forms a core trimer that associates with the 3 MHC II $\alpha\beta$ subunit-dimers and results in a nonameric complex incapable of binding antigenic peptides, which is important since endogenous peptide fragments are present in the ER during the MHC II formation (Yan et al. 2003).

The Ii chain secures the MHC II's peptide binding groove and the MHC

II-li complex undergoes extensive glycosylation to reach the late endosomal/lysosomal's vesicle antigen processing compartments after leaving the Golgi apparatus (Yan et al. 2003, Janeway et al. 2001). A series of highly regulated proteolytic cleavages along with a HLA-DM accessory protein catalyzes the final release of the last li chain fragment, altogether known as the Class II associated invariant peptide (CLIP), and facilitates the binding of antigen to the binding groove (Yan et al. 2003). With the li chain removed, the MHC II peptide-binding groove presents specific antigenic peptide fragments (Yan et al. 2003, Mahnke et al. 2000).

The crystal structure of an MHC II shows that the antigenic peptide-binding groove can accommodate peptide fragments between 12-25 amino acids in length (De Groot et al. 2008, Mangalam et al. 2013). The core 9mer peptide binds non-covalently, but is held in place at key anchor sites within the binding groove (positions 1, 4, 6, and 9) (De Groot et al. 2008, Mangalam et al. 2013, Male et al. 2006). Peptides are chosen based on stability, charge, and binding affinity to the MHC II molecule (De Groot et al. 2008, Mangalam et al. 2013, Parker et al. 2010, De Groot et al. 2009). The peptide-MHC II formation results in a stable complex that travels to the DC surface for presentation to its target cell population, CD4⁺ T cells (Holling et al. 2004, Steinamn et al. 2000, Yan et al. 2003, Mahnke et al. 2000).

DC Migration

Now DCs undergo a maturing differentiation process, which is

facilitated by the uptake of antigen and subsequent generation of peptides (Steinman et al. 2000, Steinman et al. 2007, Tacke et al. 2007, Lahoud et al. 2012, Wilson et al. 2003). Antigen processing primes the immature DCs to initiate intracellular signaling, secrete cytokines, modulate cell surface receptor expression, and further signals the DCs to migrate from the tissue periphery via the lymphatic system to the draining lymph node, the site of antigen localization and naïve T cell priming (Steinman et al. 2007, Janeway et al. 2001, Lahoud et al. 2012, Lipscomb et al. 2002, Mahnke et al. 2003). During this process, immature DCs outwardly change their appearance to possess more finger-like structures that protrude from the main body to increase surface area multifold; this morphological change better represents their new primary role of antigen presentation via MHC molecules (Lipscomb et al. 2002, Bonifaz et al. 2002, Steinman et al. 2006, Tacke et al. 2007, Wilson et al. 2003).

Immunological Synapse (IS)

Once DCs mature, up-regulation of various “signaling” molecules, representative of the current cell state appear, such as increased expression of MHC molecules, cell surface receptors (CD80, CD86, CD40), secretion of chemokines and cytokines, and adhesion molecules (Steinman et al. 2000, Lahoud et al. 2012, Lipscomb et al. 2002, Tacke et al. 2007). The current state of cellular and environmental affairs is represented by the critical expression of these molecules and is meant to inform other immune cells, especially T cells whilst the DC is in the lymph node (Steinman et al. 2000,

Janeway et al. 2001). The naïve T cells present in the lymph node are continuously circulating around the DCs, searching for antigenic peptides that are displayed on MHC II molecules (Steinman et al. 2000).

T cells contain on their surface T cell receptors (TCRs), which are cell surface disulfide-linked heterodimer receptors similar to the antibody's F'ab region (Broere et al. 2011, Janeway et al. 2001, Rudolph et al. 2006). At any one time, approximately 30,000 TCRs are present on the T cell's surface with the majority of TCRs containing a α and β chain (Janeway et al. 2001). Antigen specificity is determined by TCRs, which recognize and bind to specific peptides presented by the MHC II molecules, on DC surfaces (Broere et al. 2011). However, T cells are only capable of recognizing peptides if they are apart of the peptide-MHC complex (Figure 5) (Broere et al. 2011, Josefowicz et al. 2012, Janeway et al. 2001). T cell lineage-specific accessory molecules and adhesion molecules bind to DCs (CD4 or CD8) and facilitate the process by providing stability to the DC-T cell interaction (Janeway et al. 2001, Broere et al. 2011). Nonetheless, immediately after binding, the naïve T cell initiates a cascade of events, such as intracellular signaling with -function-associated protein 1 (LFA-1) and the intracellular adhesion molecule 1 (ICAM-1), up-regulates and modulates cell surface receptors (CD28) (CTLA-4), and secretes cytokines and chemokines (Rudolph et al. 2006, Janeway et al. 2001, Broere et al. 2011).

This activation process of the T cell informs the DC whether to assume an inflammatory or regulatory phenotype hereafter, alerts other immune cells

to the antigens presence, and polarizes T cells, which can result in activation and expansion of T effector cells and B cells or T regulatory cells (Tregs) (Broere et al. 2011, Janeway et al, 2001, Mennechet et al. 2006). Overall, this DC-T cell interaction creates positive and negative feedback loops filled with information on how to proceed with the antigen-specific immune response (Broere et al. 2011, Janeway et al. 2001, Steinman et al. 2007).

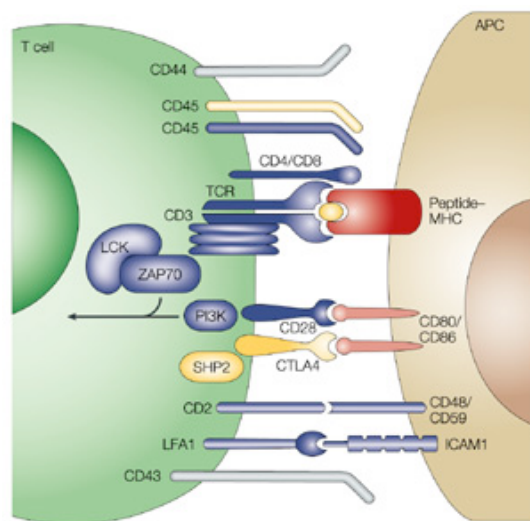


Figure 4. A mature Immunological Synapse (IS). The IS represents the events leading up to and the formation of the nanometer scale gap interaction between APCs and T cells, as well as the subsequent T cell polarity and signaling pathways activated by this synapse formation. Image from Huppa et al. 2003.

This fluid and dynamic interaction between the DC's MHC II-peptide complex and the T cell's TCR is the basis for the immunological synapse (IS) (Figure 4) (Griffiths et al. 2010, Bromley et al. 2001, Viola et al. 2010, Steinman et al. 2007, Huppa et al. 2003). The IS encompasses the environment generated by the coupling of DCs and T cells, such as it relates to the membrane structure, T cell polarity, signaling compartmentalization, the

antigen-presenting cell, and quality of antigen presented (Bromley et al. 2001, Viola et al. 2010, Huppa et al. 2003, Grakoui et al. 1999). This interaction results in T cell activation and the initiation of an antigen-specific T cell mediated adaptive immune response (Janeway et al. 2001, Male et al. 2006, Broere et al. 2011, Bromley et al. 2001, Huppa et al. 2003). Thus, antigen trafficked to the DEC-205 receptor can lead to the generation of either a T cell effector or regulatory antigen-specific immune response (Steinman et al. 2007, Tacke et al. 2007, Bonifaz et al. 2002).

Adaptive Immunity

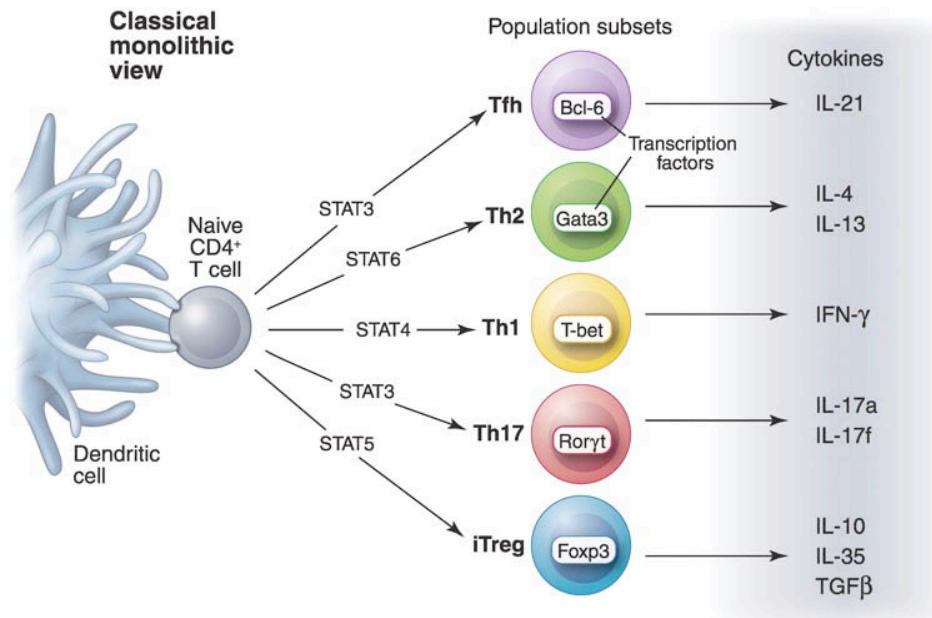


Figure 5. Naïve T cell differentiation. After a naïve T cell recognizes and binds to a peptide-MHC complex, a cascade of events occurs intracellularly and extracellularly that allows the naïve T cell to undergo differentiation. Image from O'Shea and Paul, 2010.

Adaptive immunity refers to the immune responses generated to a specific peptide-MHC complex that are then recognized by antigen-specific T cells (Steinman et al. 2006, Janeway et al. 2001). Adaptive immunity is slow acting, but it is more specific and targeted against a particular pathogen (Janeway et al. 2001, Steinman et al. 2007). From adaptive immunity, two types of IS induced immune responses are possible: A tolerogenic immune response or an effector immune response (Janeway et al. 2001, Steinman et al. 2007).

Generally, an effector immune response is generated after T cell recognition and activation to peptides presented in context of MHC molecules and co-stimulatory signals provided by APCs (Figure 5) (Steinman et al. 2007, Janeway et al. 2001). Signal 1 consists on the foreign peptide presented by the MHC II molecule and signal 2 is provided by the co-stimulatory proteins produced, up-regulated, or secreted during the IS interaction, such as CD28, CD80, and CD86 (Janeway et al. 2001, Rudolph et al. 2006, Bromley et al. 2001, Viola et al. 2011). An effector immune response would result in a robust pro-inflammatory immune response characteristic of pro-inflammatory cytokines, such as IL-1, TNF α , IFN- γ , IL-2, IL-4, or IL-12 (Hart, 1997, Dinarello et al. 201, Lipscomb et al. 2002, Wilson et al. 2003, Desombere et al. 2004).

A tolerogenic immune response is very similar in process to the effector immune response as it too is controlled directly or indirectly by the up-regulated and secreted proteins resulting from a chain of cellular interactions initiated by the presentation of bound peptide (Barbosa et al. 2007). However,

there are a few key differences: The bound peptide is from “self-protein” and there are limited numbers of cytokines and cell surface receptors present (Janeway et al. 2001). Those key differences, overall, result in a regulatory immune response with either the interacting T cell or DC secreting regulatory cytokines such as TGF- β or IL-10 (Josefowicz et al. 2012). T helper cells will become activated via the cytokine signaling of TGF- β or IL-10 and differentiate into Tregs (Male et al. 2006, Josefowicz et al. 2012).

Regulatory T cells

Regulatory T cells (Tregs) are the mediators of the immune system. Tregs, after activation, secrete regulatory cytokines that inhibit effector T cell responses. They are featured prominently in autoimmune diseases, inflammatory disorders, peripheral tolerance, and immune homeostasis (Josefowicz et al. 2012, Lio et al. 2010, Lee et al. 2014, Burchill et al. 2008). Tregs are one way for the immune system to protect itself against reacting to “self-protein”, as well as have the capacity to minimize the effects of inflammation and damage induced by pathogens (Josefowicz et al. 2012, Male et al. 2006, Janeway et al. 2001, Lio et al. 2008, Burchill et al. 2008). Tregs are characterized as suppressive immune cells whose phenotypes are CD4⁺CD25⁺FoxP3⁺. They require the presence of CD28, IL-2, and to a lesser extent IL-7 and IL-15, in order to become activated and fully differentiated (Josefowicz et al. 2012, De Groot et al. 2008, Lio et al. 2008, Burchill et al. 2008). FoxP3 is a forkhead family transcription factor that has come to be

associated with Tregs, and, though not a definitive marker, its presence still supports a dedicated function in Treg cell differentiation, as was reported in FoxP3 reporter mice cells $CD4^+CD25^+FoxP3^+$ (Josefowicz et al. 2012, Ohkura et al. 2012).

Induction of a Tolerogenic Immune Response

As reported in past studies, OVA antigen delivered by the α DEC205:OVA antibody to the DEC-205 receptor is internalized and processed by DCs (Bonifaz et al. 2002). Processing of the protein complex generates peptide fragments that are then loaded, if possible, onto the MHC II molecule for presentation to T cells (Bonifaz et al. 2002, Tacken et al. 2007, Steinman et al. 2006). Normally, delivery of antigen with this targeted vehicle induces a tolerogenic immune response when no maturation factor is used (Bonifaz et al. 2002, Steinman et al. 2006, Tacken et al. 2007, Uto et al. 2013).

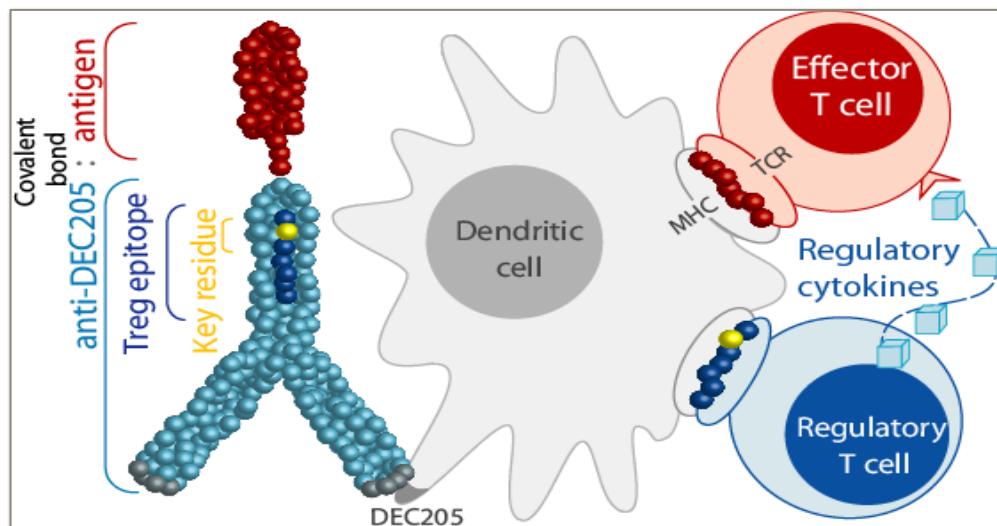


Figure 6. The Tregitope-effect concept. This figure depicts the concept behind the induction of a tolerogenic immune response that is observed after the α DEC-205:OVA antibody, without a maturation factor, is used during immunizations. Provided by Weber et al., 2008.

Presentation of tolerogenic signals or natural T regulatory cell epitopes (Tregitopes) is hypothesized to induce the tolerogenic immune response when the α DEC205:OVA antibody is used in immunizations (De Groot et al. 2008, De Groot et al. 2013). Derived from IgG molecules, Tregitopes are potentially found in both the F'ab (framework) and Fc regions of an antibody (De Groot et al. 2008, Cousens et al. 2013). Tregitopes are highly conserved in IgG molecules and considered highly promiscuous due to binding to various HLA alleles (De Groot et al. 2008, Cousens et al. 2013). Due to the “foreign” (not seen in thymic development) somatic hypermutations at the variable region's antigen-binding site in IgG molecules, it is hypothesized that highly conserved sequences (Tregitopes) were retained throughout evolution for self-protein (IgG molecules) to escape immune system recognition (De Groot et al. 2013, Janeway et al. 2001, Livesay et al. 2004).

Published findings show an induction of tolerance owing to Treg expansion ($CD4^+CD25^+FOXP3^+$) when Tregitope sequences are co-cultured with peripheral blood mononuclear cells (PBMCs) (De Groot et al. 2013). These studies were eventually replicated by Zambidis and Scott (1996) and found that an IgG's heavy chain fused to antigen induced tolerance as well, where current human Tregitopes (167 and 289) are found in the constant heavy chain FC region (De Groot et al. 2008). Thus, Tregitopes, found within the α DEC-205 antibody's sequence, are thought, once presented by MHC II molecules, to activate Tregs, upregulate Treg associated cytokines and

chemokines, and subsequently induce an antigen-specific regulatory immune response (De Groot et al. 2008, Singh et al. 2012, Cousens et al. 2013).

Proposed Objective

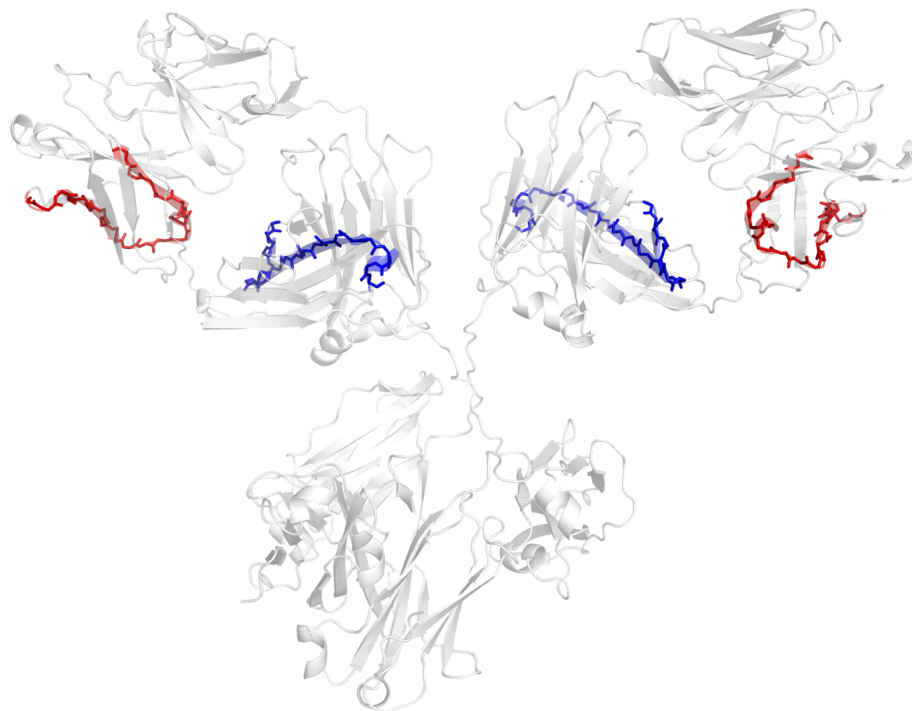


Figure 7. A 3-D model of the α DEC-205 antibody with the targeted Tregitopes highlighted in blue and red. Tregitopes or natural T regulatory cell epitopes are potentially highly conserved sequences found in all IgG molecules. When Tregitopes are presented they are believed to induce CD4+CD25+FoxP3+ regulatory T cells. Two Tregitope sequences, HC54 and VH77, are highlighted in red and blue, respectively. HC54 sequence (red) is found in the constant heavy chain region and the VH77 sequence (blue) is located in the variable heavy chain region. Model provided by Dr. Yoonjoo Choi and Dr. Chris Bailey-Kellogg (Dartmouth).

Adjuvants are believed to be required for the α DEC205:OVA antibody's targeted antigen delivery due to Tregitope presence in the IgG delivery vehicle (De Groot et al. 2008). Adjuvants are used to induce a robust and sustained,

strong T cell mediated immune response to the specific antigen fused to the antibody (Steinman et al. 2000, Hill et al. 2007). Therefore, selected Tregitope sequences within the α DEC-205:OVA antibody are modified to diminish tolerogenicity, which will inhibit Treg activation, and promote immunogenicity to less immunogenic peptides, such as the vaccine test antigen, ovalbumin. This approach removes the need for non-specific activation of the immune system (De Groot et al. 2008, Moise et al. 2012).

The proposed objective is to compare the variant α DEC-205:OVA immunization conditions' immunized splenocytes' responses to the non-modified α DEC-205:OVA-ORG immunization condition's immunized splenocytes' responses. Splenocyte responses are observed in two immunogenicity assays to determine if a greater pro-inflammatory immune response, characteristic of T cell proliferation and pro-inflammatory cytokine signaling, is observed when immunizing mice with the Tregitope modified antibody sequences. This will help to uncover whether Tregitopes are the cause for tolerogenic effects when antigen is administered in the absence of an adjuvant.

CHAPTER 3

MATERIALS AND METHODS

Mice

HLA-DRB1*0401 (DR4) mice are a transgenic humanized HLA mouse model derived from a C57BL/6 background that are used to test vaccine efficacy and T cell responses specific to the DR4 allele (Ito et al.1996). The DR4 mice are brought in from Taconic and cared for in-house in the bio-safety level 2 (BSL-2) trailer located at Peckham Farm on the University of Rhode Island's Kingston campus (Ito et al.1996, Grusby et al.1991). The mice founders of this transgenic line were initially crossed with MHC II deficient mice (Genpharm C2d line), which has the I-A beta gene inactivated and the unexpressed I-E alpha allele found in the C57BL/6 haplotype (Ito et al. 1996, Grusby et al. 1991). Mice contain hybrid MHC II molecules that contain the peptide binding domain specific to the HLA-DRA and HLA-DRB1*0401 allele (Ito et al. 1996, Grusby et al.1991).

Antibodies

The α DEC-205 antibody recombinant expression constructs were obtained from Dr. Ralph Steinman (The Rockefeller University). Aldevron produced the high titers (1 mg/mL) of the original wild type α DEC-205 antibody (ORG) and modified antibodies α DEC-205:OVA-CH54-MOD1, α DEC-

205:OVA-VH77-MOD1, α DEC-205:OVA-VH77-MOD2 (MODs). α DEC-205 is a chimeric, monoclonal, rat- α -mouse antibody that is transfected and expressed in the Chinese Hamster Ovary (CHO-S) cells mammalian expression system (Invitrogen-Life Technologies R800-07). The ovalbumin whole protein (45 kD), a major component of chicken egg white, was recombinantly fused via linker protein sequence to the C-terminus of the α DEC-205 heavy chain (Yasushi).

The staining antibodies used in the immunogenicity assays are purchased from eBioscience, BDBiosciences, or Life Technologies (Affymatrix) and can be viewed in the table below (Table 1-2). The fluorescent cell-staining dye, CFSE (Carboxyfluorescein succinimidyl ester) was purchased from Life Technologies.

Table 1. The JAWS II DC binding assay antibody staining panel.

Antibody/Stain	Manufacturer	Clone
α ms-IgG-FitC	eBiosciences	GK5.1
α CD11c-PE-Cy7	eBiosciences	145-2C11

Table 2. The T cell proliferation assay antibody staining panel.

Antibody/Stain	Manufacturer	Clone
α CD4-BV 421	BD Biosciences	GK5.1
α CD8a-APC	BD Biosciences	53-6.7
α CD3e-PE	BD Biosciences	145-2C11
CFSE	Life Technologies	n/a

JAWS II Dendritic Cells

A bone marrow derived-C57BL/6 background dendritic cell line, JAWS II DCs (ATCC-CRL-11904), are passaged once weekly at a 2:1 (cells: culture media) ratio utilizing α MEM culture media supplemented with deoxy and ribonucleosides, 5% L-glutamine 100x (Invitrogen 25030-156), 5% Sodium-pyruvate (Hyclone-SH3023901), 10% Fetal Bovine Serum (Invitrogen 10100-147), 5% Penicillin/Streptomycin (Invitrogen 15140-122), and Granulocyte Macrophage-Colony Stimulating Factor-Recombinant Mouse (R&D systems: 415ML010) in T-75 flasks, as per manufacturer's (ATCC) instructions (ATCC-CRL-11904).

Immunoinformatics Analyses: The EpiMatrix System

EpiMatrix

The α DEC-205 sequence was computationally screened for putative HLA-Class II restricted conserved T cell epitopes with the use of an in silico tool, EpiMatrix (Table 1). EpiMatrix identifies which 9 amino acid frames are predicted to bind to specific HLA alleles and are highly conserved in existing databases of IgG sequences (De Groot et al. 2013, De Groot et al. 2014). Protein antigens are parsed into overlapping 9-mer frames. Each 9-mer frame overlaps the last frame by eight amino acids. 9-mer frames are scored against a panel of 8 class II "supertype" alleles that comprise at least 90% of the world's human HLA genetic makeup to determine the epitope's binding potential to each of the 8 common class II alleles: DRB1*0101, *0301, *0401, *0701, *0801, *1101, *1301, and *1501 (De Groot et al. 2008, Cousens et al.

2013, Singh et al. 2012). The EpiMatrix algorithm compares the amino acid sequence of each 9-mer frame to the coefficients contained in the matrix, which produces a raw score for each frame. The raw score is then converted to a normalized z-scale. The EpiMatrix z-scale provides z-scores, allowing for comparison of potential epitopes to multiple class II HLA alleles (De Groot et al. 2008, Singh et al. 2012). Peptides scoring ≥ 1.64 (top 5th percentile) on the EpiMatrix “Z”-scale are predicted MHC II ligands. The higher the EpiMatrix “Z” score, the more likely the peptide sequence is an MHC ligand and a T cell epitope (De Groot et al. 2008). The EpiMatrix Cluster analyses reported predictions for each 9mer frame sequence with top 10% z-scores (hits) or greater. The relevant z-scores can be read as follows, top 10% hits are highlighted in the lightest blue, the top 5% hits in a bluish-grey, and the top 1% hits are highlighted in dark blue (Table 5). The top 1% and 5% hits reported in all the EpiMatrix Reports have z-scores ≥ 2.30 and scores between 1.64-2.29, respectively (Table 5).

ClustiMer

ClustiMer is a ancillary algorithm used with EpiMatrix analyses and identifies areas densely laden with putative T cell epitope clusters with high-scoring EpiMatrix z-scores throughout the entire antibody sequence (Sintechenko et al. 2010). More specifically, ClustiMer maps the EpiMatrix motif matches along the length of a protein and calculates the density of motifs against the panel of eight HLA alleles (De Groot et al. 2008, Sintechenko et al. 2010). T cell epitope clusters with ClustiMer scores ≥ 10 indicate a significant

potential for immunogenicity for a given epitope sequence. Clusters usually range between 9-25 amino acids in length and can have 4-40 binding motifs (De Groot et al. 2008). All high scoring putative T-cell epitope clusters ultimately are compared to human IgG sequences and only cluster sequences showing homology to human IgG, potentially regulatory sequences, were selected for targeted modification, to reduce binding potential to the HLA-DRB1*0401 allele (De Groot et al. 2008, Caspi, 2008, Parker et al. 2010).

OptiMatrix

OptiMatrix, the final algorithm, identifies amino acids contributing the most to the T cell epitope's HLA binding affinity to a specific allele and targets the amino acids for select modifications, to reduce binding potential (De Groot et al. 2008). Each epitope sequence modification is dependent on the individual amino acid's binding affinity to the MHC II molecule and the predicted effect it will have on the HLA binding affinity (De Groot et al. 2008). Only amino acid substitutions that resulted in the reduction of the epitope sequence's EpiMatrix z-score were chosen for potential incorporation into the full-length α DEC-205:OVA-ORG antibody. A reduction in the predicted EpiMatrix z-score correlated to that particular epitope sequence's decrease in predicted binding affinity for a given HLA-allele (Moise et al. 2012, De Groot et al. 2008). Generally, amino acids located within the MHC II anchoring peptide positions are modified first, since those amino acids are key to that peptide's affinity to the MHC molecule (De Groot et al. 2008). Normally, both alanine or glycine are chosen as amino acid replacements because they are least likely

to interact with the surrounding peptides within the MHC II molecule's binding groove and provide less steric hindrance (De Groot et al. 2008). Alanine substitutions have shown in previous work by Warmerdam *et al.* to result in de-immunization of the epitope and reduce or eliminate a subsequent T cell response (Schönbach et al. 2008).

Site Directed Mutagenesis

The suggested modifications were incorporated into the full-length α DEC-205:OVA sequence by Site Directed Mutagenesis to produce an array of α DEC-205 antibody variants via Stratagene's QuikChange® XL II Site-Directed Mutagenesis Kit, per manufacturer's instructions. The procedure enables site-specific mutations within double-stranded plasmids and eliminates the need for specialized vectors and unique restriction sites. Normal thermo-cycling procedures involving denaturation of plasmid at 95°C for 1 cycle, annealing of the mutagenic primers complementary to opposite strands of the plasmid, and subsequent elongation of the mutagenic primers for 18 cycles, resulting in the synthesis of mutant strands (Quik Change XL II-200522). Left over hemi-methylated and methylated parental DNA strands were removed by Dpn I endonuclease digest. Resulting nicked-mutant strands were then transformed into XL10 gold bacterial cells where ligation and subsequent transformation into bacterial cells occur (Quik Change XL II-200522). Newly transformed XL 10 gold bacterial cells containing the mutant strands were streaked on Agarose gel plates containing ampicillin. Immediately following, streaked plates were placed in a 37°C incubator for

overnight cell growth. Only bacterial cell colonies that had properly taken in the plasmid and potentially the mutagenic strands would have been able to grow due to antibiotic resistant genes present within the plasmid. Thus, colonies were chosen the following morning for plasmid purification purposes. The very same day, plasmid DNA was isolated from the top 10 cells via lysing techniques and purified using Qiagen-mini prep techniques (QIAGEN-Kit Cat. No. 27104). The purified plasmids containing the select amino acid modifications to targeted potential Tregitope sequences were sent for confirmatory sequencing and high production yields (1 mg/mL) of low endotoxin plasmid to Aldevron.

Transfection of CHO-S cells

Chinese Hamster Ovary-S cells (CHO-S) (R800-07) were passaged day prior to transfection, at 5×10^6 cell/mL in Freestyle™ CHO Expression Media (12651-014) as per manufacturer's instructions (Invitrogen-Life Technologies-K9000-20). Flasks were placed in an orbital shaker platform (120-135 rpm) at 37°C, 8% CO₂. On the day of transfection the CHO-S cell density must range between $1.2\text{-}1.5 \times 10^6$ cell/mL. Cells were counted via Cellometer and the live cell viability is recorded. A 95% cell viability or greater is also required to ensure high transfection results. 30 million cells were taken from the repository culture flask and were loaded into a new 125 mL culture flask that is placed into a 37°C incubator for acclimation purposes.

Immediately following, 37.5 ug of each respective plasmid DNA is aliquoted into FACS test tubes and mixed with 0.6mL/test tube of OptiPro™ SFM (123-9-050). Concurrently, in separate tubes, the Freestyle™ MAX Transfection Reagent (16447-100) is diluted with up to 0.6mL/test tube of OptiPro™ SFM. Plasmid DNA preparations were mixed with their respective transfection reagent-OptiPro solutions, yielding mixed transfection reagent-DNA solutions. Mixed DNA-transfection solutions incubated for 10 minutes (min.) at room temperature, enabling formation of plasmid DNA-reagent complexes. The DNA-reagent complex solutions were added to the respective previously acclimated 125 mL culture flasks. Culture flasks were then loaded into the orbital shaker platform within the 37°C, 8% CO2 incubator for a maximum of 7 days (Invitrogen). During the 7-day time frame, cell viability is checked everyday to determine the optimal “harvest” day. Generally, cells are harvested on the 7th day due to 60-80% cell viability throughout the entire transfection period. Once the cells were harvested, the cell pellet was separated from the supernatant and discarded. Supernatants are stored at -20°C until protein purification.

Protein Purification

Supernatants were processed one of two ways: By normal flow filter concentration prior to column purification or just column purification. At times, if supernatant volumes surpassed a certain volume, the flow filter concentration is used to concentrate the supernatants prior to loading on the

AKTA Avant liquid chromatography system. A collection of supernatants, 300 mL volume maximum, were thawed and loaded into the protein concentrator. A specific size exclusion membrane is used that only allows protein sizes up to the molecular weight of the α DEC-205:OVA-ORG antibody to pass. The concentrated supernatant was then collected into a secondary stationed 50 mL test tube for column purification on the AKTA Avant. All larger extraneous protein is prevented from passing and maintained in the protein concentrator.

If that step was not warranted and was by passed, then supernatants were purified using the AKTA Avant liquid chromatography system as per manufacturer's instructions (GE Healthcare Life Sciences). The supernatant was streamed over a protein G column that contains bound Fc-receptors that bind specifically to the protein of interest. Immediately after, a wash buffer was ran over the protein G column to ensure the extraneous protein was washed off the column and out of the machine. A second buffer containing competing ligands was ran over the protein G column and the ligands bind to the Fc receptors, outcompeting the protein of interest. The bound protein was eluted off the column and into waiting micro-centrifuge tubes. A sudden spike in protein concentration, represented as peaks on the AKTA Avant's display screen, alert to the presence of eluted protein in micro-centrifuge test tubes. Fraction samples collected at various time points during the purification process, such as prior to supernatant loading into the AKTA Avant, supernatant (flow through) run-off once ran over the protein G column, wash cycle run-off, and finally a sample of from the final purified eluate (Figure 8).

These fraction samples were ran on a sodium dodecyl sulfate (SDS) polyacrylamide (PAGE) gel and western blot to confirm purity of protein, molecular weight of protein, and if any protein was lost throughout the procedure. After protein purification confirmation, the newly purified protein's concentration was analyzed via Nano Drop. The protein was aliquoted into working stock solutions and stored at 4°C until further use.

Dendritic Cell Binding Assay

DC binding assays were performed to determine if the variant α DEC-205:OVA antibodies, after modification, were capable of targeting and binding to the DEC-205 receptor found on DCs after modification. JAWS II DCs were aliquoted into FACS test tubes at 1×10^6 /mL. Instantly, 5 ug/mL of purified wild type and variant α DEC-205 antibodies were added to respective FACS test tubes. The DC-antibody mixtures were cultured for 30 min. at room temperature; this allows time for the antibodies to target the DEC-205 endocytic receptor. After the 30 min. incubation, DCs were washed twice more. Afterwards, the FACS test tubes containing the wild type and variant α DEC-205:OVA antibodies were stained with a rat- α -mouse IgG-PE antibody for 1hr at 4°C. This antibody only detects the α DEC-205:OVA antibody attached to the DEC-205 receptor on the DC surface. Any α DEC-205:OVA antibody not bound to the DEC-205 receptor was washed away after the staining incubation in subsequent washes. DCs were then immediately analyzed on the BD LSRII Flow Cytometer. The rat- α -mouse IgG-PE staining

antibody allows for fluorescence intensity quantitation based on antibody targeting capacity to the DEC205 receptor.

Immunization and Splenocyte Isolation Procedure

Immunizations to the 16 HLA-DR4 mice were performed using a 27” gauge needle for subcutaneous injections to the left hind flank. Immunizations occur at the BSL-2 trailer at Peckham Farm on the University of Rhode Island’s Kingston Campus. Immunizations and splenocyte culturing procedures were followed according to a previously described protocol (Bonifaz et al. 2004) with minor procedural adjustments.

16 HLA-DRB1*0401 mice are immunized on Day 0 and after a 7-day time period mice were anesthetized with isoflurane (inhalant) via an oxygen chamber and then euthanized with Ketamine (100µl/mouse). After euthanasia, spleens were immediately harvested, put on ice, and transported back to URI’s Feinstein Providence Campus (Bonifaz et al. 2004). Once back at the lab, the splenocyte isolation procedure begins.

Splenocytes were isolated from their tissue matrix via maceration of spleens over a 40µm nylon mesh filter that sits atop a 50 mL test tube. This is performed for each respective immunization condition. Resulting splenocytes are not pooled together and remain in their respective 50 mL collection test tube. Afterwards, the filters atop of the 50 mL collection test tube were washed with 10 mL RPMI-1640 culture media to displace residual splenocytes. A Cellometer is then used to count the number of living splenocytes there are

per milliliter of solution. Trypan blue, a viability dye exclusion method is used to identify live and dead cells (Tran et al. 2011). Trypan blue was added to an aliquot of cells and then loaded onto a counting chamber specific to the Cellometer, which then determines the live cell concentration and viability of the splenocyte samples. Splenocytes were separated according to number requirements for each immunogenicity assay. Both memory response immunogenicity assays, a T cell proliferation assay and a Murine (Mu)-Interferon-gamma (IFN- γ) enzyme-linked immunospot assay (ELISpot), were used to detect splenocyte pro-inflammatory tendencies observed from antigen re-stimulation (Desombere et al. 2004, Desombere et al. 2005).

Table 3. HLA-DRB1*0401 mice immunization conditions.

Group ID	Mouse ID	Immunization Conditions
A	A1	Negative CTL (PBS)
	A2	
B	B1	Antibody CTL (α DEC:OVA-ORG)
	B2	
C	C1	α DEC:OVA + CD40
	C2	
D	D1	α ISO:OVA + CD40
	D2	
E	E1	Adjuvant CTL (CD40)
	E2	
F	F1	α DEC:OVA-HC54-MOD1 (MUT1)
	F2	
G	G1	α DEC:OVA_VH77-MOD1 (MUT2)
	G2	
H	H1	α DEC:OVA_VH77-MOD2 (MUT3)
	H2	

Ex vivo T cell proliferation assay

Splenocytes were resuspended in prewarmed PBS/0.1% Fetal Bovine Serum (FBS) at a final concentration of 1×10^7 cells/mL. CFSE labeling ($1\mu\text{M}$) was performed per manufacturer's instructions (Life Technologies) prior to culture. Splenocyte containing FACs test tubes receive $1\mu\text{L}$ of the $1\mu\text{M}$ CFSE solution and were incubated for 10 min. at 37°C . With the addition of ice-cold culture media at five times the volume, the CFSE staining process was quenched. Immediately following, splenocytes were incubated for 5 min. in an ice bath. After splenocytes are washed three times, they were plated in a 48-well flat-bottomed plate at 1×10^6 cells/0.5 mL. Prior to 72hr 37°C incubation, splenocytes were re-stimulated with ovalbumin at $500\mu\text{g/mL}$. Ovalbumin antigen re-stimulation is done to identify if splenocytes can recall antigen-specific T cell response due to initial immunizations with ovalbumin (Bonifaz et al. 2004).

Following the 72hr incubation, splenocytes were harvested and stained with fluorophore-conjugated antibodies (Table 2) for 1hr at 4°C . After the 1hr incubation, excess staining antibody was removed with splenocyte washing. Splenocytes were then analyzed for T cell proliferation on the BD LSR II Flow Cytometer. For this particular experiment, $\text{CD3}^+\text{CD4}^+$ T helper cells and $\text{CD3}^+\text{CD8}^+$ Cytotoxic T cells were identified amongst the splenic population and measured for percent T cell proliferation. Of particular interest is the percent T cell proliferative responses of the $\alpha\text{DEC-205:OVA-ORG}$ antibody immunized splenocyte responses to ovalbumin re-stimulation in comparison to

the proliferative responses of the variant α DEC-205:OVA antibody immunized splenocytes.

Ex vivo ELISpot assay

The capture and detection antibodies were supplied by MABTECH and the ELISpot assay protocol was followed per manufacturer's kit instructions (Ngai et al. 2007). Splenocytes were loaded into Mu-IFN- γ 96-well round-bottomed plates pre-coated with an IFN- γ binding capture antibody. Plates were then washed four times with 1x Phosphate Buffer Saline (PBS) and loaded with 150 μ L RPMI-1640 culture media for 30 min. at room temperature. Following this, splenocytes were then plated at 250,000cells/well. All stimulations were performed in triplicate; the negative controls were represented with 6 wells each comprising splenocytes and media alone. Afterwards, each well was re-stimulated, if necessary according to the experimental plan. Antigen stimulations included OVA class I₍₂₅₇₋₂₆₄₎ and OVA class II₍₃₂₃₋₃₃₉₎ peptides (10 μ g/mL) as per the previously described protocol by Bonifaz *et al.* (2004) (Table 4). The Class I and II OVA peptides were predicted to bind to the DR4 allele and have shown to be capable of detecting and inducing activation and proliferation of CD8⁺ Cytotoxic T cells and CD4⁺ T helper cells, respectively (Bonifaz et al. 2004, Sun et al. 2010). Concanavalin A (CON A) was added to the positive control wells only (2 μ g/mL). CON A is normally used as a positive control treatment condition because it readily triggers T cell activation and proliferation (Palacios, 1982). Plates were then

placed in a 37°C incubator for 48 hr.

After the 48hr incubation, plates were washed five times and IFN- γ production was detected according to the MABTECH development protocol. The biotinylated-detection antibody (R4-6A2-biotin) was then diluted to 1 μ g/mL in PBS containing 0.5% FBS and 100 μ L was added to each well. Following this step, plates were incubated for 2hr at room temperature. After incubation, plates were re-washed and 100 μ L Streptavidin-Horseradish Peroxidase (HRP) conjugate (diluted 1:1000) was added to each well. Plates were then incubated at room temperature for 1hr. Following incubation, to visually observe if IFN- γ secretion occurred due to antigen re-stimulation, plates were washed and then loaded with 100 μ L TMB substrate per well. The plates were immediately left to develop for a maximum of 10 min. at room temperature, until distinct blue spots emerged. The colorimetric TMB substrate forms an insoluble precipitate when catalyzed by the Streptavidin-HRP enzyme and acts as a visible representation of a single activated cell secreting the IFN- γ cytokine (MABTECH). Each plate's color development was stopped after extensive plate rinsing with tap water. Plates were left to dry overnight and inspected for spots the following day, utilizing the ELISpot Reader (Cellular Technologies Limited, Cleveland, OH).

In general, a positive T-cell response was defined by a significantly elevated spot count in antigen-stimulated wells over the non-stimulated media only-control (negative-control), usually, 50 spot forming units above background was considered a positive response (Dittrich et al. 2012). The

data generated by the ELISpot Counter was then compiled into an excel spreadsheet. Each triplicate's spot data, not including the negative controls, was added together and then averaged, resulting in one value representative of the immunization condition's positive control and antigen stimulated samples. As for the negative controls, all 6 wells per respective immunization condition were added together and then averaged. The resulting negative control values represent the background "noise" of the assay. Each immunization condition's negative control values were then subtracted from their respective antigen stimulated averaged sample values. The newly generated averaged data was then normalized to 1 million cells. The resulting spot count data now represents an actual IFN- γ response due to antigenic peptide re-stimulation.

Table 4. OVA Class I and Class II peptide sequences. Specific peptide sequences were found within the whole protein, Ovalbumin, which was used in the Mu-ELISpot IFN- γ assay as a method of antigen re-stimulation.

OVA Peptide ID	Sequence
Class I (257-264)	SIINFEKL
Class II (323-339)	ISQAVHAAHAEINEAGR

Flow Cytometry

The BD LSRII Flow Cytometer identifies and functionally characterizes various cellular population subsets (BDBiosciences). Prior to all experiments,

compensation must be performed to inform the machine of each antibody stain's fluorophore fluorescence intensity (FI) and to confirm each fluorophore's FI matches perfectly with the specified cell marker-antibody stain used. Compensation is also necessary to perform when multiple fluorophores are used per assay and per cell because it also addresses the issue of spillover, which is the physical overlap of emission spectra (BD Biosciences). Filters are in place in the machine to try and prevent as much spillover of fluorescence spectra as possible, however, most fluorophores emit over a broad range of wavelength so a compensation process is required to subtract the fluorescence spectral spillover from other channels (BD Bioscience). Data, for all samples, was acquired on the same day using the BD LSRII Flow Cytometer to assure consistency among samples. Once sample acquisition was complete, samples are analyzed with BD's FACS Diva Software V8.0 and Tree Star's FlowJo Analysis Software V7.6.5.

Statistical Analyses

Data generated from both ex vivo memory immune response assays is taken and compiled into an excel spreadsheet. Analyses are performed per experiment using the Mann-Whitney U test in the GraphPad Prism 6 software, a non-parametric t-test that measures for the significant difference between two groups of independent samples (Doerge et al. 2009). This test is normally used when the data set does not meet the requirements for a parametric t-test, such as the data set is not normally distributed (Doerge et al. 2009). Each

sample condition's data set was tested for normal distribution using the Shapiro-Wilk frequency distribution test. All data sets were shown to not have normal distribution (not shown). Both data sets for the two conditions that were compared were compiled together and then ranked from lowest to highest. If identical values were present within the data set, each of the values respective ranks are added together and then averaged to break the tie (Doerge et al. 2009). From now on, only the ranking values are used rather than the measured data value. Therefore, the ranks are separated back into their respective sample conditions and the sum is taken. Each sample condition's rank total is then used to observe the differences between the two conditions. Generally, if there is a systematic difference, the sample condition receiving the significantly greater immune response will tend to have the higher-ranking values, while the other sample condition will tend towards the lowering ranking values (Doerge et al. 2009). However, if both sample conditions generate a similar immune response then both high and low ranking values are distributed fairly amongst the two sample conditions, and both rank totals will be fairly similar (Doerge et al. 2009).

One of the statistics generated from the ranked data set is a p-value. The p-value is a probability and measures the strength of evidence against the null hypothesis. The assumption being the null hypothesis is true (Stills, 2005). Thus, it measures the likelihood of significance and whether the difference observed between the two data sets likely is to occur by random chance (Stills, 2005). All Mann-Whitney-U statistical analyses performed for these

assays are one-tailed with a 95% confidence interval, therefore, the p-value significance level threshold is set at 0.05. Any p-value score that is lower than the 0.05 significance level threshold is considered significant and the null hypothesis can be rejected. Generally, the lower the p-value score, the more significant and the greater the difference observed between two sample conditions (Stills, 2005).

CHAPTER 4

FINDINGS

In silico Analyses

In order to first create a de-tolerized antibody, tolerogenic signals must be located within the α DEC-205 antibody's sequence. Specific amino acids within the Tregitope sequence, at key positions, were selected for modification. The aim was to perform the least amount of substitutions as possible to minimize the likelihood of disruption to structure and function of the antibody sequences, as well as, reduce binding potential to the respective HLA allele (Moise et al. 2012). Therefore, the entire EpiMatrix suite of tools were used to identify T cell epitopes, potentially regulatory in nature, based on criterion defined in the epitope-mapping algorithms and suggest and substitute in key amino acid replacements in order to reduce the binding potential of the tolerogenic signals to the HLA allele (De Groot et al. 2008).

EpiMatrix Cluster report analyses showed EpiMatrix z-scores for each of the 8 alleles; however, only the DR4 allele was of interest due to future in vivo immunizations taking place in HLA-DR4 mice (Tables 5-9). In total, only two out of the six Tregitope sequences within the α DEC-205 antibody's sequence were targeted for modification (VH77 & HC54) based on previous research performed by De Groot *et al.* 2008 indicating that VH77 (unpublished) and HC54 Tregitope sequences are the most immunogenic out

of the six Tregitopes. These two Tregitope sequences comprise the most potential for driving this tolerogenic immune response based on prior research showing ex vivo expansion of CD4⁺CD25⁺FoxP3⁺ T cells when Tregitopes are used as stimulation conditions for PBMCs (De Groot et al 2008, De Groot et al. 2013, Cousens et al. 2013). Tregitope sequences HC54 and VH77 were defined as 22 and 23 amino acid length sequences, respectively (Table 10).

Both HC54 and VH77 Tregitope sequences, with respect to the HLA-DR4 allele, contain four hits in total, with three of the four hits in at least the top 5th percentile for peptides likely to bind to the DR4 allele for each sequence (Tables 5,7). This suggests there are three potentially regulatory sequence frames within both Tregitope sequences (HC54 and VH77) that are predicted to bind to the HLA-DR4 allele. The EpiMatrix Cluster reports showed Tregitope sequences, α DEC-205:OVA-HC54-ORG and α DEC-205:OVA-VH77-ORG, have 2.5 Epibars and 4 Epibars when compared against the panel of 8 class II alleles, respectively (Tables 5, 7), suggesting the presence of promiscuous alleles, which further suggests that these sequence frames are potentially regulatory in nature and are predicted to bind to the DR4 allele (De Groot et al. 2008). More specifically, the α DEC-205:OVA-HC54-ORG (original) sequence contains 1 top 1% hit and 2-top 5% hits (Table 5). The sequence does contain a top 10% hit, which is considered to be non-significant and least likely to bind to the DR4 allele. The Tregitope α DEC-205:OVA-VH77-ORG (original) sequence (VH77-MOD1) contains 1 top 1% hit, 2-top 5% hits, and 1 top 10% hit (non-significant) (Table 7).

Modification to the (22 amino acid-length) Tregitope α DEC-205:OVA-HC54-MOD1 (HC54-MOD1) sequence involved the replacement of amino acids, leucine (position 4 & 11) and tyrosine (position 9), with all alanine amino acids. The resulting modifications to the HC54-MOD1 Tregitope sequence decreased the possible number of sequence frames likely to bind to the DR4 allele, from three sequence frames to one (Table 10). The EpiMatrix z-score also decreased after subsequent modification, however, the single retained top 5% hit sequence frame suggests this sequence frame is still predicted to have a significant chance of binding to the DR4 allele, which could potentially inhibit effector, pro-inflammatory immune responses if presented and activates T cells (Table 6).

In terms of the (23 amino acid-length) Tregitope VH77-ORG sequence, substitution of leucine (position 12) to alanine resulted in the VH77-MOD1 Tregitope sequence and further substitution of tyrosine (position 6) to alanine resulted in the VH77-MOD2 Tregitope sequence. After modification to the VH77-ORG Tregitope sequence, the number of 9-mer frames predicted to bind to the DR4 allele decreased, similar to the HC54-ORG Tregitope sequence, from three sequence frames (ORG) to one top 1% hit-sequence frame within the VH77-MOD1 Tregitope sequence and zero frames in the VH77-MOD2 Tregitope sequence (Table 7-9). After modification, the EpiMatrix z-scores for all sequence frames relevant to the DR4 allele also decreased, similar to the HC54-ORG modification. However, in regards to the VH77-MOD1 Tregitope sequence, there is still one sequence frame, after

modification, still predicted to bind to the DR4 allele. Though the EpiMatrix z-scores decreased after modification, this sequence frame is still considered a top 1% hit and predicted to have an extremely likely chance of binding to the DR4 allele. The resulting VH77-MOD2, after modification, did not retain any sequence frames predicted to bind to the DR4 allele.

EpiMatrix Cluster Detail Report															
File: STEINMAN_DEC205_MODS Sequence: MUDEC205_CH_54_ORG Cluster: 54															
November 04, 2011 (Epx Ver. 1.2)															
Frame Start	AA Sequence	Frame Stop	Hydrophobicity	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits	I-AB Z-Score	I-AD Z-Score	I-ED Z-Score
54	PAVLQSDLY	62	0.32	0.45	-0.19	-0.06	-0.77	-1.45	-0.43	-0.48	-0.49	0	-0.5	0.76	1.16
55	AVLQSDLYT	63	0.42	-0.01	-0.04	0.63	-0.04	-0.12	0.49	0.24	1.07	0	-1.05	0.26	-1.06
56	VLQSDLYTL	64	0.64	0.3	1.38	0.02	1.07	0.36	0.88	0.89	1.48	0	-0.04	0.18	-0.84
57	LQRDLYTLS	65	0.09	1.69	2.13	1.79	0.33	0.83	-0.08	1.01	0.99	3	1.09	-0.45	0.44
58	QSDLYTLSS	66	-0.42	0.51	-0.52	1.22	-0.32	0.17	0.8	-0.13	-0.01	0	0.6	0.65	-0.82
59	SDLYTLSSS	67	-0.12	-1.33	-0.34	-0.84	-1.05	0.28	-0.14	0.89	0.68	0	-1.06	0.76	-1.84
60	DLYTLSSSV	68	0.43	0.96	-0.08	0.65	1.86	-0.02	-0.3	0.64	1.11	1	0.56	1.23	-2.42
61	LYTLSSSVT	69	0.74	1.87	0.22	1.55	1.84	1.64	1.25	0.34	1.8	3	0.34	1.01	-0.86
62	YLSSSVTV	70	0.79	2.7	2.15	2.29	3.23	1.32	1.52	1.77	2.02	6	1.85	1.35	-0.59
63	TLSSSVTVF	71	0.76	-0.12	0.02	0.02	0	-0.55	-0.58	-0.11	-0.2	0	0.49	-0.35	0.21
64	LSSSVTVFS	72	0.74	1.86	2.25	2.33	1.33	1.24	1.38	1.88	1.39	4	1.12	0.18	-1.04
65	SSSVTVFSS	73	0.23	0.39	0.34	0.73	-0.36	0.34	1.04	0.64	0.21	0	0.4	2.25	-1.59
66	SSVTVFSST	74	0.24	0.25	-1.19	-0.1	0.71	-0.39	-0.79	-0.46	0.27	0	0.77	1.81	-1.68
67	SVTVFSSTW	75	0.23	-0.15	-0.23	-0.33	1.21	-1.11	-0.61	-0.11	0.28	0	0.6	-0.03	-1.82
Summarized Results (04-NOV-2011)				DRB1*0101	DRB1*0301	DRB1*0401	DRB1*0701	DRB1*0801	DRB1*1101	DRB1*1301	DRB1*1501	Total			
Maximum Single Z score				2.7	2.25	2.33	3.23	1.32	1.52	1.88	2.02	--	1.85	2.25	1.16
Sum of Significant Z scores				8.12	6.54	6.42	6.93	0	0	3.65	3.82	35.48	1.85	4.06	--
Count of Significant Z Scores				4	3	3	3	0	0	2	2	17	1	2	0
Total Assessments Performed: 112				Hydrophobicity: 0.30			EpiMatrix Score: 23.94			EpiMatrix Score (w/o flanks): 23.94					
Scores Adjusted for Tregitope:				--			EpiMatrix Score: 23.94			EpiMatrix Score (w/o flanks): 23.94					

Table 5. The αDEC-205:OVA-HC54-ORG EpiMatrix Cluster Report analysis. Significant EpiMatrix z-scores ≥ 1.64 (hits), for all 8 alleles, are highlighted in the medium blue and dark-blue colors. An EpiMatrix z-score indicates potential for that particular 9mer frame sequence to bind to a given HLA allele. There are three hits relevant to the DRB1*0401 allele and are predicted to bind, prior to modification.

EpiMatrix Cluster Detail Report

File: STEINMAN_DEC205_MODS Sequence: MUDEC205_CH_54_MOD1 Cluster: 54
November 04, 2011 (Epx Ver. 1.2)

Frame Start	AA Sequence	Frame Stop	Hydrophobicity	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits	I-AB Z-Score	I-AD Z-Score	I-ED Z-Score	
54	PAVLQSDLY	62	0.32	1.95	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0	-0.5	0.76	1.16	
55	AVLQSDLYT	63	0.42	-0.01	-0.64	0.93	-0.04	-0.12	0.49	0.24	-1.07	0	-1.05	0.26	-1.06	
56	VLQSDLYTG	64	0.18	-0.03	1.18	0.23	-0.32	0.84	1.09	0.69	1.16	0	0.07	1.14	-0.74	
57	LQSDLYTGS	65	-0.38	1.35	1.79	1.46	-0.01	0.48	-0.43	0.67	0.66	1	1.09	-1.04	-0.21	
58	QSDLYTGSS	66	-0.89	0.21	-0.58	0.46	-0.41	0.1	0.32	-1.42	-0.3	0	0.63	0.41	-0.6	
59	SDLITGSSS	67	-0.59	-0.1	-0.8	0	-0.92	0.01	-0.4	0.64	0.68	0	-0.17	0.32	-1.1	
60	DLITGSSSV	68	-0.03	0.89	-0.35	0.39	1.8	0.3	0.86	0.37	0.35	0	0.56	-0.1	-1.95	
61	LYTGSSSVT	69	0.28	0.97	0.29	0.99	1.19	0.03	0.29	-0.29	1.43	0	0.46	0.99	-1.4	
62	YTGSSSVTV	70	0.32	2.41	1.86	2.02	2.95	1.02	1.23	1.49	1.74	5	2.27	1.68	-0.91	
63	TGSSSVTVV	71	0.29	-0.65	-0.52	-0.1	-0.22	-1.11	-1.13	-0.84	-1.78	0	0.61	0.11	0.22	
64	GSSSVTVPS	72	0.28	0.74	0.97	1.24	0.22	0.05	0.23	0.62	0.16	0	0.78	-0.35	-2.73	
65	SSSVTVPS	73	0.23	0.39	0.34	0.73	-0.36	0.54	1.04	0.64	0.21	0	0.4	2.25	-1.59	
66	SSVTVPSST	74	0.24	0.25	-1.19	-0.1	0.71	-0.39	-0.79	-0.46	0.27	0	0.77	1.81	-1.68	
67	SVTVPSSTW	75	0.23	-0.16	-0.23	-0.33	1.21	-1.11	-0.61	-0.11	0.28	0	0.6	-0.03	-1.82	
Summarized Results (04-NOV-2011)				DRB1*0101	DRB1*0301	DRB1*0401	DRB1*0701	DRB1*0801	DRB1*1101	DRB1*1301	DRB1*1501	Total				
Maximum Single Z score				2.41	1.86	2.02	2.95	1.02	1.23	1.49	1.74	--	2.27	2.25	1.16	
Sum of Significant Z scores				2.41	3.65	2.02	2.95	0	0	0	1.74	12.77	2.27	5.74	--	
Count of Significant Z Scores				1	2	1	1	0	0	0	1	6	1	3	0	
Total Assessments Performed: 112				Hydrophobicity: 0.10			EpiMatrix Score: 1.24			EpiMatrix Score (w/o flanks): 1.24						
Scores Adjusted for Tregitope:				--			EpiMatrix Score: 1.24			EpiMatrix Score (w/o flanks): 1.24						

Table 6. The α DEC-205:OVA-CH54-MOD1 EpiMatrix Cluster Report analysis. Significant EpiMatrix z-scores ≥ 1.64 (hits), for all 8 alleles, are highlighted in the medium blue and dark-blue colors. An EpiMatrix z-score indicates potential for that particular 9mer frame sequence to bind to a given HLA allele. After modification, there is a single 9mer sequence frame considered a top 5% hit that is predicted to have a significant chance of binding to the DRB1*0401 allele.

EpiMatrix Cluster Detail Report

File: STEINMAN_DEC205_MODS Sequence: MUDEC205_VH_77_ORG Cluster: 77
November 04, 2011 (Epx Ver. 1.2)

Frame Start	AA Sequence	Frame Stop	Hydrophobicity	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits	I-AB Z-Score	I-AD Z-Score	I-ED Z-Score	
77	TQNILYLQM	85	0.17	0.73	0.43	-0.24	0.96	0.79	0.12	0.99	0.94	0	0.23	1.42	-0.24	
78	QNILYLQMN	86	-0.14	0.23	-0.3	-0.1	0.02	1.14	0.83	-1.26	0.54	0	-0.63	0.53	0.56	
79	NILYLQMS	87	0.16	-0.44	-0.62	-0.26	-0.67	0.71	0.44	-0.22	0.38	0	-1.71	-0.17	-1.07	
80	ILYLQMSL	88	0.97	1.69	1.38	1.21	1.92	1.29	1.56	0.81	1.61	2	-0.89	0.69	-0.36	
81	LYLQMSLR	89	-0.03	1.92	2.33	1.89	1.06	2.02	1.57	2.91	1.9	6	-1.2	-1.46	1.57	
82	YLQMSLR	90	-0.26	3.3	2.01	3.41	2.9	2.23	3.25	1.97	2.82	6	1.6	1.42	-1.44	
83	QLQMSLR	91	-0.5	0.93	1.72	0.88	0.8	1.89	1.52	0.24	0.35	6	0.72	-0.6	0.33	
84	QMSLRADD	92	-1.31	-0.09	0.12	-0.95	-0.19	0.93	0.95	0.04	-0.82	0	0.26	-1.15	0.68	
85	MNSLRADD	93	-1	2.01	0.53	1.62	1.66	1.39	1.52	0.29	1.42	2	1.15	1.21	-0.02	
86	NSLRADDTA	94	-1.01	-0.93	-1.69	-1.57	-1.3	-1.33	-0.87	-1.12	-1.51	0	-0.92	-0.8	-1.11	
87	SLRAEDTAI	95	-0.12	0.04	0.87	0.44	0.43	-0.13	-1.17	0.84	1.12	0	-0.79	-0.65	-0.41	
88	LRAEDTAIY	96	-0.18	1.23	1.7	1.66	-0.12	-0.47	0.85	1.67	-0.25	3	1.36	0.38	1.15	
89	RAEDTAIYY	97	-0.74	-0.8	1.12	-0.21	-1.74	-1.64	-1.8	-0.01	-0.83	0	-0.56	1.13	-0.13	
90	RDTAIYYC	98	0.03	-2.19	3.06	1.99	-0.91	1.81	-2.48	-2.32	-1.45	0	1.45	-0.26	-0.96	
91	EDTAIYYCA	99	0.03	-0.42	-1.25	-0.71	-0.56	-1.29	-1.02	-1.37	-0.29	0	0.81	1.27	-0.32	
Summarized Results (04-NOV-2011)				DRB1*0101	DRB1*0301	DRB1*0401	DRB1*0701	DRB1*0801	DRB1*1101	DRB1*1301	DRB1*1501	Total				
Maximum Single Z score				3.3	2.33	3.41	2.9	2.23	3.43	2.91	2.82	--	1.6	1.42	1.57	
Sum of Significant Z scores				8.92	7.76	6.96	6.47	6.14	3.43	6.55	4.71	50.94	--	--	--	
Count of Significant Z Scores				4	4	3	3	3	1	3	2	23	0	0	0	
Total Assessments Performed: 120				Hydrophobicity: -0.06			EpiMatrix Score: 38.59			EpiMatrix Score (w/o flanks): 38.59						
Scores Adjusted for Tregitope:				--			EpiMatrix Score: -0.05			EpiMatrix Score (w/o flanks): -0.05						

Table 7. The α DEC-205:OVA-VH77-ORG EpiMatrix Cluster Report analysis. EpiMatrix z-scores ≥ 1.64 (hits), for all 8 alleles, are highlighted in the medium blue and dark-blue colors. An EpiMatrix z-score indicates potential for that particular 9mer frame sequence to bind to a given HLA allele. There are three 9mer frame sequences predicted to bind to the HLA-DRB1*0401 allele. The frames highlighted in green are the conserved IgG sequences and are chosen for modification.

EpiMatrix Cluster Detail Report																
File: STEINMAN_DEC205_MODS Sequence: MUDEC205_VH_77_MOD1 Cluster: 77																
November 04, 2011 (Epx Ver. 1.2)																
Frame Start	AA Sequence	Frame Stop	Hydrophobicity	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits	I-AB Z-Score	I-AD Z-Score	I-ED Z-Score	
77	TQNLILQGM	85	0.17	0.73	-0.43	-0.04	0.52	-1.69	0.12	0.55	0.59	0	0.23	1.42	-0.24	
78	QNILALQGM	86	-0.14	0.23	-0.3	-0.1	0.92	1.34	0.53	-1.26	0.54	0	-0.63	0.53	0.56	
79	NILYLQMS	87	0.16	-0.44	-0.62	-0.26	-0.67	0.71	0.44	-0.22	0.36	0	-1.71	-0.17	-1.07	
80	ILYLQMSG	88	0.5	1.36	1.16	1.43	0.53	1.77	1.78	0.61	1.29	2	-0.78	1.65	-0.25	
81	LVLQMSGR	89	-0.5	1.58	1.99	1.56	0.74	1.66	1.22	2.57	1.57	3	-1.2	-2.06	0.91	
82	YLQMSGRA	90	-0.72	2.99	1.95	2.64	2.81	2.15	2.96	0.68	2.52	7	1.64	1.18	-1.21	
83	LQMSGRAE	91	-0.97	1.26	1.46	0.92	0.67	1.62	1.26	0.99	0.34	0	1.61	-1.04	0.42	
84	QMSGRAED	92	-1.78	-0.36	-0.12	-1.24	-0.46	0.65	0.07	-0.23	-1.08	0	0.26	-1.47	1.15	
85	MNSGRAEDT	93	-1.47	1.11	0.58	1.05	0.99	0.37	0.52	-0.34	1.05	0	1.27	1.19	-0.56	
86	NSGRAEDTA	94	-1.48	-1.21	-1.98	-1.85	-1.58	-1.63	-1.16	-1.41	-1.78	0	-0.5	-0.47	-0.43	
87	SGRAEDTAL	95	-0.59	-0.49	0.34	-0.07	-0.09	-0.68	-1.71	0.32	0.61	0	-0.67	-0.19	-0.39	
88	GRAEDTALY	96	-0.64	0.1	0.41	0.57	-1.23	-1.65	-0.3	0.41	-1.48	0	1.01	-0.15	0.46	
89	RAEDTALYY	97	-0.74	-0.8	1.12	-0.21	-1.74	-1.64	-1.8	-0.01	-0.83	0	-0.56	1.13	-0.13	
90	AEDTALYYC	98	0.03	-2.15	-3.06	-1.99	-0.91	-1.81	-2.48	-2.32	-1.43	0	1.45	-0.26	-0.96	
91	EDTALYYCA	99	0.03	-0.42	-1.25	-0.71	-0.56	-1.29	-1.02	-1.37	-0.29	0	0.81	1.27	-0.32	
Summarized Results (04-NOV-2011)				DRB1*0101	DRB1*0301	DRB1*0401	DRB1*0701	DRB1*0801	DRB1*1101	DRB1*1301	DRB1*1501	Total				
Maximum Single Z score				2.99	1.99	2.64	2.81	2.15	2.96	2.57	2.52	27.79	1.64	1.65	1.15	
Sum of Significant Z scores				2.99	3.94	2.64	2.81	5.58	4.74	2.57	2.52	27.79	1.64	1.65	--	
Count of Significant Z Scores				1	2	1	1	3	2	1	1	12	0	1	0	
Total Assessments Performed: 120				Hydrophobicity: -0.24			EpiMatrix Score: 15.45			EpiMatrix Score (w/o flanks): 15.45						
Scores Adjusted for Tregitope:				--			EpiMatrix Score: 15.45			EpiMatrix Score (w/o flanks): 15.45						

Table 8. The α DEC-205:OVA-VH77-MOD1 EpiMatrix Cluster Report analysis. EpiMatrix z-scores ≥ 1.64 (hits), for all 8 alleles, are highlighted in the medium blue and dark-blue colors. An EpiMatrix z-score indicates potential for that particular 9mer frame sequence to bind to a given HLA allele. After modification, there is a single-9mer frame sequence considered a top 1% hit that is predicted to bind to the HLA-DRB1*0401 allele.

EpiMatrix Cluster Detail Report																
File: STEINMAN_DEC205_MODS Sequence: MUDEC205_VH_77_MOD2 Cluster: 77																
November 04, 2011 (Epx Ver. 1.2)																
Frame Start	AA Sequence	Frame Stop	Hydrophobicity	DRB1*0101 Z-Score	DRB1*0301 Z-Score	DRB1*0401 Z-Score	DRB1*0701 Z-Score	DRB1*0801 Z-Score	DRB1*1101 Z-Score	DRB1*1301 Z-Score	DRB1*1501 Z-Score	Hits	I-AB Z-Score	I-AD Z-Score	I-ED Z-Score	
77	TQNLILQGM	85	0.51	1.16	0.43	1.03	0.98	-0.8	0.75	0.92	0.94	0	0.89	2.01	-0.29	
78	QNILALQGM	86	0.2	0.16	-0.37	-0.17	-0.05	1.06	0.56	-1.33	0.47	0	-0.63	-0.14	0.27	
79	NILALQMS	87	0.5	0.46	0.66	0.66	-0.21	0.97	0.75	-0.4	0.61	0	-0.87	0.37	-1.22	
80	ILALQMSG	88	0.84	1.5	1.33	1.57	0.89	1.92	1.94	0.76	1.43	2	-0.46	1.74	-0.03	
81	LALQMSGR	89	-0.16	1.32	1.72	1.3	0.88	1.38	0.85	2.31	1.31	2	-1.26	-2.25	0.52	
82	ALQMSGRA	90	-0.38	1.53	0.98	1.22	1.37	1.62	1.46	-0.36	1.51	0	0.4	0.82	-1.73	
83	LQMSGRAE	91	-0.97	1.26	1.46	0.92	0.67	1.62	1.26	0.99	0.34	0	1.61	-1.04	0.42	
84	QMSGRAED	92	-1.78	-0.36	-0.12	-1.24	-0.46	0.65	0.07	-0.23	-1.08	0	0.26	-1.47	1.15	
85	MNSGRAEDT	93	-1.47	1.11	0.58	1.05	0.99	0.37	0.52	-0.34	1.05	0	1.27	1.19	-0.56	
86	NSGRAEDTA	94	-1.48	-1.21	-1.98	-1.85	-1.58	-1.63	-1.16	-1.41	-1.78	0	-0.5	-0.47	-0.43	
87	SGRAEDTAL	95	-0.59	-0.49	0.34	-0.07	-0.09	-0.68	-1.71	0.32	0.61	0	-0.67	-0.19	-0.39	
88	GRAEDTALY	96	-0.64	0.1	0.41	0.57	-1.23	-1.65	-0.3	0.41	-1.48	0	1.01	-0.15	0.46	
89	RAEDTALYY	97	-0.74	-0.8	1.12	-0.21	-1.74	-1.64	-1.8	-0.01	-0.83	0	-0.56	1.13	-0.13	
90	AEDTALYYC	98	0.03	-2.15	-3.06	-1.99	-0.91	-1.81	-2.48	-2.32	-1.43	0	1.45	-0.26	-0.96	
91	EDTALYYCA	99	0.03	-0.42	-1.25	-0.71	-0.56	-1.29	-1.02	-1.37	-0.29	0	0.81	1.27	-0.32	
Summarized Results (04-NOV-2011)				DRB1*0101	DRB1*0301	DRB1*0401	DRB1*0701	DRB1*0801	DRB1*1101	DRB1*1301	DRB1*1501	Total				
Maximum Single Z score				1.53	1.72	1.57	1.37	1.92	1.94	2.31	1.51	--	1.61	2.01	1.15	
Sum of Significant Z scores				0	1.72	0	0	1.92	1.94	2.31	0	7.89	--	3.75	--	
Count of Significant Z Scores				0	1	0	0	1	1	1	0	4	0	2	0	
Total Assessments Performed: 120				Hydrophobicity: -0.11			EpiMatrix Score: -4.47			EpiMatrix Score (w/o flanks): -4.47						
Scores Adjusted for Tregitope:				--			EpiMatrix Score: -4.47			EpiMatrix Score (w/o flanks): -4.47						

Table 9. The α DEC-205:OVA-VH77-MOD2 EpiMatrix Cluster Report analysis. Significant EpiMatrix z-scores ≥ 1.64 (hits), for all 8 alleles, if present, are highlighted in the medium blue and dark-blue colors. An EpiMatrix z-score indicates potential for that particular 9mer frame sequence to bind to a given HLA allele. There are not any 9mer frame sequences predicted to bind to the DRB1*0401 allele, after modification.

Table 10. Potential Tregitope sequences selected for modification. Non-modified and modified predicted Tregitope sequences showing the number of epitope sequence frames predicted to bind to the HLA-DRB1*0401 allele. The table also shows which sequences were successfully expressed into recombinant protein.

Antibodies	Cluster Address	Cluster Sequence	HLA-DRB1*0401 EpiMatrix Hits	Produced
αDEC-205:HC54-ORG	54-75	PAVLQSDLYTLSSSVTVPSSTW	3	YES
αDEC-205:HC54-MOD1	54-75	PAVAQSDLATASSSVTVPSSTW	1	YES
αDEC-205:HC54-MOD2	54-75	PAVAQSDLATASQSVTVPSSTW	0	NO
αDEC-205:VH77-ORG	77-99	TQNILYLQMNSLRAEDTAIYYCA	3	YES
αDEC-205:VH77-MOD1	77-99	TQNILYLQMNSGRAEDTAIYYCA	1	YES
αDEC-205:VH77-MOD2	77-99	TQNILALQMNSGRAEDTAIYYCA	0	YES
αDEC-205:VH77-MOD3	77-99	TQNI GALQMNSGRAEDTAIYYCA	0	NO

Protein Purification

In total, recombinant expression of six variant α DEC-205:OVA antibodies was attempted, however only three of the six variant antibodies were successfully expressed. The other three variant antibodies showed either no or low expression levels, which suggested that the epitope modifications performed were deleterious to the structure. Purified recombinant protein was determined via gel electrophoresis analyses, which showed the presence of strong bands at the predicted molecular weights for a single light (25 kD) and a single heavy chain attached to ovalbumin (95 kD) (Ito et al. 1996, Janeway et al. 2001, Sun et al. 2010). The resulting recombinant antibodies successfully expressed were α DEC-205:OVA-HC54-MOD1, α DEC-205:OVA-VH77-MOD1, and α DEC-205:OVA-VH77-MOD2 (Table 10).

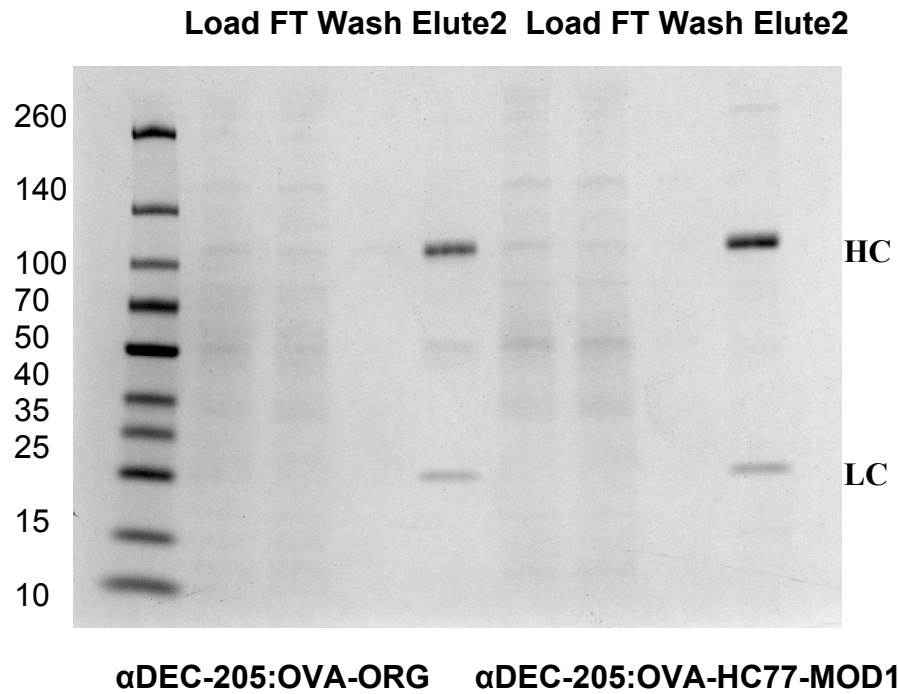


Figure 8. A gel electrophoresis image showing the purity check points taken during protein purification. A sample of protein supernatant is taken prior to purification, during purification over protein G column, wash through, and the final eluting of the concentrated sample's heavy and light chain. The expected molecular weight of a single heavy chain attached to Ovalbumin is 95 kD. The molecular weight of a single light chain is 25 kD.

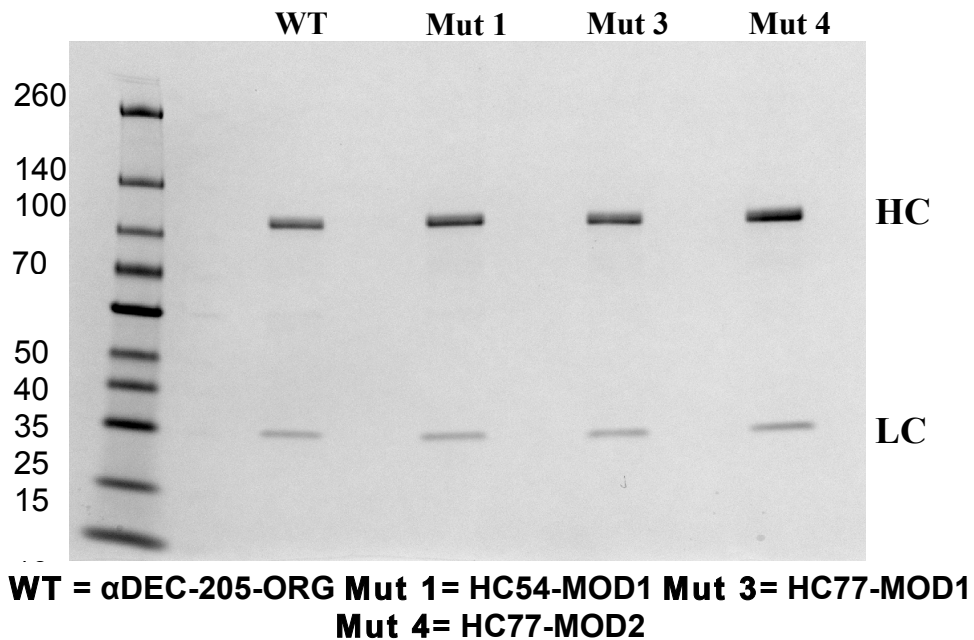


Figure 9. A gel electrophoresis image showing the production of wild type and modified α DEC-205 antibodies.

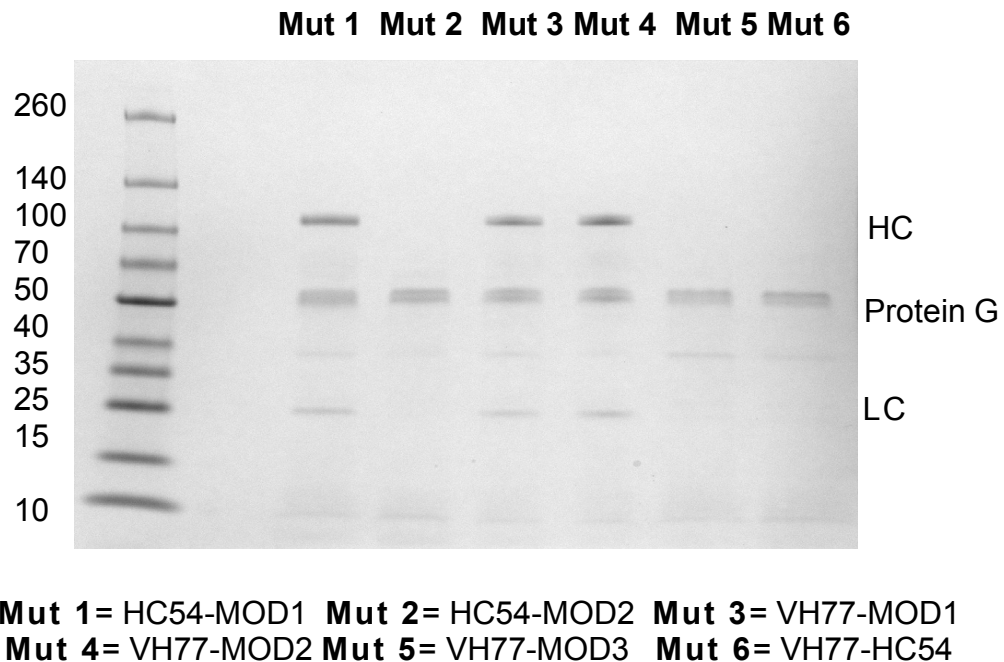


Figure 10. A gel electrophoresis image showing the correct or partial production of the variant α DEC-205:OVA antibodies. Mutant antibodies, HC54-MOD1, VH77-MOD1, VH77-MOD2, were produced correctly during transfection, where as, mutant antibodies HC54-MOD2, VH77-MOD3, and VH77-HC54 were only partially produced due to lack of heavy chain presence on the gel. .

Dendritic Cell Binding Assay

Using the flow cytometer analysis software (BD FACs Diva V8.0) DC subsets negative for the CD11c⁺ marker (DC lineage marker) were gated on to establish the negative control DC population (Figure 11). Further selective gating for the α IgG-FITc staining antibody was performed. Any DCs migrating from the initial negative control population to the “CD11c⁺ α IgG-FITc⁺” labeled quadrant indicates double positive DC staining (Figure 12). Migration into the double positive quadrant further indicates α DEC-205:OVA antibodies (ORG and variant) are present and targeting the DEC-205 receptor, within their respective samples.

The negative control JAWS II DC population was not pulsed with any antibody, but was stained with the α IgG-FITc antibody. However, 3.3% of the CD11c⁺ DCs were shown to have positive staining for the α IgG-FITc antibody. This suggests that the α IgG-FITc antibody is capable of non-specifically binding to DCs. That non-specific binding percentage was then subtracted from all resulting positive binding data. As for the DCs that were pulsed with the α IsO:OVA antibody, similar results to the negative control are observed. Only 3.1% of the JAWS II CD11c⁺ DC total population are targeted by the non-specific antibody and positively stained with the α IgG-FITc antibody (Figure 11).

The column graph (Figure 13) demonstrates that all three variant antibodies (HC54-MOD1, VH77-MOD1, VH77-MOD2) and the α DEC-205:OVA-ORG antibody have very similar targeting capacities for the DEC-

205 receptor. 60-65% of the total JAWS II CD11c⁺ DC population were targeted by the variant α DEC-205:OVA antibodies, whereas 59% of the DC population was targeted by the α DEC-205:OVA-ORG antibody.

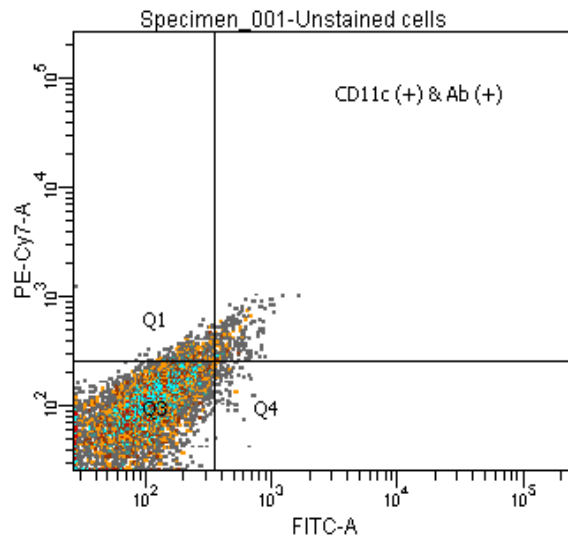


Figure 11. A DC binding assay negative control density plot showing only JAWS II CD11c⁺ DCs. There is no positive staining for the α IgG-FITc antibody, which only targets IgG molecules.

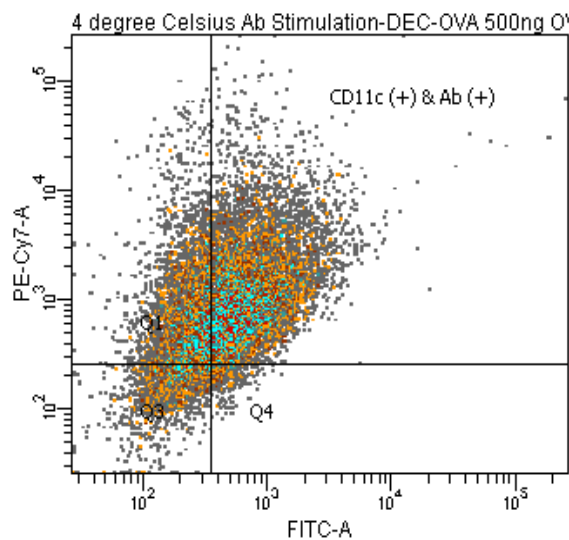


Figure 12. A DC binding assay positive control density plot showing JAWS II CD11c⁺ DCs pulsed with α DEC-205:OVA. The main population of JAWS II DCs migrate from the initial "Q3" quadrant to the right as is expected for α IgG-FITc positively stained DCs.

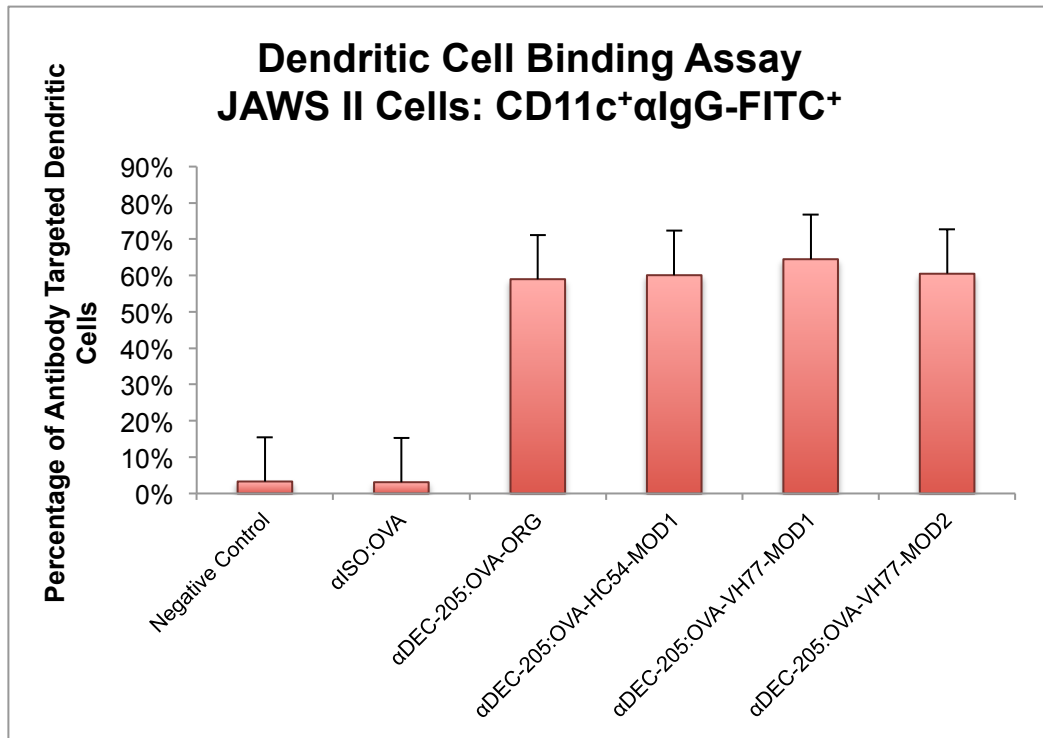


Figure 13. A dendritic cell binding assay column graph depicting the percentage of JAWS II CD11c⁺ αIgG-FITC⁺ DCs. Flow cytometer data of the percentage of JAWS II CD11c⁺ αIgG-FITC⁺ DCs is taken for each sample and formatted in a column graph output. All αDEC-205:OVA antibodies (ORG and variant) showed similar targeting capacities to the DCs' DEC-205 receptors.

In vivo Immunizations

HLA-DR4 mice were immunized with control and modified antibodies (HC54-MOD1, VH77-MOD1, VH77-MOD2), both with and without a maturation factor (α CD40) and IFN- γ secretion and antigen-specific T cell expansion were measured to characterize a pro-inflammatory immune response using an ex vivo ELISpot assay and a T cell proliferation memory response assay (Tacken et al. 2007, Hochrein et al. 2001, Letsch et al. 2003).

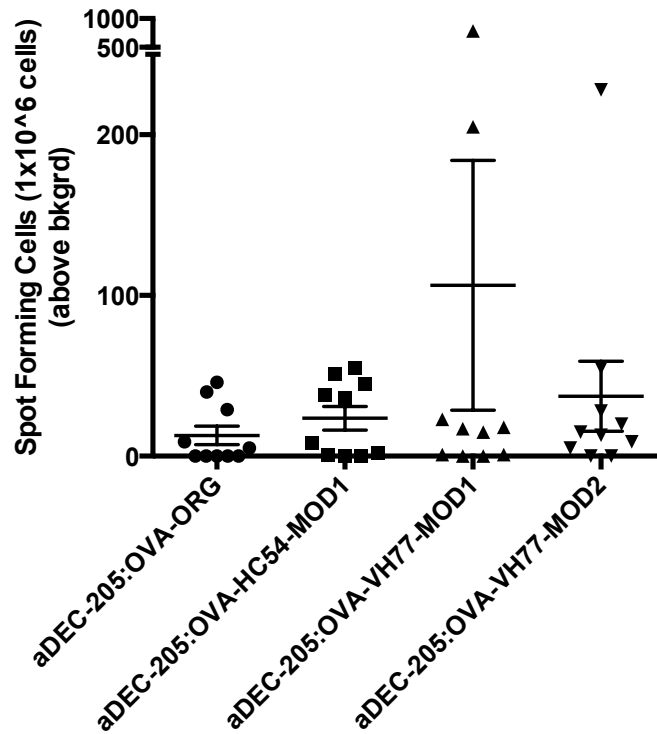
Ex vivo ELISpot Assay Results

Splenocytes previously immunized with the α DEC-205:OVA-ORG antibody and agonistic α CD40, after re-stimulation with the OVA class I peptide (257-264), elicited significant OVA-specific CD8⁺ T cell immune responses as defined by IFN- γ secretion. Splenocytes immunized with the α DEC-205:OVA-ORG antibody alone secreted significantly less IFN- γ overall with 12.9 spots on average per mouse subject in comparison to 2404.5 spots on average for mice treated with α DEC-205:OVA-ORG (Mann-Whitney U test analysis, p-value < 0.0001) (Figure 14). The same result was obtained (p value \leq 0.0001) when comparing the IFN- γ responses generated by splenocytes immunized with the α DEC-205:OVA-ORG antibody + α CD40 condition and splenocytes immunized with the non-specific, non-targeting α Isotype:OVA + α CD40 condition (Figure 14). Splenocytes from mice immunized with α DEC-205:OVA-HC54-MOD1, α DEC-205:OVA-VH77-MOD1, and α DEC-205:OVA-VH77-MOD2 antibodies produced similar levels of IFN- γ

when re-stimulated with OVA Class I ₍₂₅₇₋₂₆₄₎ ($p > 0.05$) than the splenocytes from mice immunized with α DEC-205:OVA-ORG (Figure 15). This suggests the variant α DEC-205:OVA antibodies, though modified, failed to activate antigen-specific T cells at greater levels than the α DEC-205:OVA-ORG immunized splenocytes.

Another experiment was done using the OVA Class II peptide ₍₃₂₃₋₃₃₉₎ to re-stimulate splenocyte cultures. This peptide tested for OVA specific CD4⁺ T cell activation. However, all mice immunization conditions failed to elicit a memory recall response characterized by IFN- γ secretion, including the positive control (α DEC-205:OVA antibody + CD40). This result is unexpected because this peptide is predicted to bind to the HLA-DRB1*0401 allele.

Splenocyte IFN- γ Secretion
OVA₍₂₅₇₋₂₆₄₎ Class I Re-stimulation



Experimental Plot

Figure 15. Splenocyte IFN- γ Secretion: OVA₂₅₇₋₂₆₄ Re-stimulation: Experimental Plot. This experimental plot shows that splenocytes immunized with either variant α DEC-205:OVA antibodies did not elicit higher elevated IFN- γ responses than splenocytes immunized with the non-modified α DEC-205:OVA immunization condition. (Mann-Whitney U test; $p > 0.05$).

Ex vivo T cell Proliferation Assay

The T cell proliferation assay was performed to test if ovalbumin-specific CD4⁺ and CD8⁺ T cell expansion occurred in response to immunization with the variant antibodies. Significantly more CD4⁺ and CD8⁺ T cell expansion occurred with α DEC-205:OVA antibody combined with the α CD40 co-stimulatory signal, compared to the weak proliferation levels for both CD4⁺ and CD8⁺ T cell populations when mice were immunized with the α DEC-205:OVA-ORG antibody alone (Mann-Whitney U test; $p < 0.0001$; Figures 16,18). Minimal T cell proliferation was observed when the non-specific, non-targeting control antibody plus maturation factor were used in immunizations (p -value = 0.003 (CD4), $p > 0.0001$ (CD8)) (Figures 16,18).

Immunization of mice with variant antibodies, α DEC-205:OVA-HC54-MOD1 and α DEC-205:OVA-VH77-MOD1 did not lead to statistically higher CD4⁺ T cell proliferation percentages in splenocytes than splenocytes immunized with α DEC-205:OVA-ORG ($p > 0.05$; Figure 19). However, mice immunized with α DEC-205:OVA-VH77-MOD1 (but not α DEC-205:OVA-HC54-MOD1) showed significantly higher CD8⁺ T cell proliferation when compared to the α DEC-205:OVA-ORG immunization ($p < 0.05$; Figure 17).

Mice immunized with the α DEC-205:OVA-VH77-MOD2 immunization condition did generate statistically higher CD4⁺ T cell proliferation percentages than splenocytes immunized with α DEC-205:OVA-ORG ($p < 0.05$; Figure 19). However, the α DEC-205:OVA-HC54-MOD1 and α DEC-205:OVA-VH77-MOD2 immunized splenocytes did not elicit statistically significant CD8⁺ T cell

proliferation percentages in comparison to splenocytes immunized with α DEC-205:OVA-ORG ($p>0.05$; Figure 17).

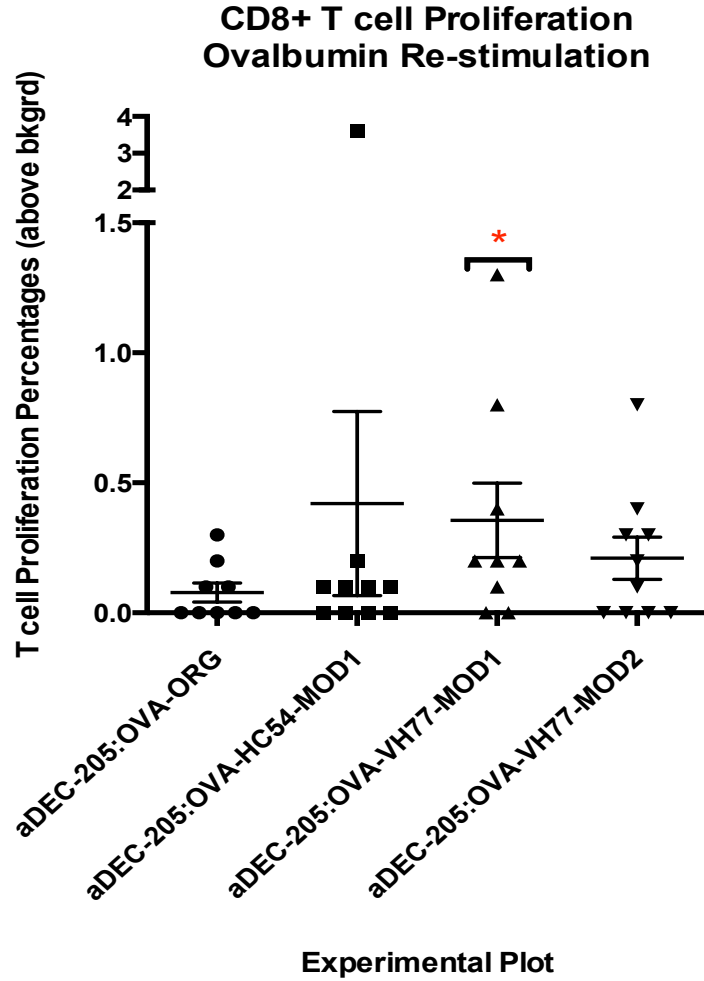


Figure 17. CD8⁺ T cell Proliferation: Ovalbumin Re-stimulation Experimental Plot. This experimental plot shows statistically higher and significant CD8⁺ T cell proliferation percentages from splenocytes immunized with the αDEC-205:OVA-VH77-MOD1 immunization condition than splenocytes immunized with αDEC-205:OVA-ORG (Mann-Whitney U test; $p < 0.0001$). Splenocytes immunized with the αDEC-205:OVA-HC54-MOD1 and αDEC-205:OVA-VH77-MOD2 did not elicit statistically higher CD8⁺ T cell proliferation percentages than splenocytes immunized with αDEC-205:OVA-ORG ($p > 0.05$).

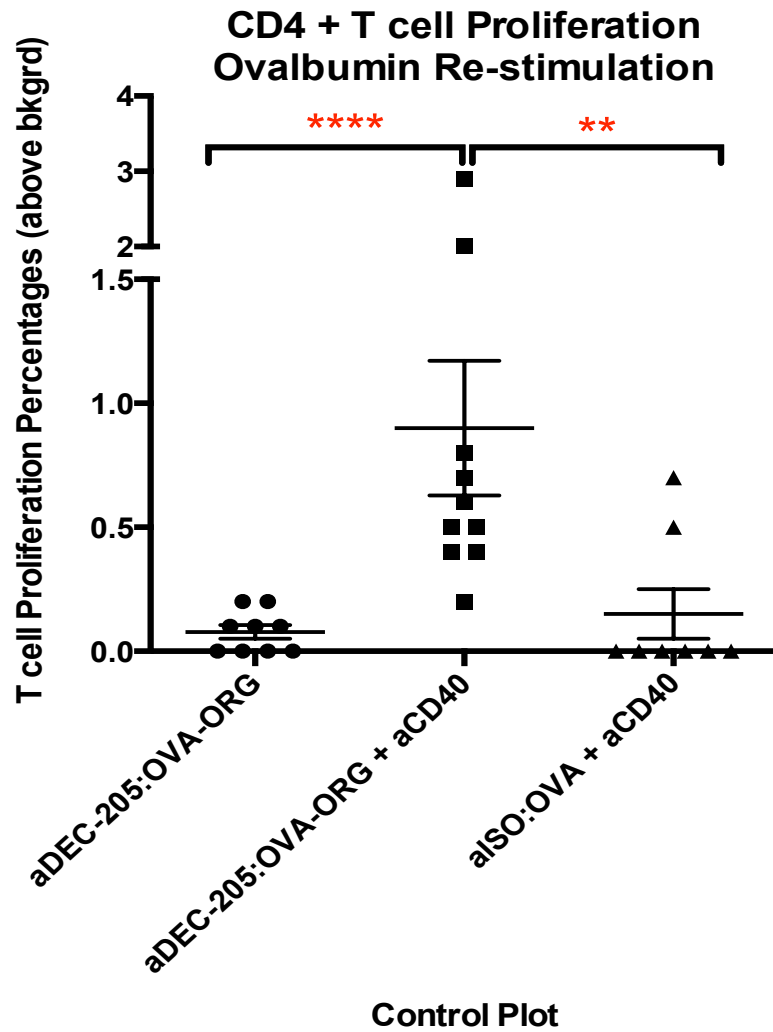
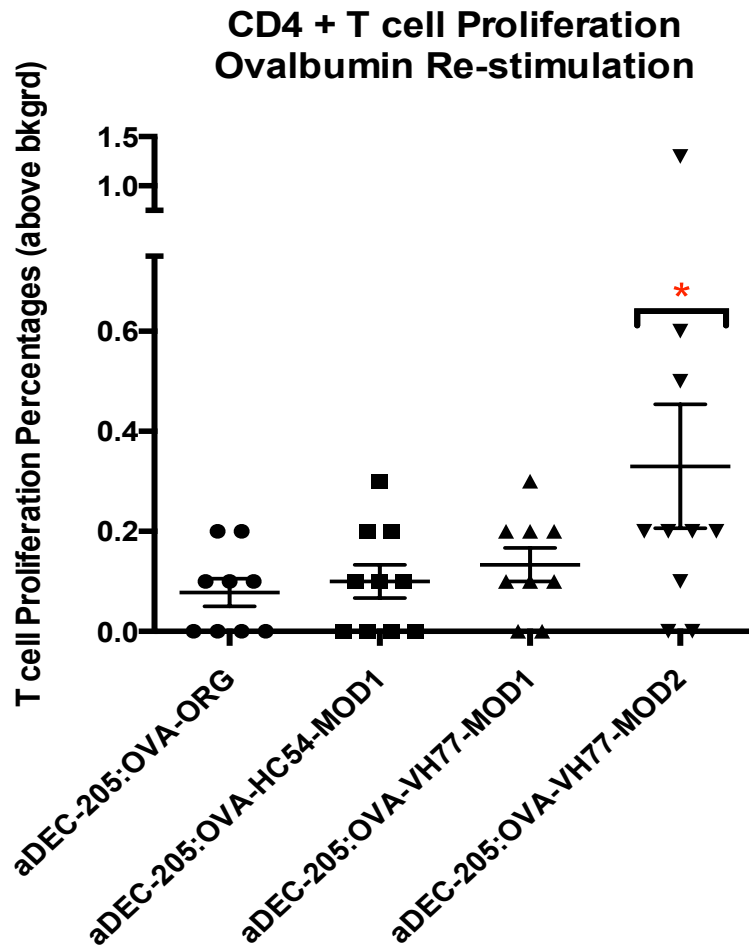


Figure 18. CD4⁺ T cell Proliferation: Ovalbumin Re-stimulation Control Plot. This control plot shows statistically higher and significant CD4⁺ T cell proliferation percentages from splenocytes immunized with the α DEC-205:OVA + α CD40 immunization condition than splenocytes immunized with α DEC-205:OVA-ORG or α ISO:OVA + α CD40 (Mann-Whitney U test; $p < 0.0001$ (ORG), $p = 0.003$ (ISO:OVA)).



Experimental Plot

Figure 19. CD4⁺ T cell Proliferation: Ovalbumin Re-stimulation Experimental Plot. This experimental plot shows statistically higher and significant CD4⁺ T cell proliferation percentages from splenocytes immunized with the α DEC-205:OVA-VH77-MOD2 immunization condition than splenocytes immunized with α DEC-205:OVA-ORG (Mann-Whitney U test; $p < 0.05$). Splenocytes immunized with the α DEC-205:OVA-HC54-MOD1 and α DEC-205:OVA-VH77-MOD1 did not elicit statistically higher CD4⁺ T cell proliferation percentages than splenocytes immunized with α DEC-205:OVA-ORG ($p > 0.05$).

CHAPTER 5

DISCUSSION

Overall, three modified antibodies (α DEC-205:OVA-HC54-MOD1, α DEC-205:OVA-VH77-MOD1, α DEC-205:OVA-VH77-MOD2) were successfully expressed and administered in in vivo immunizations to HLA-DR4 mice. Data from the in vivo immunizations indicated that statistically higher T cell proliferation was observed from splenocytes immunized with α DEC-205:OVA-VH77-MOD1 (CD8⁺ T cells) and α DEC-205:OVA-VH77-MOD2 (CD4⁺ T cells) than splenocytes immunized with the non-modified α DEC-205:OVA-ORG immunization condition. However, the data indicated that there was not a statistically elevated IFN- γ response from the splenocytes immunized with any of the three variant α DEC-205:OVA antibodies in comparison to the non-modified α DEC-205:OVA-ORG immunized splenocytes. No statistically significant elevated responses characteristic of an effector immune response was reported for splenocytes immunized with the α DEC-205:OVA-HC54-MOD1 immunization condition. Overall, this data suggests that the modifications to the potential VH77 Tregitope sequence lead to a decrease in antibody tolerogenicity and resulted in a potentially novel and increased immunogenic vaccine antigen delivery vehicle.

Our results that the α DEC-205:OVA monoclonal antibody is an effective delivery vehicle selectively targeting APCs for ovalbumin-antigen presentation

to CD4⁺ and CD8⁺ T cells confirmed previous findings presented by Bonifaz et al. 2004 and Tacke et al. 2007. Generally, antigen targeted to the DEC-205 receptor is trafficked to the MHC II presentation pathway where MHC II molecules present antigenic peptides to CD4⁺ T cells specifically. This work provides evidence that when the α DEC-205:OVA-VH77-MOD2 antibody targets the DEC-205 receptor, the receptor's bias towards CD4⁺ T cells is showcased, which is due to the proximal and distal regions of the DEC-205 receptor's cytosolic tail, which targets late endosomal vesicles containing MHC II molecules (Figure 19) (Tacke et al. 2007, Steinman et al. 2000, Lahoud et al. 2012, Mahnke et al. 2000, Harding et al. 2010) This work also provides further evidence, initially reported by Bonifaz *et al.* 2004, that though the DEC-205 receptor is biased towards the MHC II pathway, that targeting the DEC-205 receptor can also traffic antigen to MHC I molecules for CD8⁺ T cell presentation, in which our results show the use of α DEC-205:OVA-VH77-MOD1 during in vivo immunizations results in CD8⁺ T cell proliferation after splenocytes have been re-stimulated with either OVA₍₂₅₇₋₂₆₄₎ or ovalbumin (Figures 14-17). Due to the occurrence of CD8⁺ T cell proliferation in our results, this suggests that cross presentation is occurring, which is the process in which antigenic peptides make their way out into the cytosol for proteasomal degradation and are then taken back into the lumen of the Endoplasmic Reticulum for peptide loading onto MHC I molecules and take part in the MHC I pathway (Steinman et al. 2000, Janeway et al. 2001).

We found that immunization of mice with antibodies in which modifications to the VH77 Tregitope sequence, located in the variable region of the heavy chain (Table 10) (α DEC-205:OVA-VH77-MOD1 and α DEC-205:OVA-VH77-MOD2), led to significantly higher levels of CD8⁺ and CD4⁺ T cell proliferation (respectively) than mice immunized with the antibody containing unmodified Tregitopes, suggesting that the point mutations lowered the tolerogenicity level of the antibody and increased the antibody's immunogenicity allowing for a potential effector immune response, characterized by T cell proliferation and IFN- γ secretion, to ensue. This further suggests that the potential Tregitope effect was fully knocked out and allowed for the proverbial "immunological balance" with tolerance on one arm of the scale and immunogenicity on the other, to shift towards immunogenicity. Therefore, enabling the α DEC-205:OVA-VH77-MOD1 or α DEC-205:OVA-VH77-MOD2 targeting antibodies to induce a potential pro-inflammatory immune response without the need for a maturation factor or co-stimulatory molecule (Moise et al. 2012, De Groot et al. 2008).

However, the modified antibodies (VH77-MOD1 and VH77-MOD2) immunized splenocytes did not induce statistically elevated IFN- γ responses. Overall, an elevated IFN- γ response per se was not expected to occur. Though IFN- γ does not need to be seen at elevated levels to indicate an immune response, it could still be seen as an indicator that further cytokine presence should be analyzed to determine if there is a cytokine during antigen-re-stimulation that is prevalent above all other cytokines. For instance,

cytokines such as IL-2, IL-4, IL-12, and TNF- α or IL-10, and TGF- β could be reviewed in culture supernatants to identify the type of immune response that is being induced by these variant antibodies during ex vivo culture and re-stimulation of splenocytes (Steinman et al 2000, De Groot et al. 2008, Cousens et al. 2013).

Splenocytes from all of the immunized conditions were treated to not only OVA₍₂₅₇₋₂₆₄₎, but also OVA₍₃₂₃₋₃₃₉₎ during the ELISpot assay; however, the splenocytes treated with OVA₍₃₂₃₋₃₃₉₎ failed to become activated and thus did not secrete IFN- γ . OVA₍₃₂₃₋₃₃₉₎ is a class II peptide (Table 4) that can be found within ovalbumin, the whole protein, that was conjugated to the α DEC-205 antibody and administered to the DR4 mice during in vivo immunizations. Thus, if the ovalbumin had been processed and degraded into peptides by the acid proteases (Cathepsins S & L) within the acidic endosomal/lysosomal vesicles, then it is possible that OVA₍₃₂₃₋₃₃₉₎ could have been generated through this degradation process. Then OVA₍₃₂₃₋₃₃₉₎ would be presented by MHC molecules to circulating T cells for the subsequent activation and induction of a protective immune response. Some of the potential effector T cells generated from the initial immune response then differentiate into memory T cells. Memory T cells, upon recognition of the same OVA₍₃₂₃₋₃₃₉₎ antigen, are then able to rapidly turn into effector T cells once again and induce an accelerated antigen-specific T cell mediated immune response. However, because IFN- γ was not secreted after re-stimulation with OVA₍₃₂₃₋

³³⁹) that suggests that the initial breakdown of ovalbumin into peptides during in vivo immunizations did not generate OVA₍₃₂₃₋₃₃₉₎ for presentation to T cells.

Since CD4⁺ T cell proliferation was observed in the T cell proliferation assay, this means that class II peptides can be presented to T cells and T cells are able to subsequently recognize the peptide, bind to the complex, and initiate proliferation. This result is suggesting that there are potentially other class II peptides within ovalbumin that can be presented to T cells and induce an immune response. This is definitely possible because the initial in silico prediction of OVA₍₃₂₃₋₃₃₉₎ binding potential was 1.66. A score of 1.64 or greater is required for any sequence frame to have a significant chance of binding to a given HLA allele. Thus, because the OVA₍₃₂₃₋₃₃₉₎ sequence had an EpiMatrix z-score of 1.66, it is considered a top 5% hit, but it is on the EpiMatrix z-score significance level threshold, which suggests there is potential for this sequence to be a false positive binder to the DR4 allele. An HLA-DR4 binding assay that would test the strength of the OVA₍₃₂₃₋₃₃₉₎ binding potential to the DR4 allele would be required to confirm a false positive binding theory. Furthermore, because of the possibility for OVA₍₃₂₃₋₃₃₉₎ to be a false positive binder to the DR4 allele, it is therefore possible that even if the sequence was generated during proteolytic processing of ovalbumin, an immune response would not be generated. An additional issue to consider as to why a lack of immune response was generated after OVA₍₃₂₃₋₃₃₉₎ was assayed is that the working stock solution that was used potentially could have been unstable and degraded. Generally, this can occur due to repeated “freeze-thaw cycles”,

which can also lead to loss of stability (Nowatzke et al. 2003). However, measures were taken prior to peptide usage in immunogenicity assays to counteract such instances, such as aliquoting the solvated peptide in usable volumes.

As for the α DEC-205:OVA-HC54-MOD1 immunization condition, no statistically elevated levels of T cell proliferation or IFN- γ secretion was observed. Splenocytes immunized with this particular variant antibody generated data similar to the α DEC-205:OVA-ORG immunization condition. This variant antibody was generated by modifying the constant heavy chain region and is not associated with the variable heavy chain (VH77) Tregitope sequence (Figure 7). Possible reasons why pro-inflammatory immune response characteristics were not observed for the α DEC-205:OVA-HC54-MOD1 immunization condition at statistically elevated levels in comparison to the non-modified α DEC-205:OVA-ORG immunization condition are that it may not be the most tolerogenic Tregitope sequence or it may not have been fully deleted for an effector (pro-inflammatory) immune response to take hold. Unpublished reports (from De Groot et al.) indicate the human Tregitope sequence (Hu84) (mouse equivalent to VH77) is the most tolerogenic out of the three potential Tregitope sequences (Hu84, Hu167, Hu289). Therefore, if a pro-inflammatory immune response were to occur, it would have been expected when the α DEC-205:OVA-VH77 (MOD1 and MOD2) antibodies were used in immunizations, due to modifications to the VH77 Tregitope sequence, which is what we observed in T cell proliferation measurements.

However, published reports from De Groot *et al.* 2008 identified two other strong epitope clusters, Hu167 and Hu289. The same EpiMatrix suite of in silico tools used in this research project were used to identify these potential tolerogenic T cell epitopes, which showcased EpiBars (band-like patterns) or dense populations of epitope hits against a panel of eight class II alleles representative of highly promiscuous and potentially immunodominant epitopes that showed homology to human immunoglobulin. These T cell epitope sequences were found to rank highly on the human immunogenicity scale (De Groot et al. 2008, Cousens et al. 2013).

De Groot *et al.* 2008 demonstrated the effectiveness of the human Tregitope sequence Hu167, homologous to murine Tregitope sequence HC54, to induce a 2-fold increase in CD4⁺CD25⁺FoxP3⁺ T cells after Tregitope stimulation (including Hu289) to PBMCs, which had suggested the activation of Tregs and confirmed the expected activity of Tregitopes. Further, studies performed by Cousens *et al.* 2013 using the murine Tregitope equivalents to human Tregitopes Hu167 and Hu289 showed similar T regulatory cell activation and effector immune response suppression in non-obese diabetic mice (NOD). Furthermore, Tregitope activity with the co-administration of Type 1 diabetes antigen was shown to delay the onset of hyperglycemia and reduce the incidence rate in non-obese diabetic mice (NOD), as well as suppress the effects of diabetes after development in NOD mice (Cousens et al. 2013).

Previous research has demonstrated that these Tregitope sequences can generate Treg cells. Modifications to the single HC54 epitope sequence

tested here may not have been enough to overcome the tolerogenicity level of an antibody containing the other two potential highly tolerogenic Tregitope sequences (Hu84 (mouse VH77) and Hu289). Further evidence that more extensive modification is required, when the “highest” tolerogenic Tregitope sequence is not targeted for modification, is the presence of potential regulatory sequence frames. Even after modifications, the α DEC-205:OVA-HC54-MOD1 antibody retained one sequence frame predicted to have a significant chance of binding to the HLA-DR4 allele. De Groot *et al.* previously demonstrated the effectiveness of the human and murine Tregitope sequences to induce Treg cells with Tregitope stimulation; therefore, it is possible with one sequence frame still predicted to bind to the DR4 allele in the HC54 Tregitope sequence, that the full Tregitope effect has not been knocked out and if the sequence is presented it could activate Tregs (De Groot *et al.* 2008, De Groot *et al.* 2013). An HLA-DR4 binding assay could be performed to confirm whether the Tregitope sequences are positive binders, which could suggest the potential likelihood for Tregitope presentation and Treg cell activation.

Overall, this study provided data that suggests modifying specific Tregitope sequences within the α DEC-205:OVA antibody is capable of rendering the antibody less tolerogenic, allowing for the induction of statistically significant levels of pro-inflammatory immune responses after antigen re-stimulation. Consequently, the information provided by this study could add to the vaccination strategy repertoire and become a viable option to

consider when utilizing therapeutic antibodies as a means for immunotherapy in regards to autoimmunity, tumors, and potentially HIV and cancer. However, the big issue plaguing most therapeutic antibodies is the immune response generated by the antibody and associated biologic proteins that can interfere with treatment efficacy due to anti-drug antibodies (De Groot et al. 2013). Therefore, it would be necessary to confirm the immunogenicity of the antibody first prior to use in clinical studies. However, this potentially improved vaccine delivery platform system may be most efficient as a vaccination strategy prior to infection since immunity against specific pathogens could be garnered by coupling specific antigens to the targeted therapeutic antibody to generate immune system recognition and lead to memory.

CHAPTER 6

CONCLUSION

By harnessing the innate ability of the DEC-205 receptor, found ubiquitously on DCs, to ingest pathogen, process it, and present its antigenic peptides to T cells, it has become a viable candidate for novel targeted vaccination strategies. The overall goal of this study was to create an improved vaccine delivery system by modifying regulatory T cell epitopes found within the α DEC-205:OVA antibody's sequence. These modifications would in turn generate newly de-tolerized monoclonal antibodies capable of targeting the DEC-205 receptor and serve as delivery vehicles for vaccine test antigens to raise a robust antigen-specific T cell mediated protective immune responses.

Two monoclonal antibodies stood out amongst the three total variant antibody sequences, the α DEC-205:OVA-VH77-MOD1 and α DEC-205:OVAVH77-MOD2 antibody. The α DEC-205:OVA-VH77-MOD1 retained one predicted and potential regulatory sequence frame predicted to bind to the DR4 allele, while the α DEC-205:OVA-VH77-MOD2 antibody did not retain any potential regulatory sequence frames predicted to bind. However, when both antibody sequences (VH77-MOD1 and VH77-MOD2) were administered in in vivo immunizations, without co-stimulatory factors, CD8⁺ and CD4⁺ T cell proliferation was induced, respectively. These results were statistically

significant when compared to the baseline levels of the non-modified α DEC-205:OVA-ORG antibody immunized splenocytes.

An improved novel targeted vaccination strategy, raising antigen-specific immune responses, is underway and the successful first steps have been taken. The next steps to take would be to perform studies that further establish what types of T cells are proliferating. These studies have given initial estimates that the T cells that are proliferating are potentially effector in nature due to IFN- γ secretion; however, further application of splenocytes in T cell proliferation and phenotyping assays, from more in vivo immunizations, will assess if the T cells present and proliferating are effector or regulatory in nature due to the application of intracellular staining. Intracellular staining can confirm the T cell phenotype established by extracellular staining based on the identification of transcription factors, present after antigen re-stimulation, associated with either effector or regulatory immune responses.

It would be interesting to see whether levels of Treg expression increased when comparing all four antibodies (ORG and three variants) against one another. Higher Treg expression would be expected with the α DEC-205:OVA-ORG (no Tregitope modification) and α DEC-205:OVA-HC54-MOD1 (no statistically significant data) conditions. Whereas, Treg cell levels would be expected to decrease when the α DEC-205:OVA-VH77-MOD1 and α DEC-205:OVA-VH77-MOD2 conditions are used and subsequent T cell proliferation is phenotyped.

Also, further site directed mutagenesis work could be performed to the α DEC-205:OVA-VH77-MOD2 antibody. This process would be performed again to determine if further modification to additional Tregitope sequences to α DEC-205:OVA-VH77-MOD2 antibody imparted further reduction in tolerance and increased expression of pro-inflammatory immune responses.

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