MAPPING IMMUNODOMINANT $CD4^+$ T CELL EPITOPES FOR THE DEFICIENT ENZYME IN POMPE DISEASE

By

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To my family and friends for all their support and encouragement

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129 Sv	Inbred strain of mouse
AAV	Adeno associated virus
APC	Antigen presenting cells
BSA	Bovine serum albumin
CD4, CD8	Cluster of differentiation 4; 8
cDNA	Complementary DNA
CFA	Complete freunds adjuvant
CRIM	Cross reactive immunologic material
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked Immunosorbent Spot
ERT	Enzyme replacement therapy
FDA	Food and Drug Administration
GAA	Acid α Glucosidase
GAA-/-	Murine knockout model for GAA gene
H – 2	Mouse major histocompatibility complex
$H – 2^{bc}$	H-2 haplotype of 129 strain mouse
HLA	Human leukocyte antigen
IFN – γ	Interferon gamma
IgG, IgE	Immunoglobulin G; E
IL – 2, 4, 5, 10, 13	Interleukin 2; 4; 5; 10; 13
MHC	Major histocompatibility complex

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PMA	Phorbol 12- Myristate 13- Acetate
rhGAA	Recombinant human acid alpha glucosidase
RNA	Ribonucleic acid
RT – PCR	Real time polymerase chain reaction
SEB	Enterotoxin B, Staphylococcal
TGF – β	transforming growth factor beta
Th1, Th2, Th17	Thymus helper cells 1; 2; 17
TNF – α	Tumor necrosis factor alpha
Treg	Thymus regulatory cells

Abstract of Thesis Presented to the Graduate School
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Pompe disease is a type II glycogen storage disorder caused by an inherited deficiency of the lysosomal enzyme acid alpha glucosidase. Partial or complete deficiency of this enzyme affects the degradation pathway of glycogen inside the lysosomes of the cells.(1, 2) (3)The severity of the disorder is related with its phase of onset and amount of residual enzyme. (4)

Since there are no clinically available treatments for this disease, both enzyme replacement and gene replacement therapies have been employed for replacing the dysfunctional enzyme. Myozyme and Lumizyme are the currently approved clinical forms of the GAA protein which have been used in different phases of human trials. And viral vectors such as AAV and Adenovirus have been used for transducing the deficient enzyme in several parts of the body. However the effectiveness of these therapies has been short lived by the formation of antibodies or cross reactive immunologic materials against the administered enzyme. (2)

A better understanding of the adaptive immune system is required for preventing such anti-protein inhibitory antibodies and for providing an active functional enzyme to

the body. Mapping of the immunodominant CD4+ T cell epitope will present a clear picture of the interactions between the protein and antigen presenting cells and help in identifying the relationship between the cell mediated and humoral immune responses of the body. (5)

The peptide sequences IFLGPEPKSVVQ (83), HSRAPSPLYSVE (19), VLQPSPALSWRS (29) were identified as the immunodominant T cell epitopes for the rhGAA protein in 129Sv strain GAA-/- mice using in-vitro techniques such as ELISpot and flow cytometry. Additionally, the message levels and profiles of cytokines expressed by of CD4+ helper T cells were determined using RT-PCR arrays. Thus, a comprehensive study of the T cell mediated immunity for the rare and fatal classical infantile syndrome of the disorder was performed.

CHAPTER 1
INTRODUCTION

Overview

Pompe disease is a debilitating neuromuscular disorder, caused by partial or complete deficiency of the lysosomal enzyme acid alpha glucosidase. The autosomal recessive disease which leads to progressive skeletal muscle weakness has been detected with varying frequencies in several ethnic groups. The rare kind of inherited storage disorder has been observed on an average in 1 in 40,000 live births with higher frequency in females compared to males. (3, 4, 6)

Sir Johannes Cassianus, a Dutch pathologist was the first person to describe the disorder as a progressive generalized muscle weakness associated with severe cardiomegaly in a 7-month pediatric patient in the year 1932. During the same period Putschar reported a parallel observation of a patient (infant) suffering from muscle weakness and cardiac dysfunction. The actual cause of the disorder was not identified even after 20 years of its initial description by Sir Pompe. Further developments in the form of discovery of lysosome by C. DeDuve and research of catalytic mechanisms of glycogen by G. T. Cori helped in characterizing the source of the disorder as a lysosomal enzyme deficiency by H.G. Hers in the year 1955. (7)

The disorder falls among a class of 49 types of lysosomal storage disorders and is considered as the only glycogen storage disease, which is also a lysosomal storage disorder.

Biochemistry of the Enzyme

The gene encoding the lysosomal enzyme, acid alpha glucosidase (GAA) is located in the long arm of chromosome 17 in humans and in chromosome 6 of mice.

The 28kb long human GAA gene comprises 20 exons and 19 introns, and contains a 2.7kb long intron between the non-translating first exon and coding second exon. The GC rich promoter mimics the characteristics of a housekeeping gene and lacks the TATA and CCAAT motifs, which have been related to the ubiquitous expression of the gene. (8)

The 952 a.a long protein encoded by the 3.8kb long cDNA is initially synthesized in the form of a catallytiacally active 110kDa precusor protein. This pre-protein undergoes core glycosilation in the ER and obtains mannose-6 phosphate receptors from the post-ER compartment. Around 70-80% of the pre-protein then gets directed to the pre-lysososmal or lysosomal organelles by mannose-6 phospahte receptors, where subsequent cleavages of the protein helps in the production of 95-, 76- and 70- kDA forms of GAA enzyme. These cleavage steps are considered to be essential for activation of the enzyme. (1, 8)

A smaller portion of the enzyme, (10 - 20%) which is initially secreted from the cells, gets re-routed to pre-lysosomes of adjacent cells through their mannose-6-phosphate receptors. Both gene replacement and protein replacement therapies have been designed on the basis of this form of the secretory protein.(1)

The lysosomal enzyme is soluble in nature and functions optimally at a pH of 4.3. The enzyme is responsible for cleaving α-1,4 and α-1,6 linkages of glycogen into monosaccharides like glucose and maltose. Glucose is taken up by several organs of the body and is required for maintaining blood sugar levels and as a source of energy during cell turnovers. A notable feature of the GAA enzyme is its sequence similarity with both the subunits of the intestinal sucrose- isomaltase enzyme complex. (7, 8)

Pathophysiology:

The disorder arises due to genomic mutations of the enzyme. At present, 287 mutations of the gene have been reported, among which 67 have been termed to be pathogenic and 197 as non-pathogenic.

GAA is the only available pathway for degradation of lysosomal glycogen, so in the absence of the enzyme, glycogen starts accumulating in almost all the cells and tissues of the the body. Lysosomal glycogen deposits have also been detected prior birth in amniotic fluids and in muscles of 18 week old embryos. Glycogen accumulation weakens and enlarges both the cardiac and skeletal muscles of the body. Traces of glycogen have also been observed in satellite cells, liver cells, in motor neurons and glail cells and in nuclei of brain stem and anterior horns. (1, 4, 7)

Even though pathophysiology of the disease varies with the expressed phenotype, generalized and progressive skeletal muscle weakness has been observed in all the forms of the disorder.

As GSDII is a multi-system disorder, some of the organ dsyfunctions including hypertrophic cardiomyopathy, progressive skeletal myopathy, osteopenia, osteoporosis, macroglossia, and respiratory insufficiency were observed in patients. (7)

Phenotypes of GSD II

The GSD II disorder varies accordingly with the amount of residual enzyme, phase of onset, extent of organ involvement and rate of progression of muscle weakness. (4, 7)

The infantile phenotype is considered to be the most severe form of the disorder and is usually diagnosed within the first 3 months of life. One of the hallmarks of this form is the almost complete deficiency of the lysosomal enzyme in the cells. The

patient's exhibit a variety of symptoms ranging from generalized muscle weakness to ventricular outflow obstructions till feeding problems and organ failures such as cardiomegaly, hypertrophic cardiomyopathy, mild hepatomegaly, severe hypotonia, and macroglossia. Death usually occurs within a year after birth due to cardio respiratory failures. One in 1, 38,000 babies suffer from this form of fatal disorder which is also commonly referred to as Pompe disease. Alternatively the incidence of the disorder varies with the kind of ethnic population –e.g frequency of 1 among 100,000 to 1 among 200,000 in outbred Caucasian populations and 1 among 40,000 in African Americans. (3, 4, 6, 7)(9)

Both the juvenile and adult phenotypes are grouped together in the late onset form of the disease. Some of the distinguishing features of these forms are the presence of reduced but residual activity of the GAA enzyme and near total absence of cardiac dysfunctions. Patients get diagnosed within the first decade of their life and start exhibiting symptoms such as mild hepatomegaly, skeletal muscle weaknesses and respiratory insufficiencies. One in 57,000 children and adults are affected by this adult form of the disorder. (3, 6, 7)(9)

A non classical type of the disorder known as the infantile variant form has been observed in some patients. In this particular phenotype patients do not experience cardiac disorders and usually progress through symptoms similar to Pompe disease at a slower rate with milder severities. This form is also referred to as the subtype of GSDII. (6, 7)

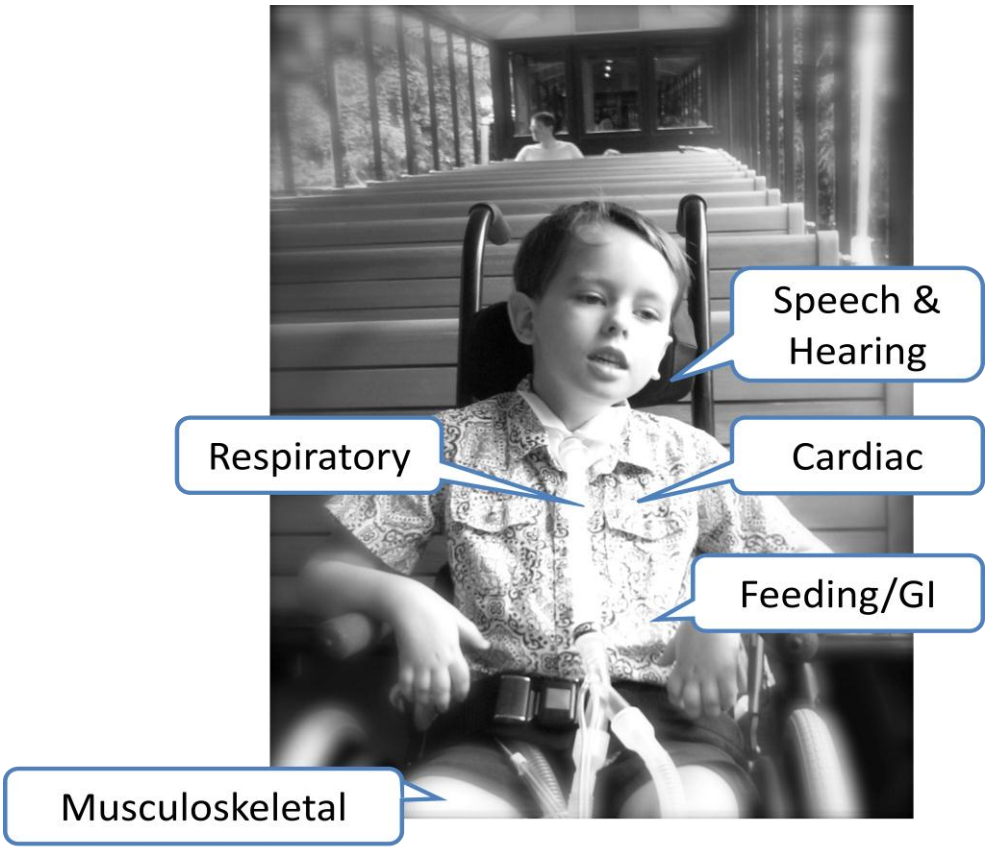

Figure 1-1. Patient diagnosed with GSD II-the tube is connected to aid with his breathing (Use of this photograph has been approved by the patient)

Diagnosis:

The rare condition of this disorder along with its non-specific symptoms has made its diagnosis harder. Nowadays Pompe disease is also being considered as a cause for floppy baby syndromes. (10)Basic diagnosis for the disorder starts with the measurement of GAA activity from either whole blood samples or muscle biopsies and skin fibroblasts. Although detection of GAA from skin fibroblasts is considered to be the gold standard, preference is being given to assays run on dried blood spots for their accuracy and rapid mode of diagnosis. (3, 6)(9).

Apart from enzyme assays several specific and non- specific diagnostic approaches including, GAA mutational analysis, electrocardiograms, echocardiograms,

chest X-rays and measurement of creatine kinase levels in serum and determining the levels of a specific glucose tetrasaccharide in urine of patients are being performed as a part of the diagnosis. Pre-natal screening of Pompe disease is also being preferred for detecting the presence of the deficient enzyme at earlier stages. These timely discoveries have led to the presymptomatic diagnosis of 30 different inborn errors of metabolism. And they could also be used for (10)improving the survival rate of patients via enzyme replacement therapies. (6, 7, 11)(9)

Animal Models for Pompe

Lapland dog, cats,sheep and Brahman and Shorthorn cattle and a particular strain of Japanese quail represent the naturally occurring animal models for GSD II. However they could not be used for research studies owing to several constraints including prolonged breeding periods, smaller litter size and evolutionary distance from human models. To overcome these discrepancies, five mouse models that more genotypically and phenotypically resembled the human form of the disorder were created. These models were generated by inducing specific disruptions in GAA gene. Both the phenotypes of GSD II were re-created in the knockout murine models by targeting Exon 6, Exon 13 and Exon 14 respectively. Generalized and progressive muscle weakness was observed along with abnormal levels of lysosomal glycogen in the heart, liver and striated muscles of these models. Nowadays exon 13 and exon 6 knockout models have been used extensively for pre-clinical studies in gene therapy. (7)

Treatments

Although there is no available cure for Pompe disease at the moment, both enzyme replacement and gene replacement therapies have been employed for replacing the dysfunctional enzyme These therapies were designed with the aim of

replacing the deficient enzyme with a functional one which would help in clearing the glycogen content from cells and successively improve their cardiac and skeletal muscle functions.

Enzyme Replacement Therapies:

Enzyme replacement therapies for GSD II were introduced in the 1960s using enzyme preparations from Aspergillus niger and human placentas. Due to lack of experience and minimal knowledge of the disease, the trials failed to provide the proposed clinical benefits. With experience, the cause of failures was then related to inappropriate choice of enzyme sources and insufficient dosage levels. (6)

Parallel studies on the mechanisms behind the uptake and functioning of the enzyme helped in unraveling the involvement of cell surface receptors with the uptake of extracellular glycoproteins. As Mannose 6 phosphate cell receptors help with the ingestion of GAA, GAA coupled with the receptor was and still being used as a means of delivering effective and efficient enzymes.

Further developments including the cloning of GAA gene helped in opening up more avenues for the large scale production of the recombinant human enzyme. The recombinant forms of the enzyme were being produced in cell cultures and Chinese hamster ovary cell lines, in milk of transgenic rabbits and even in baculovirus infected insect cells.

Currently two types of the recombinant enzymes namely Myozyme and Lumizyme were approved by the United States Food and Drug Administration system for application as therapeutic drugs in trials including infantile and late onset disorders. Myozyme or aglucosidase alfa was prepared by cloning the human GAA cDNA using a muscle specific promoter in tandem with dihrofolate reductase gene for promoting

methotraxate induced amplifications. This rhGAA was approved in the year 2006 for infantile patients. Lumizyme, which is considered to be a similar version of Myzozyme was approved by the FDA in 2010 for late- onset patients. Both the enzymes have proved to be effective in improving the conditions of the patients and have helped in increasing their survival rates.

However the effects of ERT's are highly transient and require frequent infusions for providing the desired outcome. The enzymes are administered intravenously at a dose of 20-40 mg/kg even in less than a year old patients and are usually tolerated even with their higher infusion rates. Despite these advantages, ERT's have been facing a major hurdle in the form of anti- protein inhibitory antibodies. The efficacy of the therapies have been reduced or completely halted by the formation of IgG and IgE antibodies respectively. Anapyhlactic responses are considered to be fatal counteractive events and occur rarely. Immune tolerance protocols have been and are being designed for preventing the occurrence of IgG and for providing an efficient enzyme replacement therapy to the patients. Apart from these issues, patients have also been suffering without treatments due to the cost factors and paucity of sponsoring health insurance companies. Overall 300 patients worldwide have been receiving therapeutic recombinant human alpha glucosidase enzymes. (6)

Gene Therapy:

One of the alternatives that hold a promising future for the treatment of Pompe disease is Gene Therapy. Constant supply of the functional enzyme for a prolonged period of time is the main aim of this therapy. Both In-vivo and In-vitro trials have been performed using viral vectors, cell lines and recombinant proteins. GAA enzyme has been transduced into fibroblasts using adenoviral vectors in-vitro. Transduction resulted

in an efficient expression of the enzyme and subsequent secretion of the 110kDa precursor protein of GAA. This extracellular GAA was taken up by cells which were able to use it for clearing their lysosomal glycogen. (7)(6)

Viral vectors encoding the cDNA of rhGAA have been used for transducing the enzyme in GAA-/- mice using muscle and liver specific promoters. Mutant cell lines have also been tested for expression of functional protein in skeletal and cardiac muscles and in the liver upon gene transfer. Overall it was identified that transducing and expressing in the muscles was much harder than other organs. Studies done on muscles of murines have always exhibited heterogeneous and discouraging results. Parallel developments helped in discovering the liver as an ideal site for expression and functioning of the enzyme. The protein is generally taken up effectively by the cardiac and muscle cells upon its secretion from the liver. AAV vector given through the Intravenous, Intramuscular or Intramyocardial routes were found to represent alternative gene therapy approaches. (7)

Despite encouraging results, possibilities of random integrations of the gene and the susceptibility of immune responses against the vector, transgene and its products have decelerated the development of human clinical trials on gene therapy.

Nowadays focus has also been garnered on the use of non-viral transfection agents for the treatment of Pompe disease. (6)

CRIM and GSD II:

CRIM is referred to as cross reactive immunogenic material. The enzyme replacement therapies for the infantile and adult form of the disease have been affected by the CRIM status of the patients. Patients who are incapable of producing endogenous GAA enzyme due to deleterious mutations in their genome are referred to

as CRIM negative patients, Patients who possess variable amounts of residual enzyme in any form (non-functional or partially functional) are referred to as CRIM positive patients. (2)

Replacements of the non-functional or deficient enzyme by Myzozyme in infantile patients have been less effective due to the occurrence of anti-enzyme antibodies. IgG of high titers against the enzyme were observed much earlier and were more sustained in this group of patients. As a result, they have not been receiving the desired outcomes of the treatment such as ventilation free existence, improvement of their cardiac and motor skills and survival rates. (6, 11, 12)

Although antibodies have been detected in CRIM positive and adult onset populations, the patients have been benefited with improved cardiac and motor development functions. (2, 4)

As the CRIM status of the patients has been hampering the goals of ERT, adjunct strategies that can help improve the efficacy of the treatment are currently being tested. These include immunomodulatory and immune suppressive strategies to promote tolerance to the therapeutic GAA protein.

Antibody formation often requires activation of B cells by CD4+ T cells (T helper).Identification of the immunodominant CD4+ T cell epitope will help us understand the mechanism of immune response in this disorder and could successively be used for designing of various tolerance and immune modulating protocols. (13, 14)

Clinical Trials:

Some of the current clinical trials being done on Pompe disease at University of Florida are

Phase I/II Trial of Diaphragm Delivery of Recombinant Adeno-associated Virus Acid Alpha-Glucosidase (rAAV1-CMV-GAA) Gene Vector in Patients with Pompe Disease

Effects of Immunomodulation Therapy on Anti-rhGAA Immune Response in Children with Pompe Disease Receiving rhGAA Enzyme Replacement and

A Phase I/II Open-Label Study of the Safety, Tolerability, Pharmacokinetics, Pharmacodynamic and Preliminary Efficacy of BMN 701 (GILT-tagged Recombinant human GAA) in Patients with Late Onset Pompe Disease

Need For Epitope Mapping:

Epitope:

The molecular structure of an antigenic molecule or protein, which is recognized by any individual antigen receptor or antibody, is termed as epitope.

Immunogens contain several epitopes, and they are capable of binding to various MHC class molecules. Despite the multitude of epitopes for an antigen, T cells respond only against a few of these antigenic determinants in an individual. The power of the immune system to target and regulate the immune responses to a set number of the epitopes is known as immunodominance. (15)

The immune system of the body is always going to be focused on these immunodominant epitopes for any individual, so that an initial exposure to these epitopes will help in generating an effective priming response against the antigen and will help in conferring protection against successive encounters.

Enigmatic MHC Molecules:

Major histocompatibility molecules are membrane bound glycoproteins, which are involved with the antigen presentation to thymocytes and T cells. They are highly

polymorphic and polygenic in nature and are encoded by a cluster of genes in a region called the major histocompatibility complex. Major histocompatibility molecules are termed as human leukocyte antigens in humans and as H-2 antigens in mice. There are two major classes of MHC molecules, namely the MHC class I and MHC class II molecules. Class I molecules are expressed on all nucleated cells and recognized by CD8 positive T cells, while class II molecules are expressed only on antigen presenting cells and recognized by CD4 positive T cells.

The MHC class II gene complex of mice is also known as the I region and is composed of two locis, A and E, which code for the class II molecules. These genes are also referred as Ir (immune response) genes as they determine the level of immune responsiveness of different mouse strains against several antigens. The IA and IE antigens are also jointly referred to as the Ia antigens(5, 6). (5)(16)

Class I molecules are known for binding bacterial and viral peptides residues which get degraded and processed within the cytosol of the cell. Class II molecules bind to peptides which get degraded within endocytic vesicles. Once activated, naïve CD8 T cells get converted into effector T cells or cytotoxic lymphocytes and kill the cells which are infected with foreign peptides. On the other hand CD4 T cells differentiate into different subsets such as Th1, Th2, Th17 or regulatory T cells. (15)

MHC Restriction:

A T cell can recognize an antigen only when it is presented as peptide on a specific allelic variant of a MHC molecule and will not recognize the same peptide when it is presented on another MHC. This unique property of T cells is known as MHC restriction. Specific binding of peptides to MHC molecules occurs due to the interaction of side chains of the peptides with the grooves and pockets of HLA and H-2 molecules.

The polymorphic nature of MHC molecules in the peptide binding groove justifies the preference of different MHC proteins for different sequence motifs and displays the unique manner, in which TCRs recognize MHC- allele-specific epitopes for restricted recognition of antigens.

Since there are several genes associated with the synthesis of MHC molecules and since there are several allelic variants of the same molecule, an individual is thought to posses 6 different types of MHC class I and class II molecules. So far 2215 HLA class I and 986 HLA class II allelic sequences of MHC molecules have been identified so far in humans. (15)

Systemic peptide libraries that can bind to almost all possible MHC alleles in an individual have been created. (17)The length of peptides in these libraries plays a decisive role in their binding to MHC class I and MHC class II molecules. Usually to cover the complete immunogenic sequence of a protein, peptides with a length of n-1 sequences (n = length of peptides that can be bound by MHC class I or MHC class II molecules) have been used for peptide mapping studies. Since the peptide binding groove in MHC class I molecules are closed on either ends, peptide sequences more than 10 amino acids cannot be accommodated in them. So peptide libraries containing 8-10mer sequences of peptides have been used while mapping the CD8 T cell dominant epitope. (17, 18)(15)

The peptide binding groove is open on either end and allows for binding of larger peptides. The normal length of peptides which get processed and presented to the MHC class II molecules vary from from12-25a.a. Peptide libraries consisting of 12-15 mers are generally used for CD4 epitope mapping studies. (18, 19)

MHC Haplotype:

The specific pattern of alleles found in a single chromosome is known as an MHC haplotype. The $6^{neo}/6^{neo}$ mice were generated using a mixed strain of 129/C57BL/6 mice. To avoid clinical heterogeneity between the mice, the mice were backcrossed to the 129Sv strain. (20)The haplotype of 129Sv is referred to as "H-2^{bc} or I-A^{bc}". The I-A^{bc} and I-A^{b} strains are identical in their classical H-2 and Q regions. (21, 22)

Study of CD4 T Cell Response?

CD4+ T cells play a vital role in the functioning of both innate and adaptive immune responses. They elicit various functions like promotion of cytotoxic T lymphocytes expansion, maintenance of $CD8^+$ T cells memory, communication with innate immune cells, promotion of B cell differentiation into plasma cells and their immunoglobulin class switching, assistance to function of memory cells and activation of macrophages through the secretion of effector molecules called cytokines. CD4+ T cells are required for producing a potent primary and memory $CD8^+$ T cell response and for conferring protection against bacterial and viral infections. They can also be used against viral infections by secretion of anti-viral cytokines like IFN-γ and TNF-α.

We are focused on the antigen presentation and differentiation of CD4 cells. They can branch out to various helper phenotypes like Th1, Th2 and Th17. Th1 cells function by activating the infected macrophages and by aiding B cells for IgG2a secretion and for providing help for CD8+ cells. Although Th2 cells help B cells in the production of antibodies, they are more inclined towards causing a IgG1 or IgE class switch. (15)

Expression of Cytokines

Cytokines are soluble proteins and cell signaling molecules, which participate in intracellular communications. Th1 cells secrete IFN-γ and help in activating infected

cells especially macrophages and other effector cells of the immune system. Th2 cells secrete IL-2, IL-5, IL-13, IL-10 cytokines and function by activating B cells and promoting allergic immune responses. While Th17 cells help in recruiting neutrophils to the site of infection and promotion of acute inflammation, Regulatory CD4+ T cells control and level the adaptive immune responses through the secretion of several inhibitory cytokines like IL-10 and TGF-β. These effector molecules are known for altering the behavior of target cells. (15)

Anti- GAA Formation

Both Th1 and Th2 cells are involved in the activation and functioning of B cells like class switching and antibody secretion and it can be concluded that they are responsible for the co-ordination of humoral immune responses. Some of the previous experiments carried out in Pompe mice corrobarate this view- rhGAA protein was infused through the Intravenous route at a dose of 20 mg/kg once a week for 3 weeks in wt C57BL/6 mice and CD4-/- C57BL6 mice, and their anti-GAA IgG reponses were measured.GAA specific IgG levels could be obtained only from the wt C57BL/6 mice and none were found in the CD4-/- pompe mice. The level of IgG reponses also differed with the amount of protein that was being administered to the mice

The route of immunization is directly related to the magnitude and type of immune response elicited by the antigen. Subcutaneous, intramuscular and intravenous are the most widely routes of experimental antigen administration. As antigens are taken up by Langerghan cells and expressed effectively in the lymph nodes when they are immunized subcutaneously, we chose this method for eliciting GAA specific antibodies and T cells.

The rhGAA was administered subcutaneously with complete Freunds adjuvant (CFA) for preliminary mapping studies. CFA is oil in water emulsion consisting of dead mycobaterial components. CFA works by converting the soluble antigen into particulate materials, which can be easily taken up by APC's and helps in inducing the co-stimulators of dendritic cells and macrophages. (15)

Since the route of immunization and the adjuvant confer a strong inflammatory response, the IFN-γ ELISpot was chosen for studying the effect of antigen presenting cells and for mapping the immunodominant epitopes against the antigen. Besides these choices, IFN-γ ELISpot was preferred for its high sensitivity and ability to determine the clonal size and function of T cells. (15)

Epitope Mapping Techniques:

Limiting dilution assays, direct lysis in bulk killing, MHC tetramer staining assays, intracellular cytokine staining assays, ELISpot, in-vitro cytokine staining are some of the techniques which have been used for exploring the T cell responses and for mapping the immunogenic peptides of the antigen. The first two methods require radioactively labeled matching MHC cells and are quite cumbersome to perform. Intracellular cytokine staining and MHC tetramer staining procedures were aimed to prevent the radioactive labeling of cells and serve as an improved technique, but they were also equally cumbersome and did not eschew the requirement of prior knowledge of T cell epitopes and of MHC usage. (17-19)

As ELISpot assays do not require the prior knowledge or presence of MHC specific target cells and are relatively easier to perform and analyze, this technique is currently being preferred over other available procedures. (17-19)A large collection of

peptides of a protein antigen can be studied by constructing overlapping or non-overlapping pools of peptides in the form of a library. (17)

The immunodominant epitope for human GAA in 129Sv strain GAA-/- mice has been mapped in two successive steps. The splenocytes were tested for their cytokine secreting activities by using peptide matrix pools and simple pools of peptides. Individual peptides from pools, which had emerged as positive samples in the previous step, were used in the second ELISpot assay for determing the immunodominant epitopes. Alternatively, the types of the responding T cells were also ascertained by separating them prior to stimulation in the assays.

The assay relies on the Cytokine secreting characteristics of antigen experienced T cells. Naïve T cells upon prior activation differentiate into effector and memory T cells which can secrete specific cytokines. (16)These cells do not secrete the cytokine in in-vitro conditions without prior stimulation with cognate antigens. To prevent the differentiation and expansion of naive T cells into their effector counterparts, the assay is limited to 15 hrs of stimulation period. Thus to ascertain the antigen specific response of T cells, the peptides are presented under in-vitro conditions to induce cytokine secretion from memory T cells. The imprints of these cytokines are recorded as spot forming cells, which are taken as a direct measure of antigen specific T cell response. (23)

$CD4^+$ T cell epitope mapping for the rhGAA protein in 129Sv mice will serve as a tool for analyzing both the cell mediated and humoral immune responses of the body. It will also help in studying in detail the peptide and MHC binding motifs and for making predictions on candidate T cell epitopes.

Apart from in-vitro assays, bioinformatic algorithms and in-silico tools have been designed for determining MHC class II epitopes. The in-silico tools were chosen from the immune epitope database analysis resource. (24-30)(31-36) We used these tools to compare the results obtained with the epitopes, which were identified using in-vitro assays.

Thus the CD4+ T cell immunodominant epitopes against the rhGAA protein were identified in GAA-/- mice belonging to129Sv strain.

CHAPTER 2
MATERIALS AND METHODS

Reagents

Peptide Library spanning the mature GAA sequence of 952 Amino Acids was generated in the form of 12-mers with an overlap of 2 amino acids from Anaspec in San Jose CA(purity>70%), Recombinant human acid alpha glucosidase protein was obtained from Dr. Barry Bryne's lab at the Powel Gene therapy center in UF, Mouse Interferon Gamma Development Module (R&D systems Minneapolis, MN), ELISpot Blue Color Module (R&D systems Minneapolis, MN), Mouse Interferon Gamma Secretion Assay(Miltenyi Biotec, CA), CD4(L3T4) Microbeads and CD8a (Ly-2) Microbeads for mice were purchased from Miltenyi Biotec in CA,

2- MLC and 5-MLC mouse lymphocyte culture mediums were prepared using DMEM, Sodium-Pyruvate, HEPES, Non-Essential amino acids,2-Beta Mercaptoethanol, Penicillin/streptomycin and mouse serum, Staphylococcal enterotoxin B (Sigma Aldrich, St.Louis,MO),PMA-Phorbol 12-myristate 13-acetate, Ionomycin (Sigma Aldrich, St.Louis,MO),Di-methyl sulfoxide, Recombinant Mouse Interferon Gamma(R&D systems Minneapolis, MN), 2A-54 the dominant CD4+ T cell epitope for human FIX, were used as controls for the experiments.

Some of the other reagents were Recombinant Mouse Interleukin-2 (Roche Diagnostics, Manheim, Germany),Anti-Mouse CD8 eflour450 , Anti-Mouse CD4 Alexa fluor 700 and 7-AAD(7-amino-actinomycin-D), (ebioscience, San Diego, CA) , Phosphate Buffered Saline, Freund's complete adjuvant, 70-µm cell strainer, BD- lysing buffer, Trypan blue, ELISA Wash (Tween 20, 1x PBS and water)

Equipments and Supplies:

Multiscreen Filter Plates (Millipore, MA), Haemacytometer, sterile surgical kit, Sonicator, Cellular Technology Limited ELISpot Reader, BD LSR II (BD Biosciences, MD, USA), 1ml, 5ml and 10ml syringes, 25 gage needles, sterile 1.5ml microfuge tubes, Laminar flow cabinet, Sterile pipette tips(10,100,200 and 1000µl), Single channel and Multi channel Pipettes, Sterile Pipettes (1ml, 2ml, 5ml, 25ml, 50ml),Centrifuge (Bench Top), tally counter, Cover Slip, Phase-Contrast Microscope, 37°C humidified incubator with 5% CO2 atmosphere, Vortexer, Balance.

Mouse Strain:

GAA-/- (GAA knockout) 6-8 weeks old mice were used. They had been created by targeted disruption of exon 6 using a neomycin resistance gene (6neo/6neo). A termination codon and a new EcoRV site had been introduced upstream from the neo gene in exon 6.Even though different types of knockout mice were made (e.g inframe deletion of Exon 6(Δ6/ Δ6), Exon13 and Exon 14 knockouts, only the exon 6 and exon 14 knockout mice exhibited the severe phenotypes of Pompe disease (i.e.) resembling the earlier stage of onset and rapid progression of disease. (20, 37)

6^{neo} /6^{neo} mice were generated using a mixed strain of 129/C57BL/6 mice. These mice are homozygous in nature and do not synthesize endogenous GAA protein. The strains were maintained by intercrossing the progeny of the original 129 chimeric male founders and C57BL/6 female. (20, 37) To avoid clinical heterogeneity between the mice, the mice were later backcrossed to the 129SV strain (haplotype- H-2^{bc}). (20)

The mice were obtained from Dr. Barry Bryne's Lab in the Powell Gene Therapy Center and were housed under specific pathogen free conditions in the Animal Facility

at the University of Florida. All the animal procedures were carried out as per IACUC guidelines.

Construction of the Peptide Library:

Ninety five peptides spanning the mature GAA sequence of 952 amino acids were generated as 12 mers which were overlapping by 2a.a. The peptide stocks were dissolved using Dimethysulfoxide at a concentration of 2 mg/ml. Two dimensional arrays of the peptides were designed to obtain 20 pools of peptides. (17)Each pool was composed of 8-10 peptides, and each peptide was represented in two pools. The final concentration of each peptide in the pool was 4 µg/ml. The reconstituted peptides were stored at -70°C.

Owing to the high concentration of DMSO in the stock, the peptides were re-diluted using 5-MLC media and the pools were re-designed.

Smaller Pools of peptides were constructed this time with each pool consisting of only 3 peptides. Thus 32 pools of peptides were designed and the final concentration of each peptide in the pool was 10 µg/ ml.

Immunization of Mice:

rhGAA protein mixture (50 µg/mouse) was prepared by dissolving the protein in 1 ml of Phosphate buffered saline (PBS) and 1 ml of CFA. The mixture was sonicated using a Branson Sonifier until a creamy white paste was obtained. This protein (200µl) was administered using a 1 ml syringe through the subcutaneous route to 3 mice for every set of experiment. (Figure 2-3)

Collection of Blood Samples:

The mice were anaesthetized using 2% isoflurane and heparinized microhematocrit capillary tubes were used for collecting blood from their retro orbital

plexus. Blood collected from the retro - orbital site is considered to be a representative of the venous blood. They were used for running ELISpots and in-vitro cytokine secretion assays. The results obtained were compared with the results obtained from splenocytes. Since experiments in humans can be carried out only with blood samples, pilot experiments of this kind were included in the project.

ELISpot:

ELISpot kits for Mouse IFN-γ were purchased from R&D systems and used as per manufacturer's recommendations.

Coating of Plates:

Sterile 96 well multiscreen filter plates were pre-wetted using 15 µl of 35% ethyl alcohol for a minute to overcome the hydrophobicity of the membrane.Plates were then washed thrice using 350 µl of 1x PBS and coated with anti- IFN-γ capture antibody . The antibody coated plate was left for overnight incubation at 4°C.

Isolation of Splencoytes:

The mice were sacrificed after 10-12 days of immunization, and their spleens were collected in Dulbecco's modified eagle medium (DMEM). All procedures were carried out aseptically under the laminar hood. The spleens were meshed in 70 µm nylon cell strainers using 5-10 ml of 2-MLC media and washed at a speed of 1400 rpm (revolutions per minute) at 4°C for 10 min. The supernatant was discarded, and the pellet was resuspended in BD lysing buffer (3 ml/spleen) and left on ice for 3 minutes to lyze the erythrocytes. After incubation, 10 ml of 2-MLC media was added for each spleen and spun at 1400 rpm for 10 min at 4°C. The pellet was finally resuspended in 5-MLC media and taken for counting. The final pellet was devoid of erythrocytes and composed of splenocytes.

To check the homogeneity of the spleens, they were harvested separately during the earlier experiments but pooled for the upcoming assays.

Analysis of Blood Samples:

Blood collected from the 3 mice (750-1000 µl/mice) were pooled together and lysed using BD or ACK lysing buffer to eliminate erythrocytes. The cell suspensions were centrifuged and the pellet was resuspended using 5-MLC media and taken for cell counting. Owing to the low cell count obtained by lysing the whole blood, buffy coats were used for the upcoming experiments. Blood collected from the mice were centrifuged to separate out the buffy coat from plasma and erythrocytes. Buffy coat is made up of leukocytes and platelets and used as an alternative procedure for eliminating erythrocytes from the sample. The cells were appropriately diluted to obtain a concentration of 10^6 cells/ml and added in duplicates to the ELISPOT plate. They were treated in the same manner as splenocytes. Since the volume of blood obtained was not enough to run the entire peptide library, only a few peptide pools/peptides and positive controls were tested using blood.

Cell Counting:

Dilutions of the cells at 1:10, 1:20 were made using trypan blue and 10-15 µl of the samples was loaded into the haemocytometer. Since viable cells do not take up trypan blue, this dye was used for differentiating between viable and non-viable cells. The cells were viewed under 100 x magnifications under a phase contrast microscope and counted from the four overlying 1mm2 areas of the counting chamber using a tally counter. The required cell numbers were diluted from the obtained count of live cells..

In-vitro Stimulation of Cells with Peptides:

The plates were blocked for 2 hours using (200 µl/well) ELISPOT blocking buffer (5% sucrose and 1% BSA in PBS) on day 2. Plates were then rinsed and filled with the culture medium until cells were prepared for plating.

Splenocytes were resuspended in 5-MLC medium at concentration of 10^6cells/ml and added in duplicates to the plate (200 µl/well). The culture media was also additionally supplemented with recombinant IL-2 (10 u/ml) for promoting T-cell growth. In-vitro stimulation of cells was induced by adding peptide pools at a concentration of 4 µg/ml or 10 µg/ml. appropriate controls were included to maintain high performance standards. SEB (1 µg/100 µl), PMA (0.05 g/ml)/IONOMYCIN (1 µg/ml), recombinant IFN-g and recombinant human GAA (10 µg/ml) were used as positive controls, DMSO as the negative control and 2A-54(10 µg/ml) as the irrelevant peptide. The plates were incubated in 5% CO_2 at 37˚C for 18 hours to promote cell stimulation.

Any Cytokine secreted by the cells due to stimulation during this period will be captured by the membrane bound antibodies. (23, 38)

Addition of Capture Antibody:

The cells were washed thrice using 350 µl of wash buffer and blotted against a paper towel to remove the remaining solution. The required volume of biotinylated detection antibody was calculated and diluted to its working concentration using reagent diluents. 100 µl of this antibody was then added to each well and incubated overnight at 4°C to capture the secreted cytokines (IFN-γ).

Color Development:

The Ag-Ab complex was identified by adding appropriate enzyme and substrates conjugates like Streptavidin-AP and BCIP/NBT (according to ELISPOT blue development module).

Streptavidin AP was preferred for its linear reaction rate and reduced background staining, and BCIP/NBT mixture is the most commonly used substrate for Streptavidin AP. This substrate mixture was chosen for its stable nature. Binding of AP to BCP/NBT induces the formation of intense black- blue spots. Since these spots do not fade easily, they can be reanalyzed and stored for a longer period of time. Each colored spot is representative of a cell secreting IFN-γ and the spots were enumerated using the Immunospot Analyzer.

Quantification of Spots:

The colored spots found at the bottom of the well were considered to be a quantitative measure of the cells secretion capacity. The quantitative measure was termed as Spot forming cells. The Spots were enumerated using ImmunoSpot Analyzer (Cellular Technology, Shaker Heights, OH).

In-Vitro Cytokine Secretion Assay/ Flow Cytometry:

Mouse IFN-γ secretion Assay - Detection kits were purchased from Mitenyi Biotec and used according to the recommended instructions.

In-vitro Stimulation of Cells with Peptides:

The cells were resuspended in 5-MLC medium and added in triplicates to a 96-well round bottom plate (10^6 cells/well). Peptide pools were added at a concentration of 10 µg/ml. The media was supplemented with rIL-2 at 10 u/ml for promoting T-cell proliferation. Positive and negative controls were added to standardize the procedure.

SEB (1 µg/100µl), rhGAA (10 µg/ml) were used as the positive controls and DMSO was the negative control. Apart from these controls unstained and mock-stimulated controls were also used for determining the positive cells. The cells were incubated along with the peptides in 5% CO_2 for 6 hrs at 37˚C and then transferred to 15ml tubes.

Labeling of Cells:

Cell surface staining was performed using IFN-γ catch reagent for capturing the cells capable of secreting IFN-γ. The cells were incubated with the catch reagent for 45 minutes under slow rotation at 37°c. The cells were then placed on ice and washed using cold buffer. Phycoerythrin conjugated mouse IFN-γ antibody and anti-CD4 (Alexa Fluor-700) (1 µg/10^6 cells), anti-CD8 (eflour-450) (1 µg/10^6 cells) antibodies were added to the cells and kept on ice for 10 min. The cells were washed and resuspended in cold buffer and taken for the acquiring process. The numbers of apoptotic cells were determined by adding 7-amino actinomycin D just prior to acquisition.

Acquisition and Analysis of Data:

Data acquisition was carried out in BD LSR II (BD Bioscience) Flow Cytometer and analyzed using Cell Quest and FACS DIVA 6.1.

Immunomagnetic Cell Separation:

Since we were trying to determine the CD4+ Immunodominant T cell epitopes, cell separation was carried out to choose the specific subsets of cells. Positive and negative selections are the two commonly used separation techniques. Since ELISpot is a combination of immune and bioassay, the desired cells were depleted or negatively selected from the whole cell population. (38) CD4 (L3T4) Microbeads and CD8a (Ly-2) microbeads were used for this purpose.

As soon as the cells were lysed for erythrocytes and counted, equal volume of cells were divided for separating CD4+ and $CD8^+$ cells from the whole splenocyte population. The cell suspensions were labeled with CD4 and CD8 magnetic beads and passed through cell separation (MS) columns in the presence of a magnetic field. The magnetically labeled cells were retained in the column, while unlabelled cells remained in the flow through. The labeled cells were eluted from the columns using elution buffers.

The unlabelled populations from the $CD4^+$ separation were composed of $CD8^+$ cells and antigen presenting cells and were used in the ELISPOT plate for detecting $CD8^+$ epitopes.

Conversely, the unlabelled cell populations from $CD8^+$ separation were composed of $CD4^+$ cells with antigen presenting cells and were used as samples for detecting $CD4^+$ epitopes.

After separation the cells were counted. Duplicates and triplicates of the cells at a concentration of 10^6 cells/ml and 10^7 cells/ml were used for ELISPOT and in-vitro cytokine secretion assays. The separated cells were treated identically to whole splenocytes.

To establish the cytokine expression pattern of T cells responding to GAA, RT-PCR was performed on cDNA samples extracted from splenocytes of GAA-/- mice which were immunized with the whole protein by 3 weekly IV injections at a dosage of 25 mg/kg.

Intravenous Administration of the Protein

The GAA-/-(disruption of exon 6 with neomycin resistance gene) 129 SV mice were immunized with 25 mg/kg of the rhGAA protein through the intravenous route once per week for 3 weeks for cytokine expression assays

ELISA to Determine the Antibody Levels Against rhGAA Protein in Mice:

Plasma samples of the immunized mice were obtained at the end of the intravenous immunization protocol. The levels of IgG1, IgG2a and IgG2b antibodies were determined from these samples. Plates were initially coated with 50 µl of standards and 50 µl of human GAA protein at a concentration of 1 µg/ml in coating buffer and left for overnight incubation at 4ºC. The plates were then washed and blocked with the dilution buffer for 1 hr at room temperature. The plates were again washed and incubated with the plasma samples and secondary antibodies for 2hrs at 37ºC respectively. OPD substrate was used for detection through calorimetric measurement. Color development is catalyzed by conjugated antibodies. The absorbances of the samples were read at 450 nm using ELISA reader, and the concentrations of antibodies of the experimental samples were determined. Thus ELISA was carried out for testing the immunogenicity of the whole protein administration.

Stimulation of Splenocytes with Individual Peptides and Controls for RT-PCR:

The mice were sacrificied after a period of 3 weeks and their spleens were removed for analysis the functions of antigen specific cells. Splenocytes were isolated by following the same procedure as explained above and were stimulated with the individual peptides for a time period of 48 hours.

Isolation of B and T cell Lymphocytes:

The lymphocytes were stained using antibodies against their cell surface proteins, anti-CD3 and anti- CD4 for T cells and anti- CD95 for the B cell population. The cells were sorted through a flow sorter (BD Biosciences, MD, USA) and the isolated populations were used for extraction and synthesis steps of RT-PCR.

Extraction of RNA, Synthesis of cDNA and RT-PCR

RNA was extracted from isolated subpopulation of T cells using the RNeasy kit form QIAGEN according to the instructions by the manufacturer. The extracted RNA was either stored at -80ºC or used for synthesis of cDNA on the same day.

cDNA was synthesized from 5 µg whole RNA using the RT^2 First Strand Kit from SABiosciences as instructed by the manufacturer. Two-four µl of cDNA and random oligo primers were used for the amplification steps of the PCR cycle. The baseline threshold was automatically set by the BIO-RAD instrument and 60-80 cycles of amplification were generally preferred for effective amplication of the gene of interest. The expression levels of all the cytokines were determined by obtaining the ΔΔCt values of the gene of interest from the set of housekeeping genes that were used as controls for the RT-PCR assay. Genomic DNA controls were also included to test for efficiency of the first strand of cDNA synthesis.

Table 2-1. GAA peptide library using 20 pools of peptides

	C1	C2	C3	C4	C5	C6	C7	C8	C9	c10
R1	1	2	3	4	5	6	7	8	9	10
R2	11	12	13	14	15	16	17	18	19	20
R3	21	22	23	24	25	26	27	28	29	30
R4	31	32	33	34	35	36	37	38	39	40
R5	41	42	43	44	45	46	47	48	49	50
R6	51	52	53	54	55	56	57	58	59	60
R7	61	62	63	64	65	66	67	68	69	70
R8	71	72	73	74	75	76	77	78	79	
R9	80	81	82	83	84	85	86	87	88	
R10	89	90	91	92	93	94	95			

Table 2-2. GAA peptide library from 32 simple pools of peptides

Pool 1	1, 2, 3	Pool 12	34, 35, 36	Pool 23	67, 68, 69
Pool 2	4, 5, 6	Pool 13	37, 38, 39	Pool 24	70, 71, 72
Pool 3	7, 8, 9	Pool 14	40, 41, 42	Pool 25	73, 74, 75
Pool 4	10, 11, 12	Pool 15	43, 44, 45	Pool 26	76, 77, 78
Pool 5	13, 14, 15	Pool 16	46, 47, 48	Pool 27	79, 80, 81
Pool 6	16, 17, 18	Pool 17	49, 50, 51	Pool 28	82, 83, 84
Pool 7	19, 20, 21	Pool 18	52, 53, 54	Pool 29	85, 86, 87
Pool 8	22, 23, 24	Pool 19	55, 56, 57	Pool 30	88, 89, 90
Pool 9	25, 26, 27	Pool 20	58, 59, 60	Pool 31	91, 92, 93
Pool 10	28, 29, 30	Pool 21	61, 62, 63	Pool 32	94, 95
Pool 11	31, 32, 33	Pool 22	64, 65, 66		

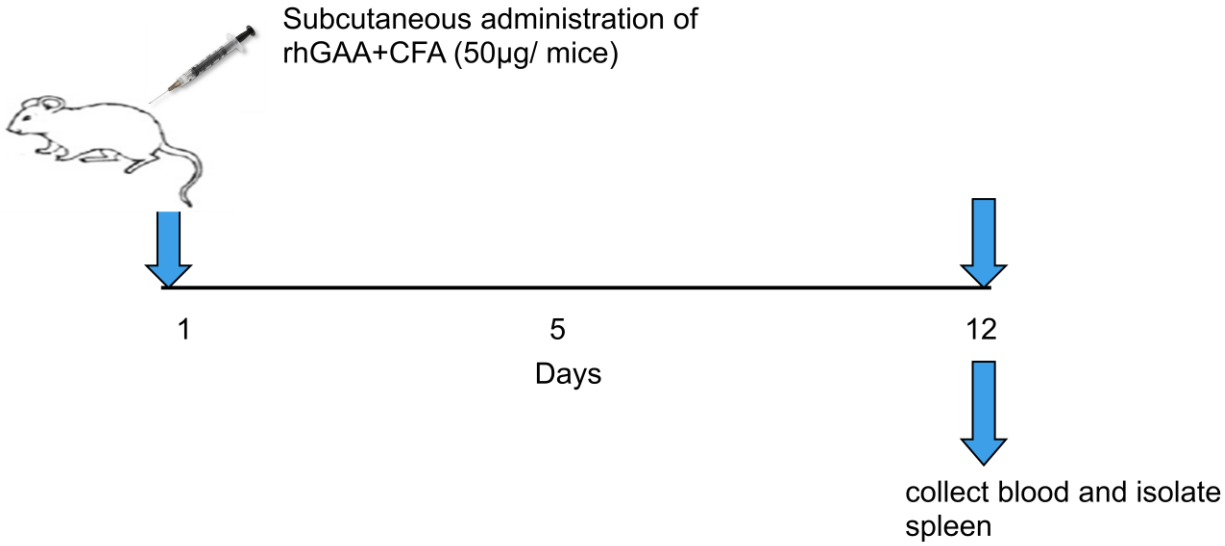

Figure 2-1. Isolation of spleen from GAA-/- 129 Sv mouse immunized with rhGAA

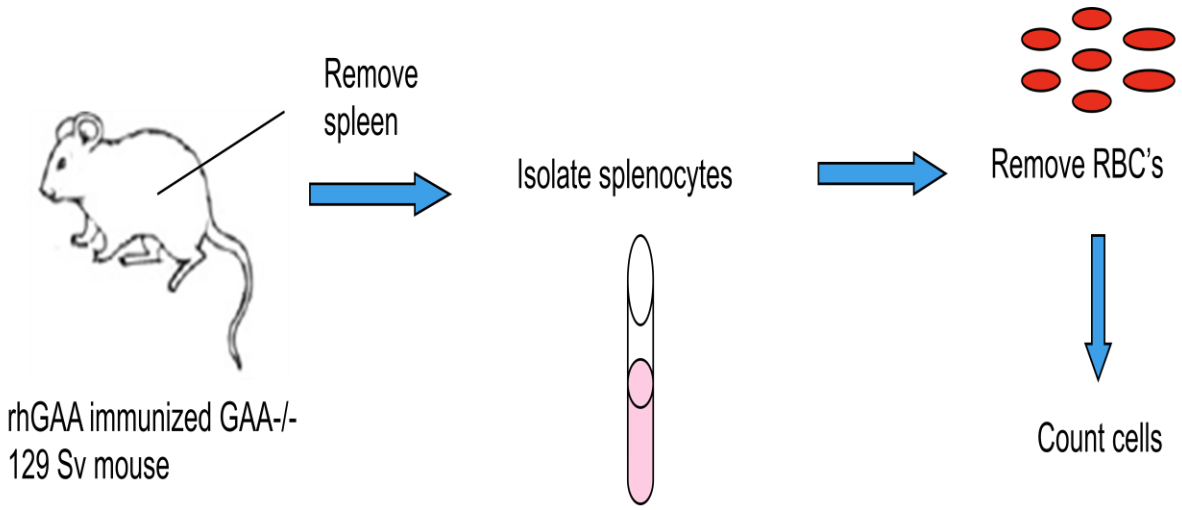

Figure 2-2. Preparation of Splenocytes

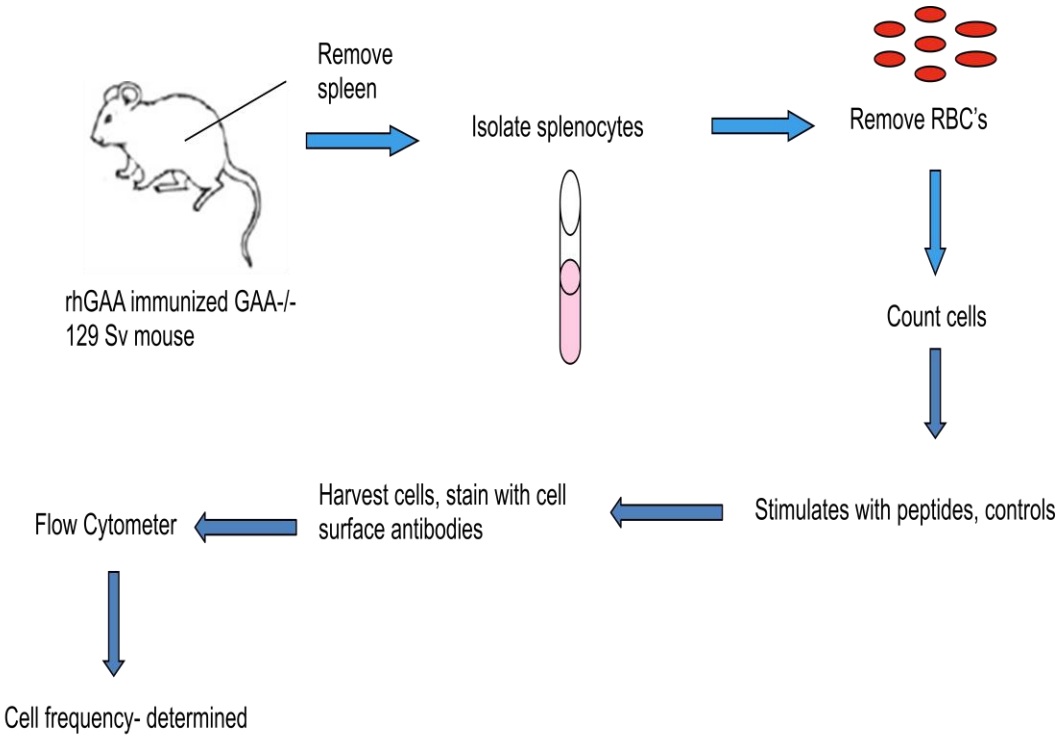

Figure 2-3. In –vitro cytokine secretion assay steps

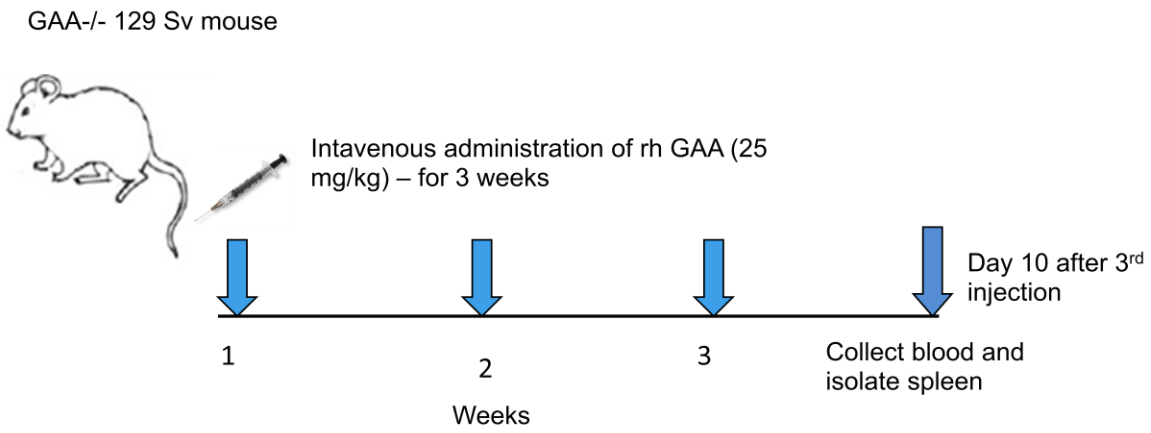

Figure 2-4. Protocol for studying cytokine expression pattern

CHAPTER 3
RESULTS

Identification of the Dominant Peptides Using ELISPOT:

This ELISpot assay was designed for detecting IFN-γ secreting cells at the single cell level and for determining their frequency. The results were quantified in the form of IFN-γ spot forming units or spot forming cells (SFC) per 10^6 input cells. (23)Usually the threshold for frequency of spot forming units is set at twice the frequency of spots formed with mock-stimulated/negative controls.

Dominant Peptide Pools:

The methodology relies on the potential of the peptides to evoke cytokine response from memory T-cells that have been previously exposed to the antigen.(17). This assay is considered to be more sensitive than ELISA and intracellular cytokine staining since they detect T cells based on their functional response to antigens. $GAA^{-/-}$ mice were immunized with recombinant human acid alpha glucosidase protein through the subcutaneous route, and their spleens were isolated after 10 days of injections. Splenocytes were isolated and cultured using 2-MLC and 5-MLC mediums and plated in duplicates on the membrane backed microplates. The cells were stimulated using the 20 peptide pools {C1-C10 and R1-R10} of the GAA protein to identify those testing positive for the secretion of the cytokine IFN-γ. For the initial experiments, pools containing peptides 54,71,74,80 and 83 (i.e pools R6, R8, R9, C1 and C4) turned out to be positive for secretion of IFN-γ.

Dominant Peptides:

Suboptimal results were obtained in the initial set of experiments, due to the high concentration of DMSO in the stock. Even though positive signals were obtained, from

the samples, clearly defined spots could not be detected from the wells. Therefore the peptides were diluted using 5-MLC media and the peptide library was redesigned. New pools with fewer peptides (total of 32 pools comprised of 3 peptides in each) were constructed and tested for the presence of IFN-γ secreting cells. The single peptides which had been identified in the previous experiments were also included in this assay,the homogeneity of the response among the mice was tested by assaying individual pooled spleens in parallel. In this experiment peptides 19,20,21,28,29,30,58,59,60,82,83,84 (i.e pools 7,10,20 and 28) were chosen. Peptide 83 was identified as the dominant one among all the single peptides, which were tested in parallel on the plate.

$CD4^+$ Peptides:

Once the single peptides were mapped, the following ELISpots were run on separated cells to determine the phenotype of the responding cells. Splenocytes were labeled using CD4 or CD8 magnetic beads and were depleted for $CD4^+$, $CD8^+$ cell populations. Duplicates of the T cell populations were added to the ELISpot plates, and the frequency of IFN-γ secreting spots was determined.

Alternatively, ELISpots were run on peripheral blood mononuclear cells to with results obtained using splenocytes. In either case peptide 19, 29 and 83 were found to be dominant among all the other sequences.

Quantification of Spots:

Quantification of spots was done with the help of CTL ELISpot reader. The reader scans the plate and saves their images in the Tiff or Jpeg format. Since all the processes are automated, the machine progresses directly from well to well and uses optical feedback for centering on each of them. Digital encoders are used for identifying

and determining the precise position of each well in the plate. User's name, date and time of scan are recorded with the help of the software.

Saved images were analyzed using the Immunospot software 4.0 version. Counting parameters, spot size gates, Minimum and Maximum spot size density, sample spot size, removal of hair and control of background are some of the features that are set to required levels to aid with the analysis. The whole procedure was done by applying identical parameters to all wells in the plate. The settings were fixed to provide a clear comparison of the cell count from every well. Despite setting these features, the results from ELISpot were always subjected to quality control to remove counts which might have risen due to artifacts. Image overlays were used to identify the spots which were actually counted from each well and for making the required modifications. All such corrections were recorded and annotated to comply with Good Laboratory Practice guidelines.

The data collected from these counts were used for identifying the $CD4^+$ dominant T cell epitope for acid alpha glucosidase protein in GAA-/- 129Sv mice.

Fluorescence Activated Cell Sorting (FACS):

Flow cytometry is used for isolating various cell populations based on their phenotypes. The phenotype of cells is usually defined by its cell surface proteins and they can be detected by using specific monoclonal antibodies which are coupled with fluorescent dyes or using flurochromes.

In our case, lymphocytes were previously stained with monoclonal antibodies specific to CD4 (Alexa Fluor-700) (1 µg/10^6 cells) or CD8 (eflour-450) (1 µg/10^6 cells). Dead cells were excluded by adding 10-15 µl of 7-amino actinomycin D just prior to acquisition. Fluorescence Activated Cell Sorter is the machine that was used for

separating cells based on their fluorescence. The mixture of labeled cells was then passed through a nozzle and a single stream of cells was created to cause the laser beam to hit one cell at a time. Laser light gets scattered when each cell passes through it and causes the excitation and emission of any fluorescent dye associated with them. The scattered light and emission spectra of cells gets captured by the photomultiplier tubes and provides information about the size and granularity of the cell and successively the expression of their cell surface proteins.

Data obtained from the flow cytometer are usually represented in the form of histograms, two dimensional scatter plots or in the form of contour diagrams where the fluorescence of one flurochrome is plotted against the other. Initially, the lymphocyte population was gated using a dot plot or contour diagram. Once the required populations were gated, the numbers of live and dead cells in the population were calculated.

The required subpopulations of cells were gated from lymphocytes and their cell numbers and fluorescence intensity were plotted against each other to obtain the required results. In our experiment the numbers of $CD4^+$ and $CD8^+$ positive cells were determined. Fluorescence intensities were compared against each other for all the tested peptides, and results were compared with those of positive and negative controls to determine the actual cell count.

Based on the above procedure and the in-vitro assays that had been carried out for this purpose, peptide 83 was confirmed to be one of the dominant peptides for the acid alpha glucosidase protein in GAA-/- 129Svmice.

Epitope Mapping Algorithms:

The peptide sequences were loaded into in-silico tools from the immune epitope database analysis resource for comparing the predicted epitope sequences with the epitopes which were obtained from in-vitro T cell assays. Fasta sequences of the whole GAA protein were loaded in all the algorithms for predicting immundominant epitopes specific for the I-A^{bc} MHC class II haplotype. The algorithms were based on neural networks and binding affinities of the peptides with MHC class II molecules. And Peptide 83 was identified as I-A^{bc} restricted immunodominant $CD4^{+}$ T cell epitope with the highest score in most of the algorithms. Therefore in-silico and experimental results were in excellent agreement.

Anti – GAA Response

Plasma samples obtained from all the 3 mice were checked for IgG1, IgG2a and IgG2b levels. And it was found that the amount of IgG1 was higher in all the samples followed by minimal amounts of both IgG2a and IgG2b. The presence of IgG1 indicates the existence of a Th2 response in the GAA-/- mice.

The results from the RT- PCR experiments will help in determining the levels of the various cytokines expressed by these cells.

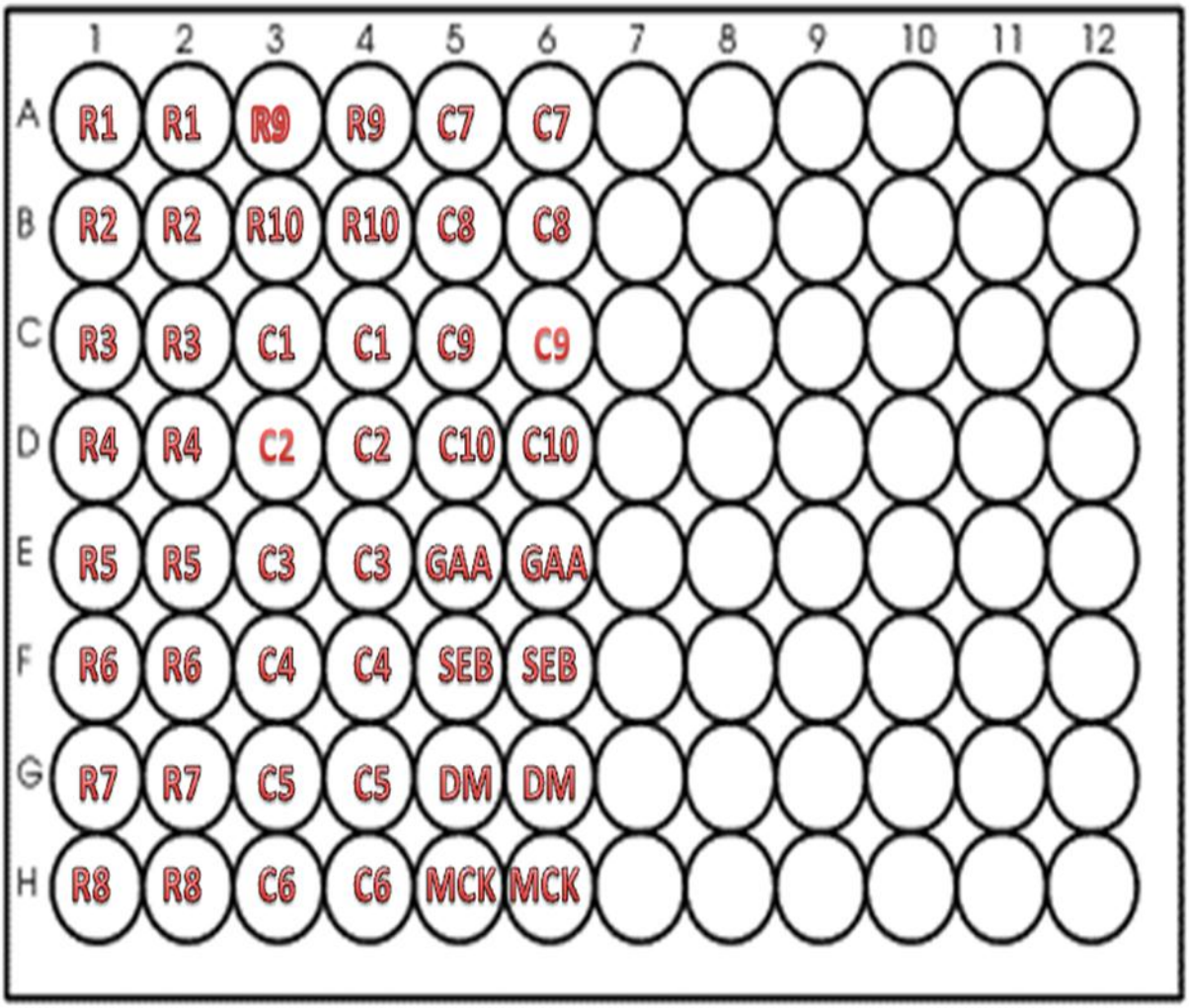

Figure 3-1. Arrangement of 20 peptide pools of 10 peptides /pool (R1- R10 and C1- C10)and controls in IFN-γ ELISpot plate;R1- R10 and C1- C10 = 20 peptide pools of 10 peptides/pool; DM- DMSO, MCK- Mock

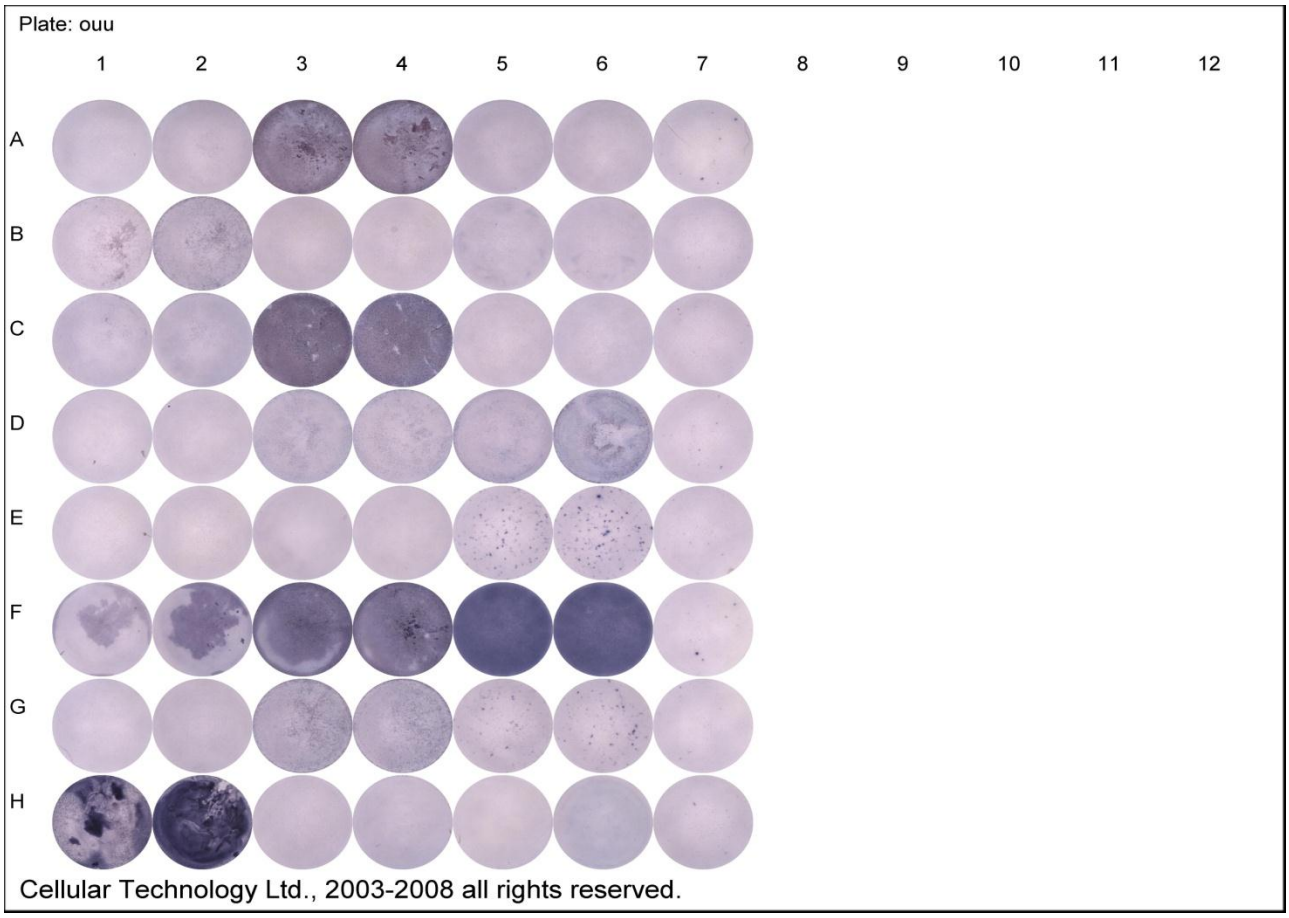

Figure 3-2. IFN-γ ELISpot plate testing 20 pools of peptide containing 10 peptides/pool (from Figure 3-1)

A (1-2) – H (1-2): Pools R1-R8
A (3-4) – B (3-4): Pools R9-R10
C (3-4) - H (3-4): Pools C1-C6
A (5-6) – D (5-6): Pools C7-C10
E (5-6) - GAA; F (5-6) - DMSO;
G (5-6) – SEB; H (5-6) - MOCK CONTROL

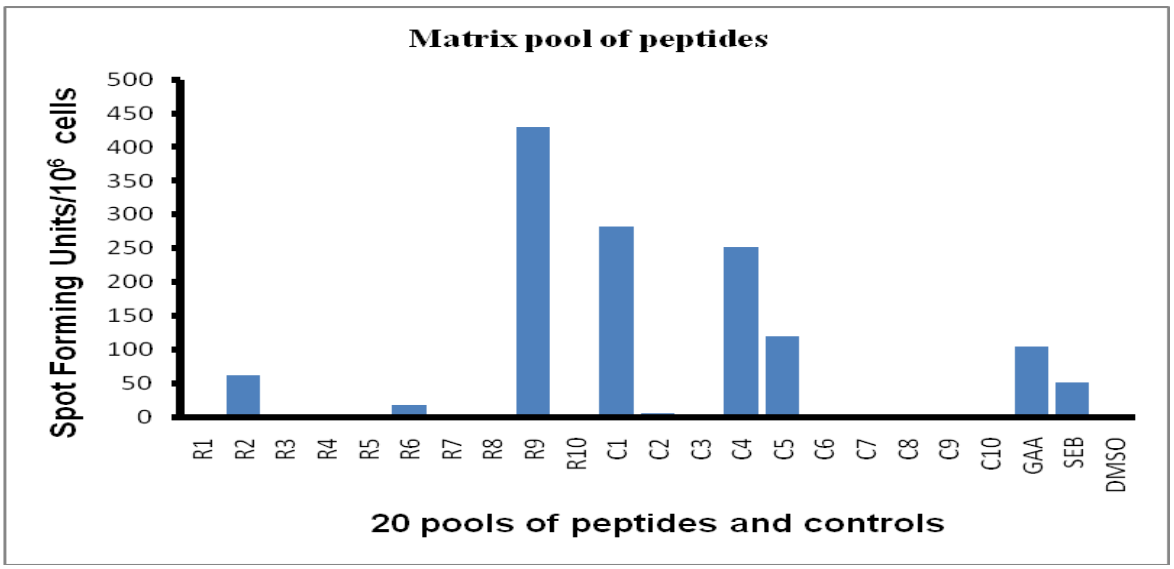

Figure 3-3. Average Count for Spot forming cells from 20 pools of peptide and controls (Quantitation of results from Figure 3-2)

Table 3-1. Average count of Spot forming units from 20 pools of peptides of Figure 3-2

	1	2	3	4	5	6
A	R1		R9		C7	
	1		429		0	
B	R2		R10		C8	
	62		0		1	
C	R3		C1		C9	
	1		270		0	
D	R4		C2		C10	
	3		6		8	
E	R5		C3		GAA	
	1		0		98	
F	R6		C4		SEB	
	33		252		40	
G	R7		C5		DMSO	
	0		122		0	
H	R8		C6			
	0		2			

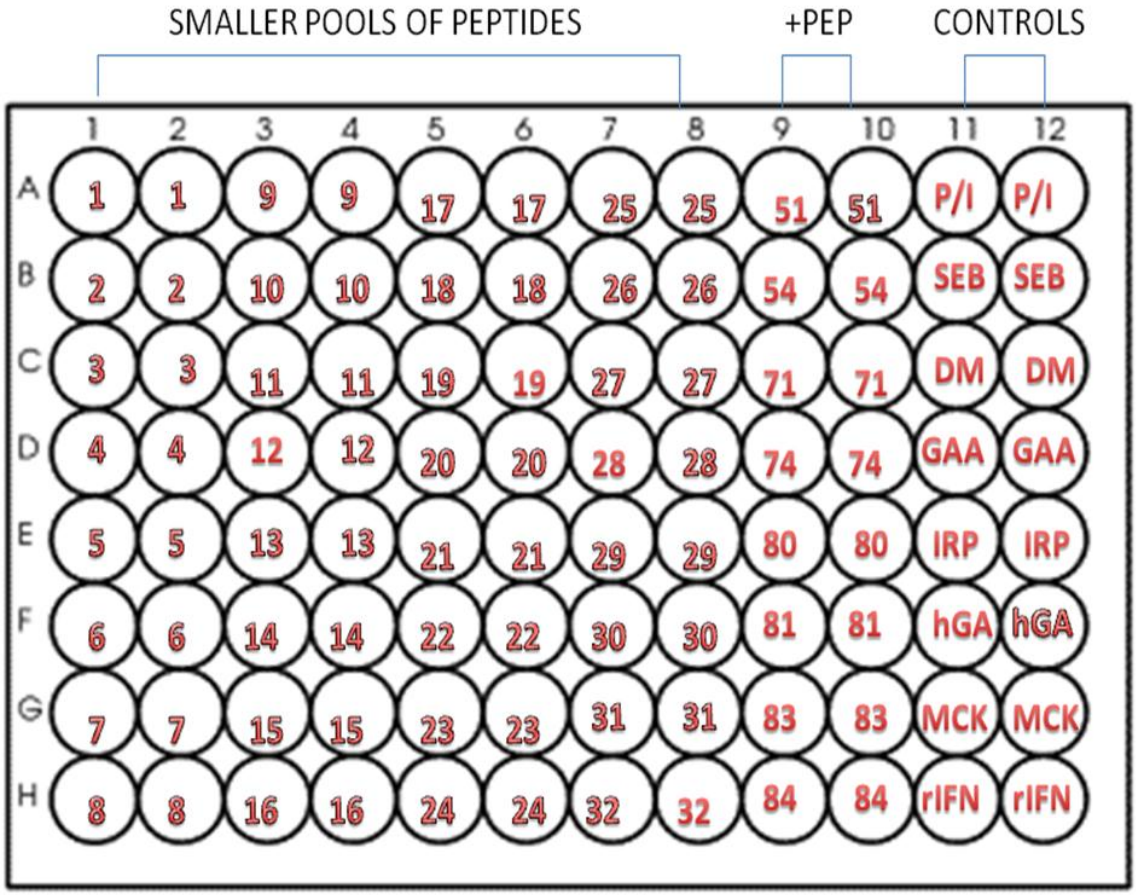

Figure 3-4. Arrangement of 32 peptide pools of 3 peptides/ pool and individual peptides tested positive from Figure 3-2 in IFN-γ ELISpot plate

1-32 (i.e A1- H8) Smaller pools of peptides containing 3 peptides/ pool
51, 54, 71, 74, 80, 81, 83, 84 – Single peptides tested positive from earlier IFN-γ ELISpot with 20 peptide pools (Ref Figure 3-2)
P/I – PMA/Ionomycin
DM- DMSO, IRP- Irrelevant peptide
hGA- Heat inactivated GAA
MCK- Mock, rIFN- Recombinant IFN- γ

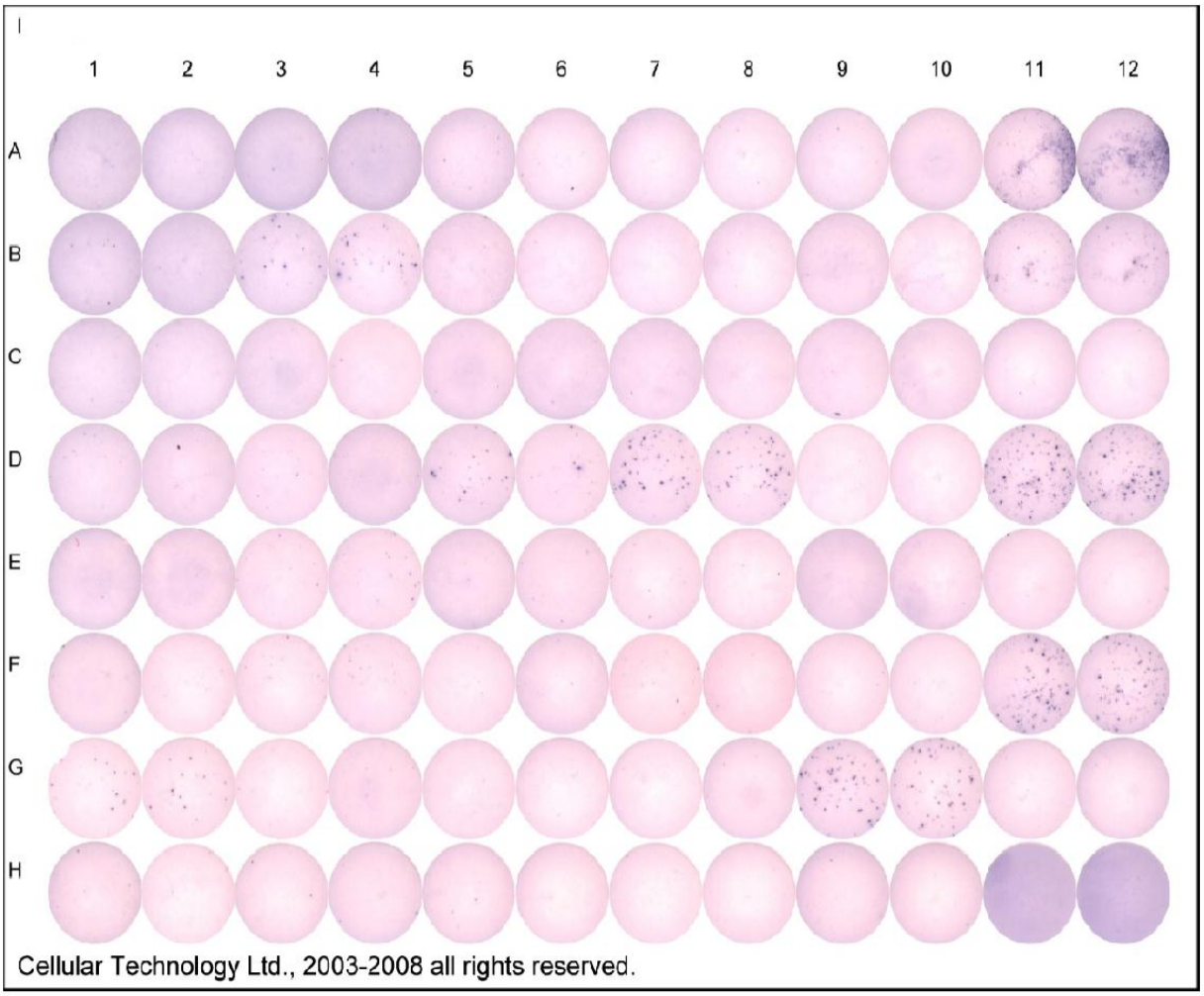

Figure 3-5. IFN-γ ELISpot plate with 32 pools of peptides containing 3 peptides/pool and 8 single peptides tested positive from initial 20 pools of peptides (Figure 3-2) containing 10 peptides each

A(1-2)-H(1-2)- Pools 1-8, A(3-4)-H(3-4)-Pools 9-16, A(5-6)-H(5-6)-Pools17-24
A (7-8)-H (7-8)-Pools 25-32
A (9-10)-H (9-10)-Peptides 51, 54,71,74,80,81,83,84
A (11-12)- PMA/IONO, B(11-12)- SEB, C(11-12)-DMSO,D(11-12)-GAA,E(11-12)-IrrPep,
F (11-12)-hiGAA, G (11-12)-MOCK,H(11-12)-rIFN-γ

Table 3-2. Average cell count from 32 pools of peptides, 8 single peptides and controls (From Figure 3-5)

	1	2	3	4	5	6	7	8	9	10	11	12
A	POOL1		POOL9		POOL17		POOL25		51		PMA/IONO	
	4.5		3		2		0.5		0.5			
B	POOL2		POOL10		POOL18		POOL26		54		SEB	
	3		17.5		1		0		0.0		20.5	
C	POOL3		POOL11		POOL19		POOL27		71		DMSO	
	0.5		0		1		1		1.0		0	
D	POOL4		POOL12		POOL20		POOL28		74		GAA	
	1.5		2		13.5		37		0.5		98	
E	POOL5		POOL13		POOL21		POOL29		80		IRR PEP	
	2		3		1		2.5		0.0		0.5	
F	POOL6		POOL14		POOL22		POOL30		81		hi-GAA	
	2		3.5		0.5		1.5		0.5		70	
G	POOL7		POOL15		POOL23		POOL31		83		MOCK	
	10.5		1		0		0.5		47.0		0.5	
H	POOL8		POOL16		POOL24		POOL32		84		rIFN-g	
	1.5		2		1.5		0		1			

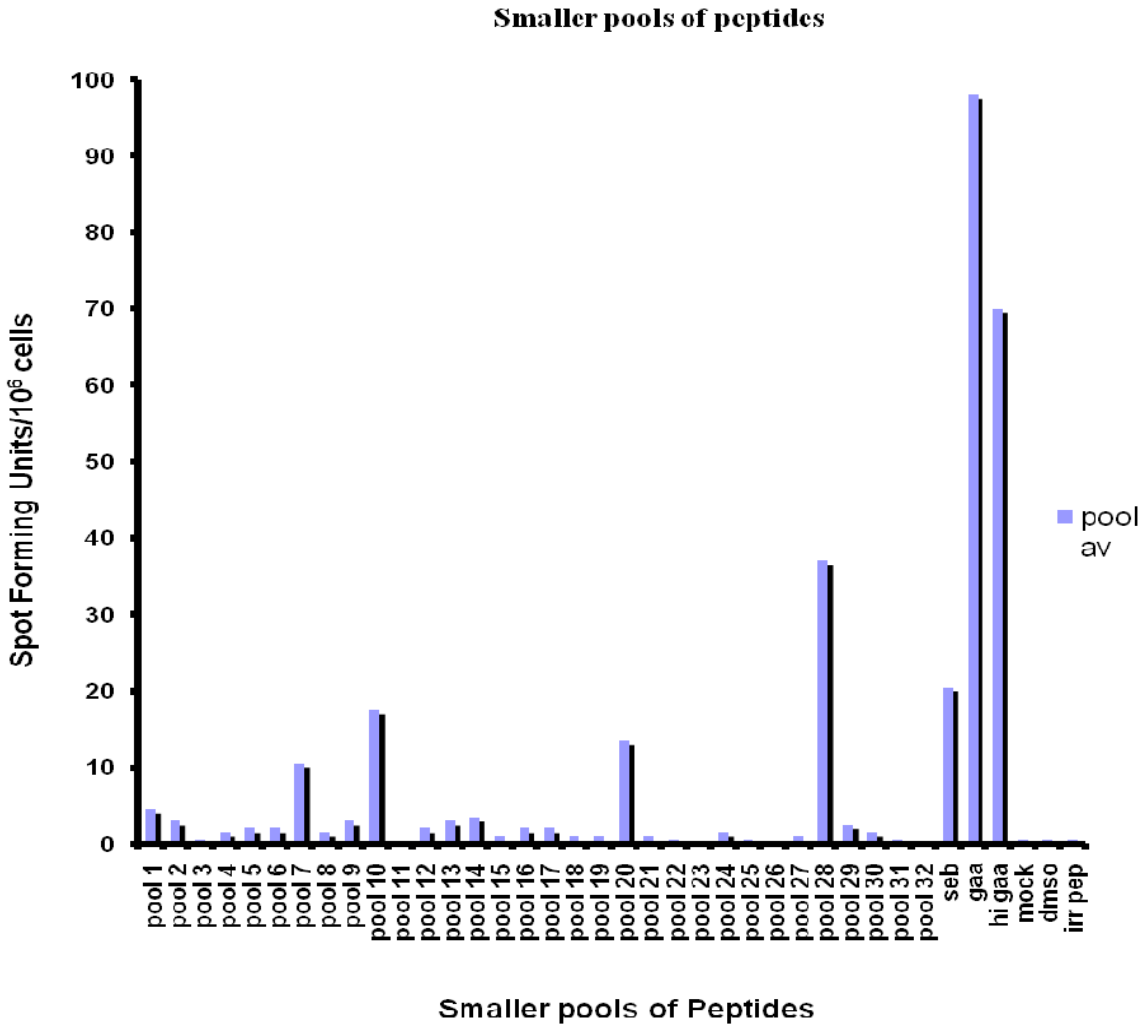

Figure 3-6. Average counts of spot forming cells from 32 pools of peptides and positive and negative controls (Quantitation of results from Figure 3-5)

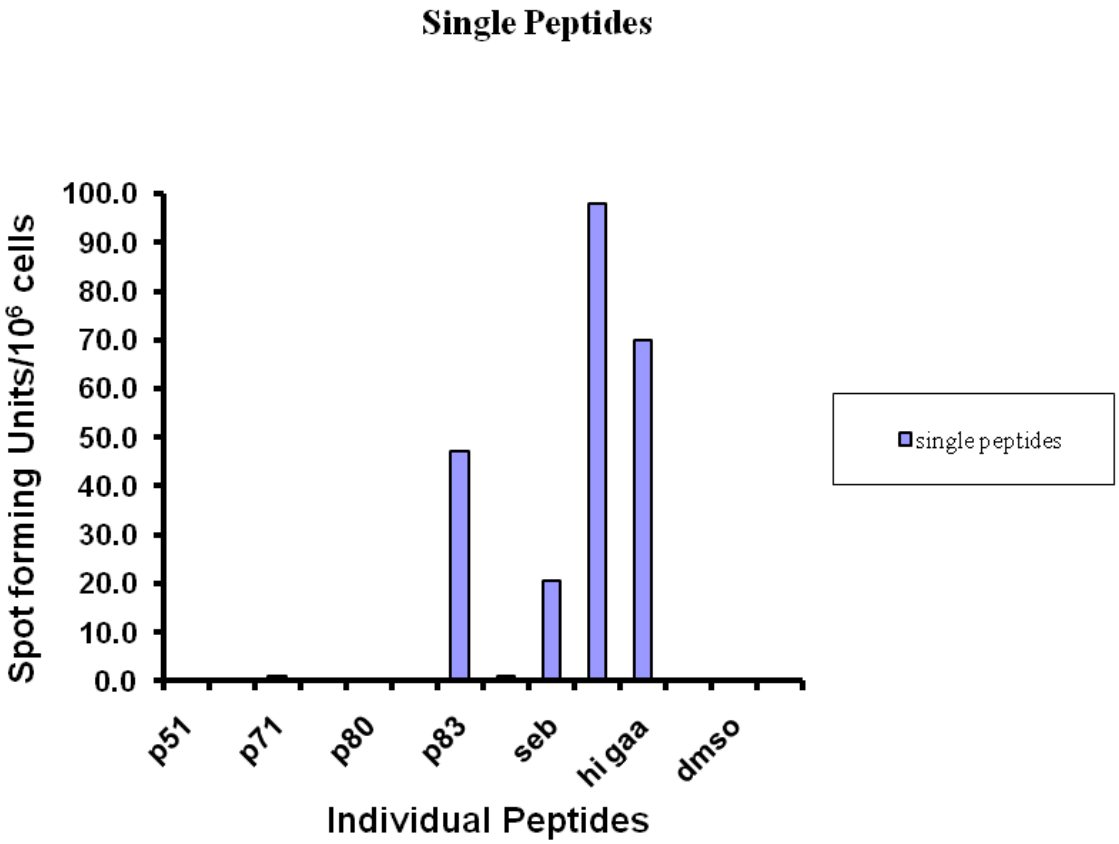

Figure 3-7. Average counts of spot forming cells from single peptides tested positive from 20 pools of peptides and controls (Quantitation of results from Figure 3-5)

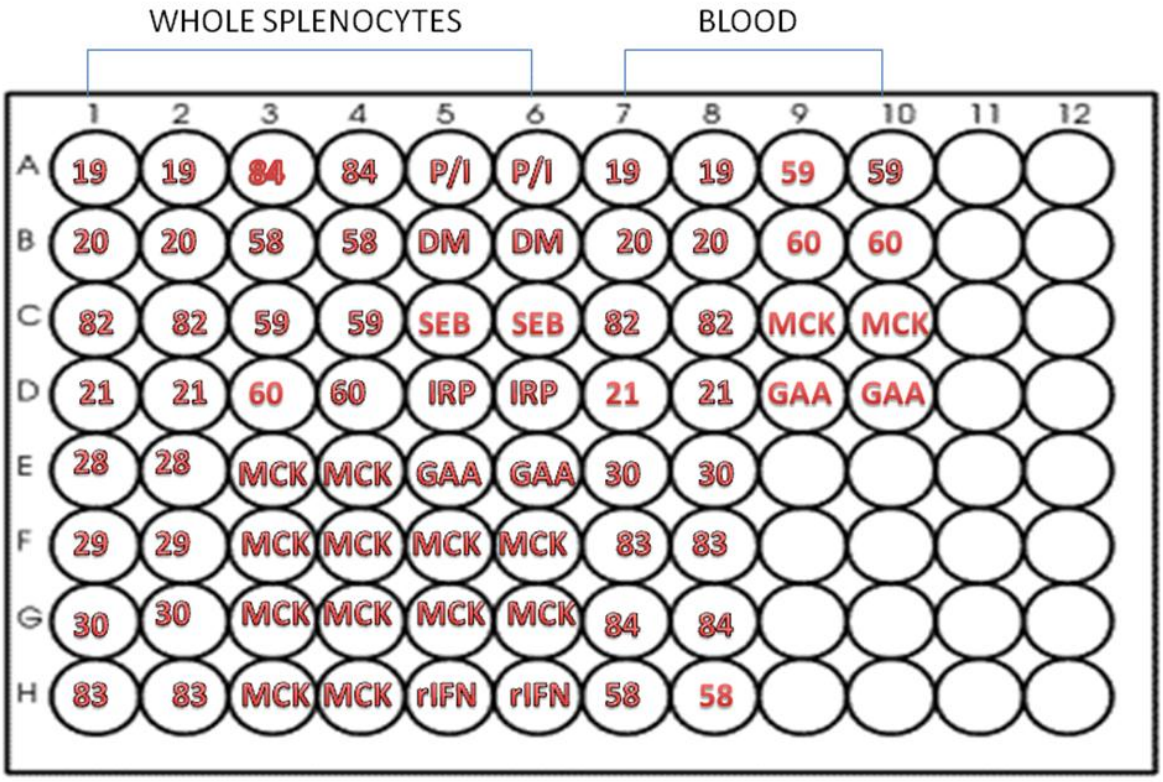

Figure 3-8. Arrangement of controls and individual peptides tested positive from Figure 3-5 in IFN-γ ELISpot plate containing splenocytes and peripheral blood lymphocytes.

19,20,82,21,28,29,30,83,84,58,59,60 – Single peptides tested positive from earlier IFN-γ ELISpot's (Ref Figure 3-5)
P/I – PMA/Ionomycin; DM- DMSO; IRP- Irrelevant peptide;
MCK- Mock; rIFN- Recombinant IFN- γ

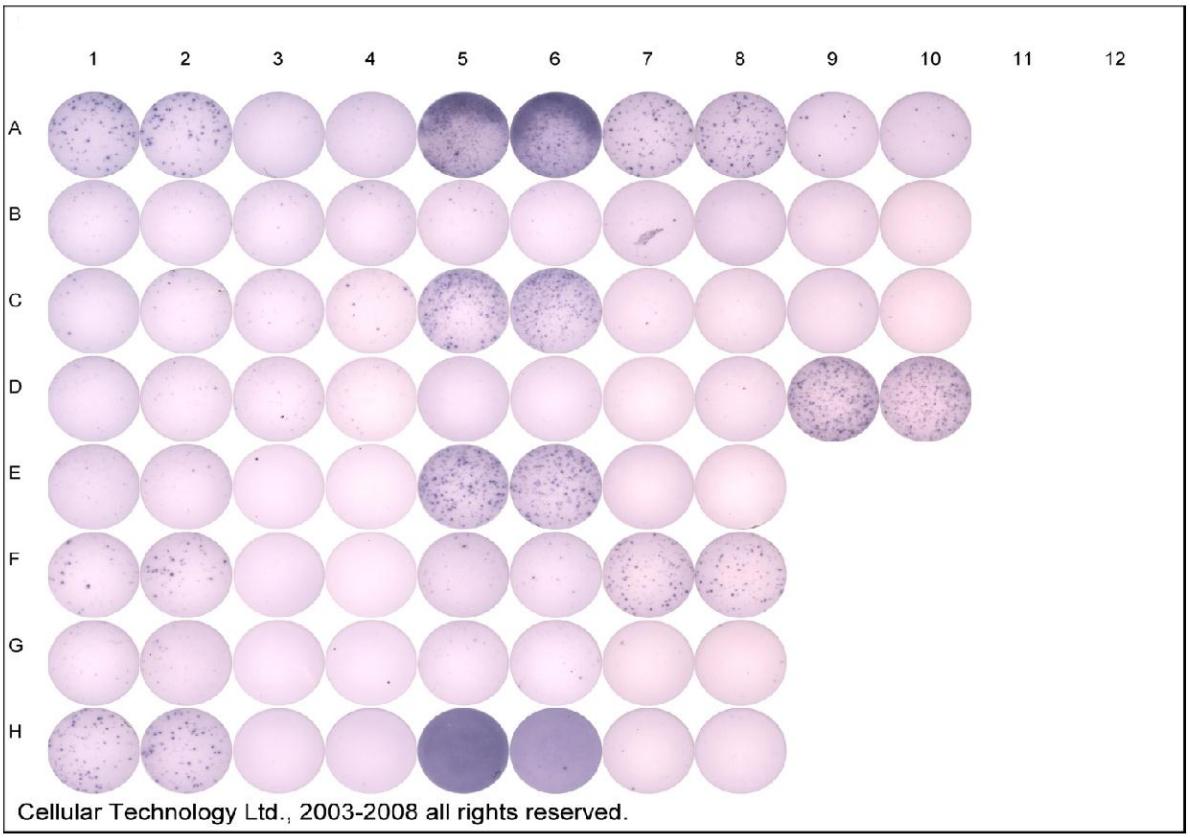

Figure 3-9. ELISpot with individual peptides that had made up the initial positive pools of 32 peptide pools containing 3 peptides/pool (from Figure 3-3)

A1- D4 Splenocytes with individual GAA peptides 19, 20, 82, 21, 28, 29, 30, 83, 84, 58, 59, 60

E3- H4 – Mock control; A5- H6 – Controls;

A7- B10- Peripheral blood lymphocytes with individual GAA peptides 19, 20, 82, 21, 28, 29, 30, 83, 84, 58, 59, 60

C9- C10 – Mock control; D9- D10- rhGAA

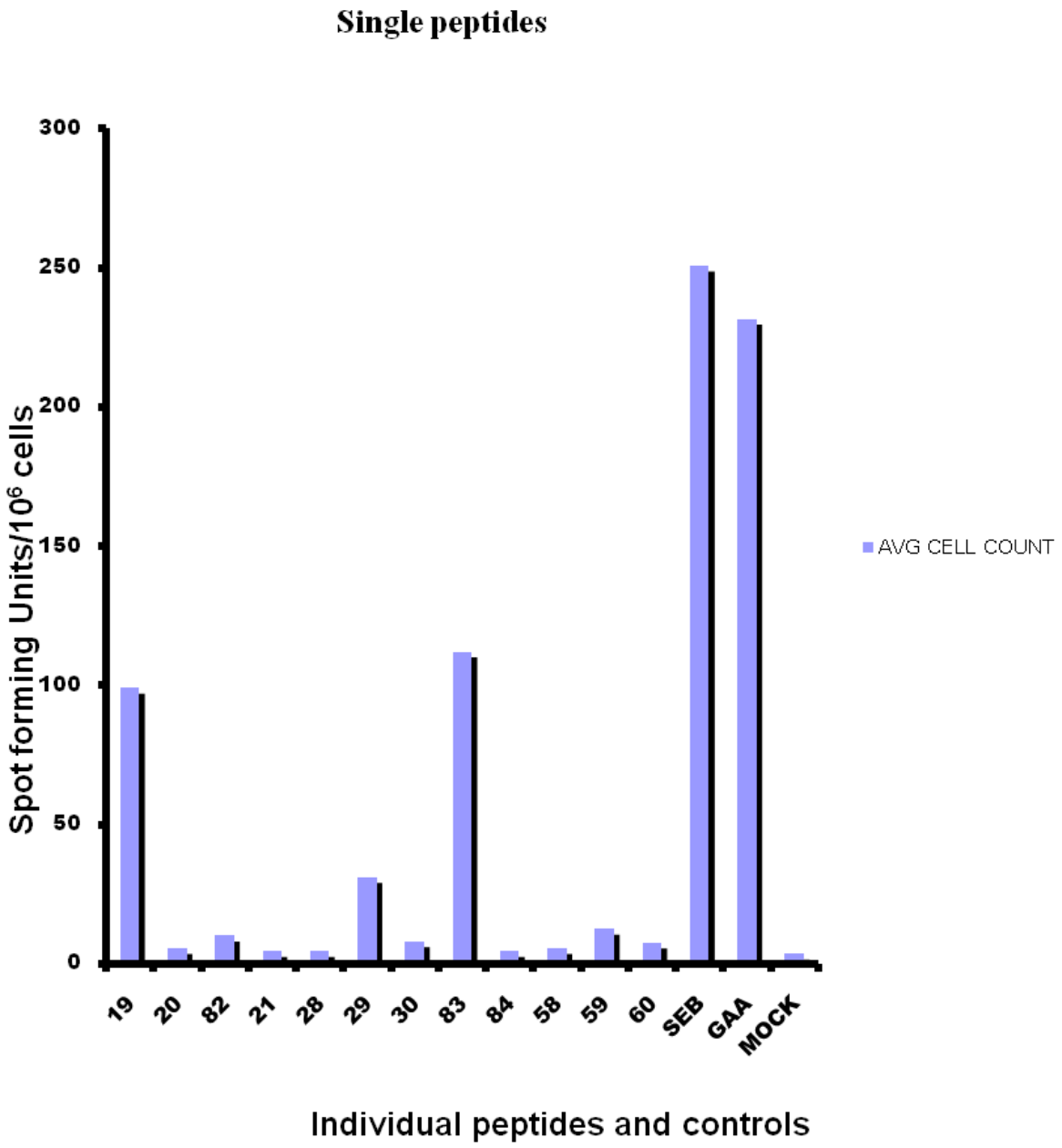

Figure 3-10. Average cell count of spot forming units from individual peptides tested positive from the 32 pools of peptides containing 3 peptides/pool and controls (Quantitation of results from Figure 3-9)

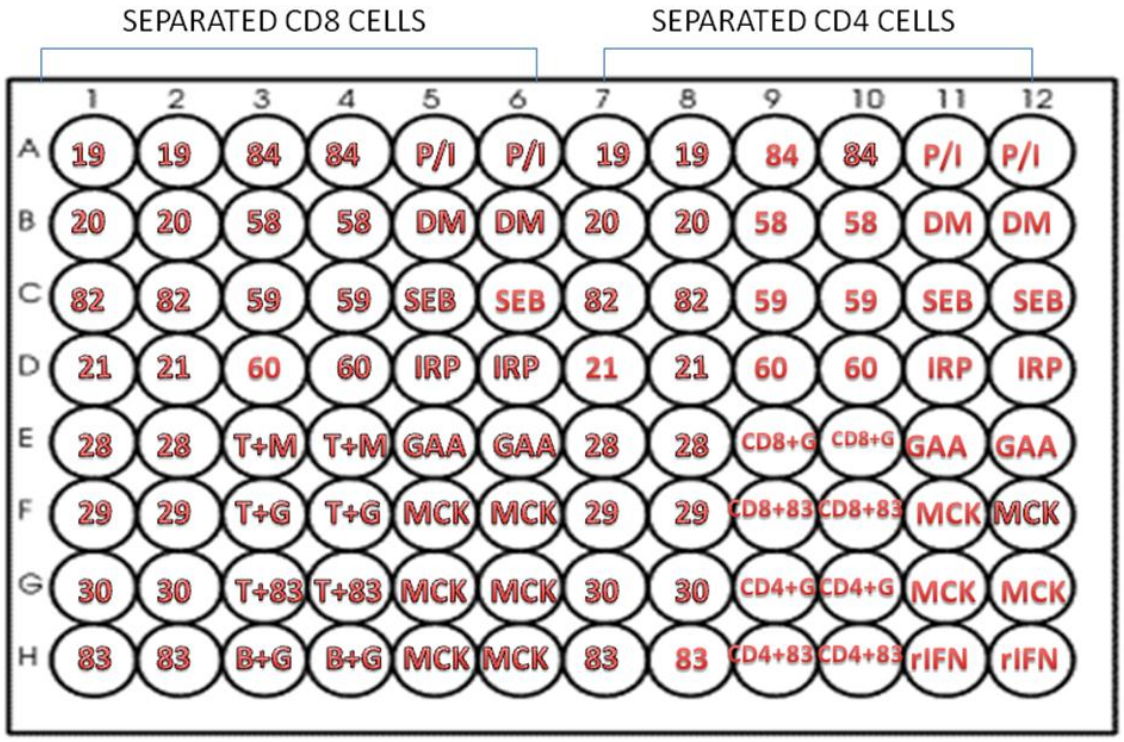

Figure 3-11. Arrangement of controls and individual peptides tested positive from Figure 3.5 in IFN- ELISpot plate containing $CD4^+$, $CD8^+$ T cells and peripheral blood lymphocytes

19,20,82,21,28,29,30,83,84,58,59,60 – Single peptides tested positive from earlier IFN-γ ELISpot's (Refer Figure 3-5)
P/I – PMA/Ionomycin; DM- DMSO; IRP- Irrelevant peptide;
hGA- Heat inactivated GAA;MCK- Mock,
rIFN- Recombinant IFN- γ
T+M = Whole splenocytes and mock control;
T+G = Whole splencoytes and rhGAA protein
T+ 83 = Whole splenocytes and peptide 83;
B+G = Peripheral blood lymphocytes and rhGAA protein;
$CD8^+$ GAA & $CD8^+$ 83 = mix of labelled and unlabelled $CD8^+$ T cell populations with rh GAA protein and peptide 83; CD4+ GAA & CD4+ 83 = mix of labelled and unlabelled CD4+ T cell populations with rh GAA protein and peptide 83

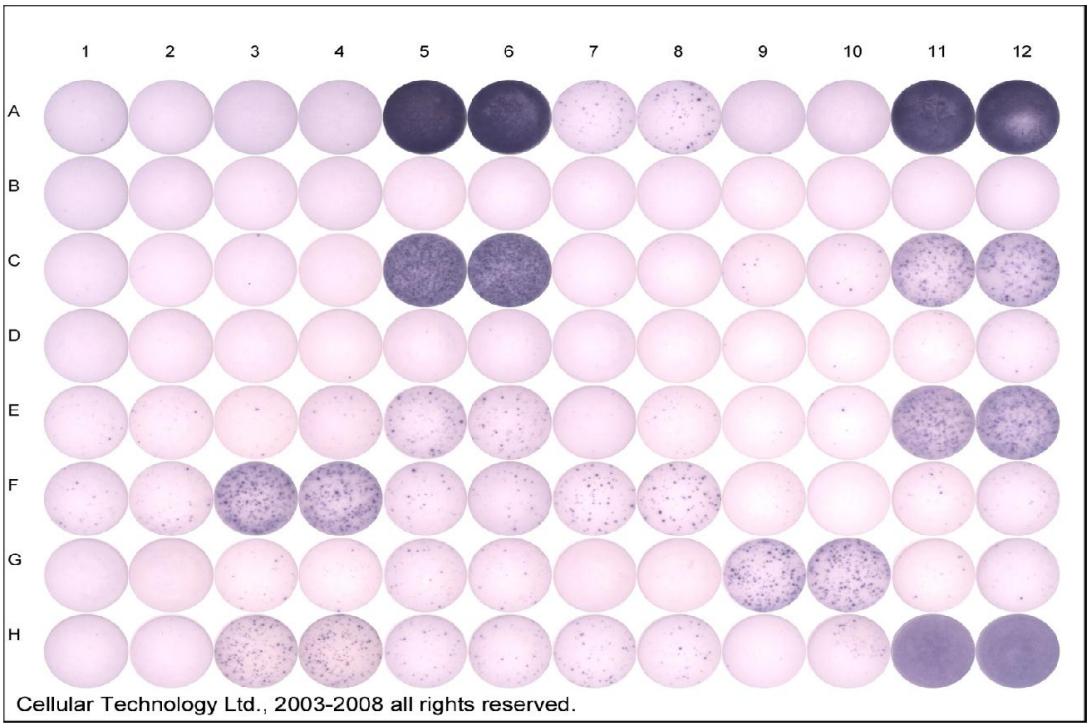

Figure 3-12. IFN-γ ELISpot plate with separated CD4+ and $CD8^+$ T cell populations from rhGAA immunized GAA-/- 129 Sv mouse

A (1-2)-H (1-2) – Peptide Pools 1-8 with 10 peptides/pool,
A (3-4)-H (3-4)-Pools 9-16, A (5-6)-H (5-6)-Pools17-24
A (7-8)-H (7-8)-Pools 25-32
A (9-10)-H (9-10)-Peptides 51, 54,71,74,80,81,83,84
A (11-12) - PMA/IONO, B (11-12) - SEB, C (11-12)-DMSO, D (11-12)-GAA,
E (11-12)-IrrPep,
F (11-12)-hiGAA, G (11-12)-MOCK, H (11-12)-rIFN-γ

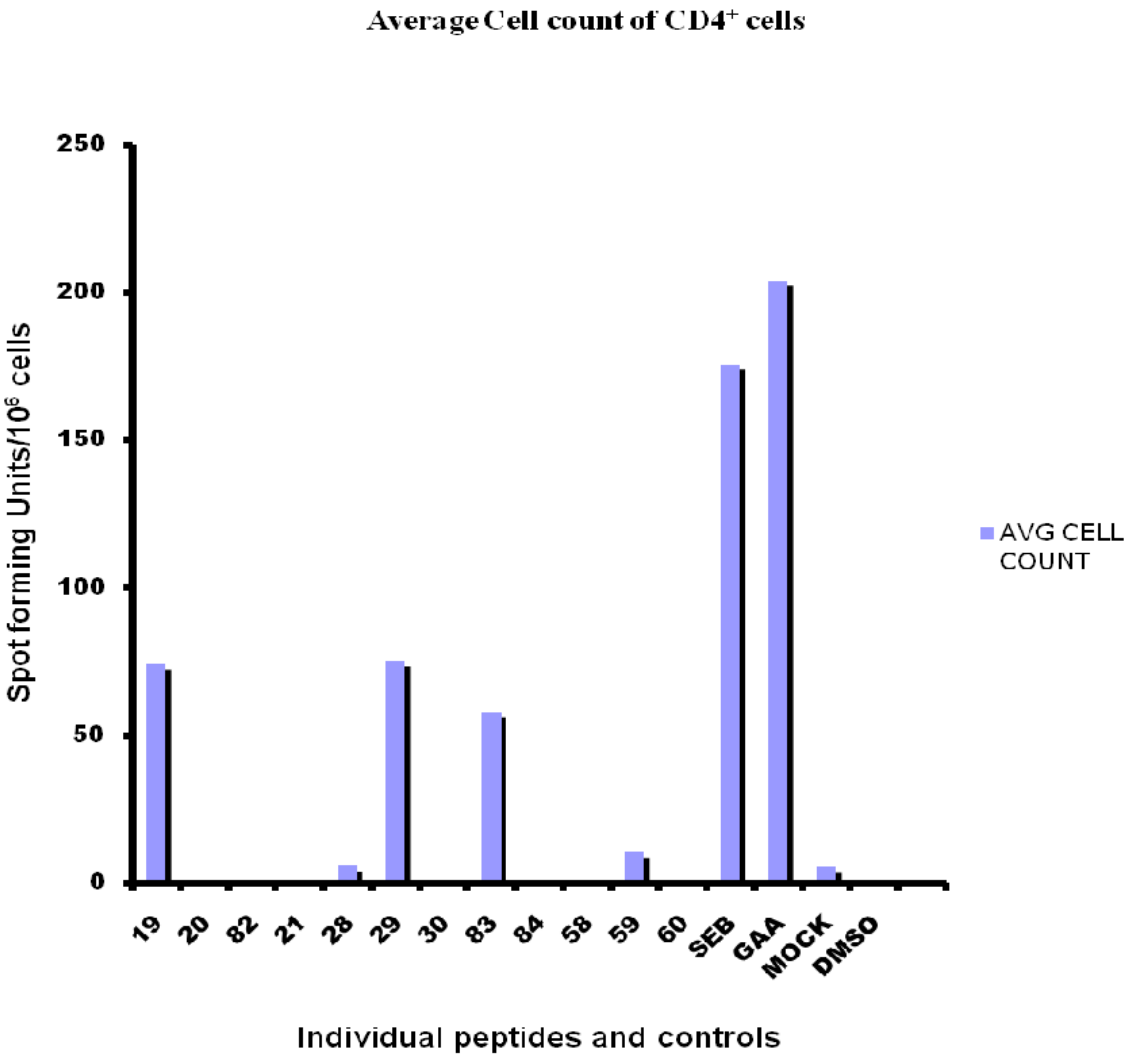

Figure 3-13. Average count of spot forming units/106 cells from CD4+positive T cells of Figure 3.12.

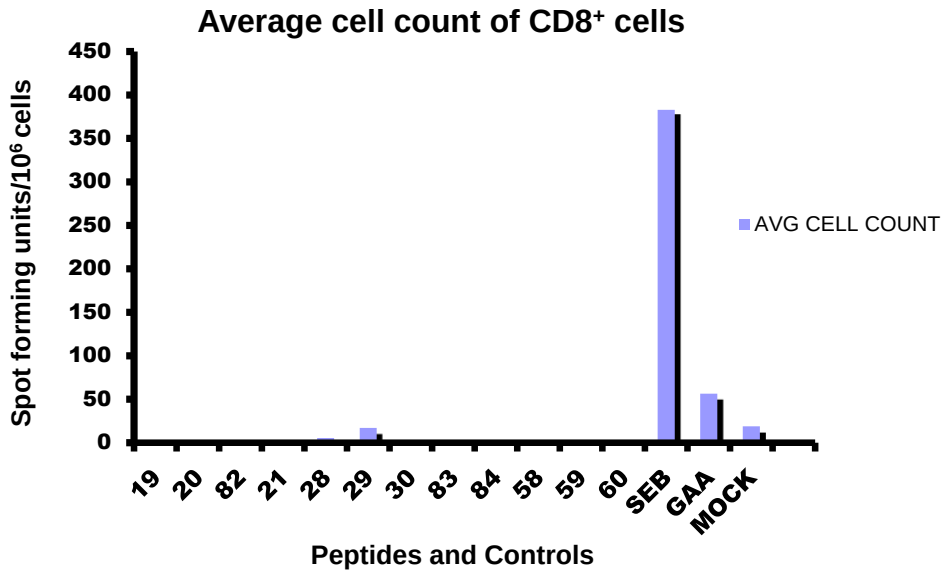

Figure 3-14. Average count of spot forming units from $CD8^+$ T positive cells of Figure 3-8

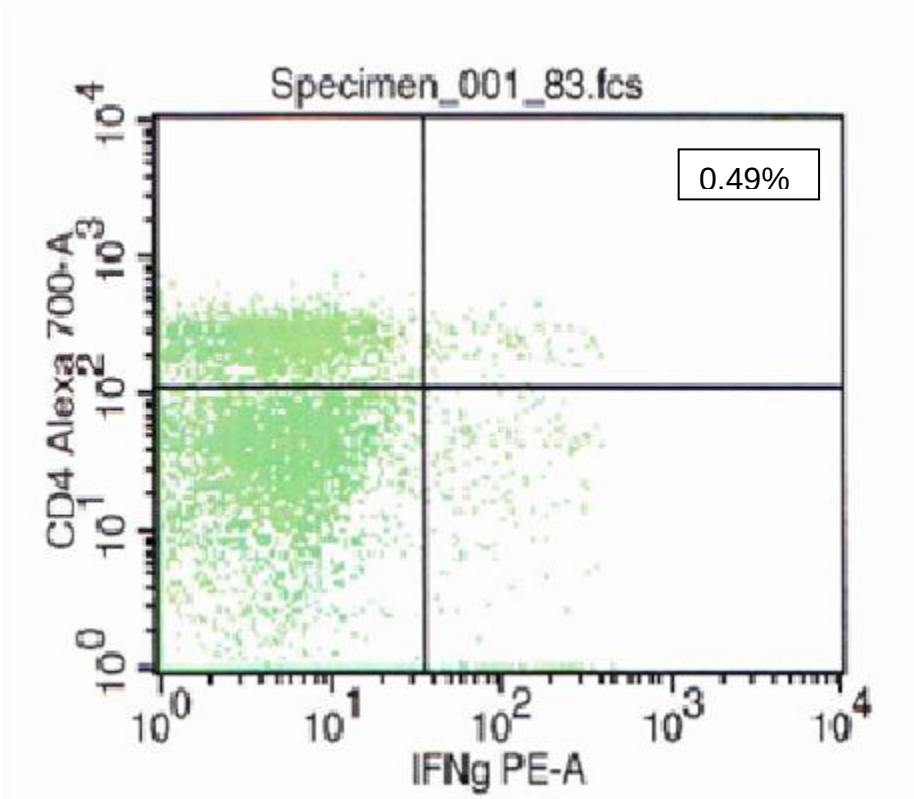

Figure 3-15. IFN-γ^+ $CD4^+$ T cell frequencies in mice using peptide 83

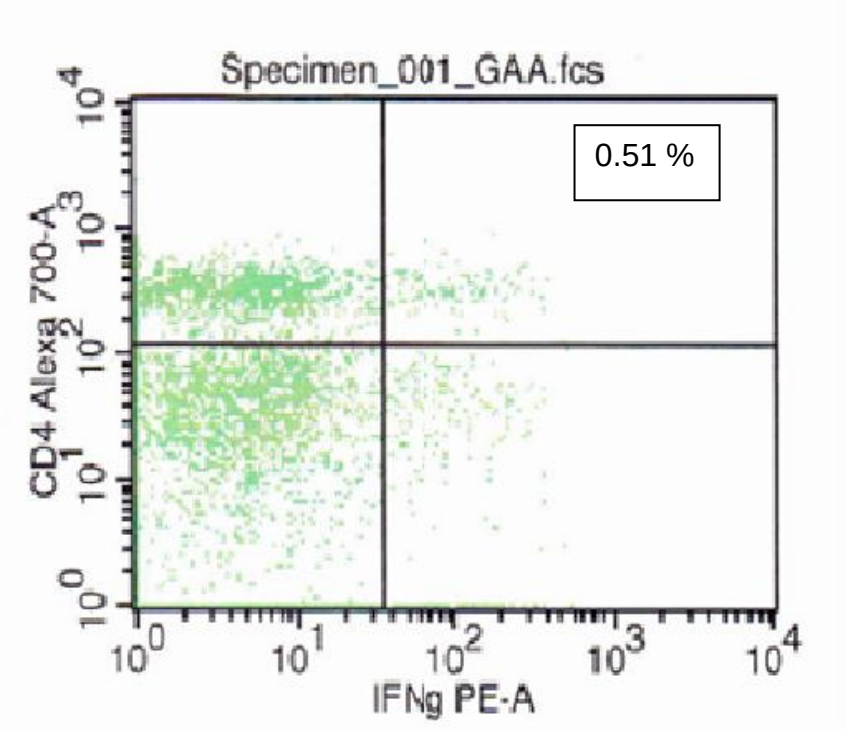

Figure 3-16. IFN-γ^+ $CD4^+$ T cell frequencies in mice with whole protein rhGAA

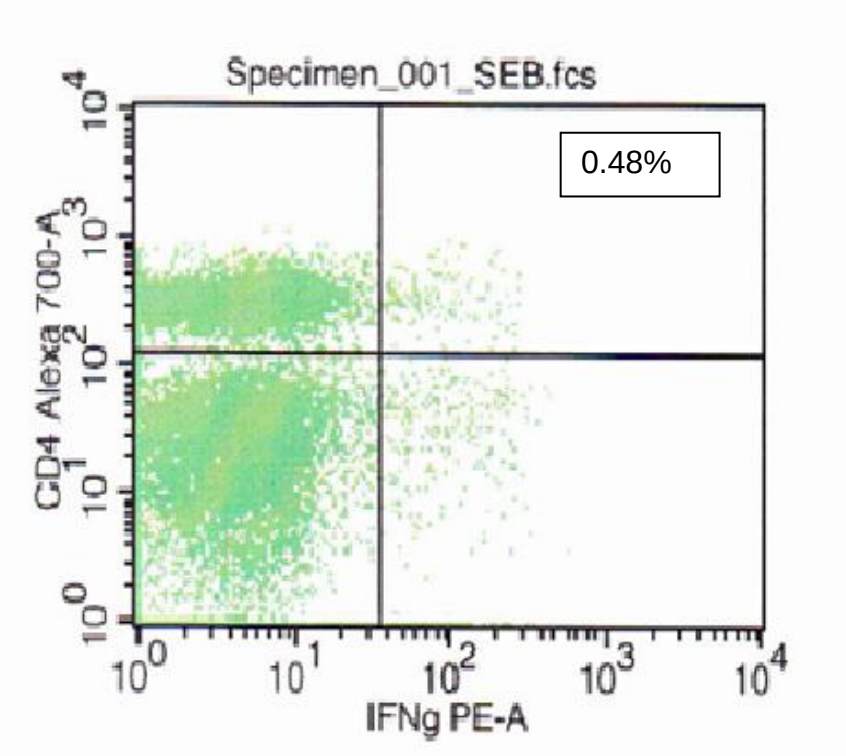

Figure 3-17. IFN-γ^+ $CD4^+$ T cell frequencies in GAA-/- mice with SEB

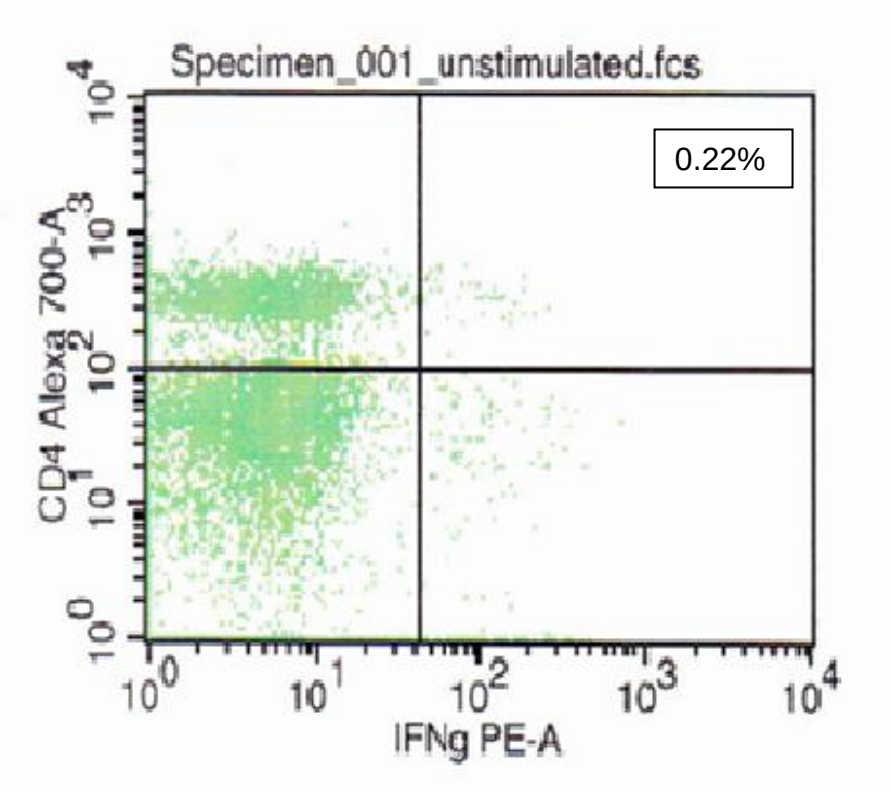

Figure 3-18. IFN-γ^+ $CD4^+$ T cell frequencies in GAA-/- mice using mock-stimulated samples

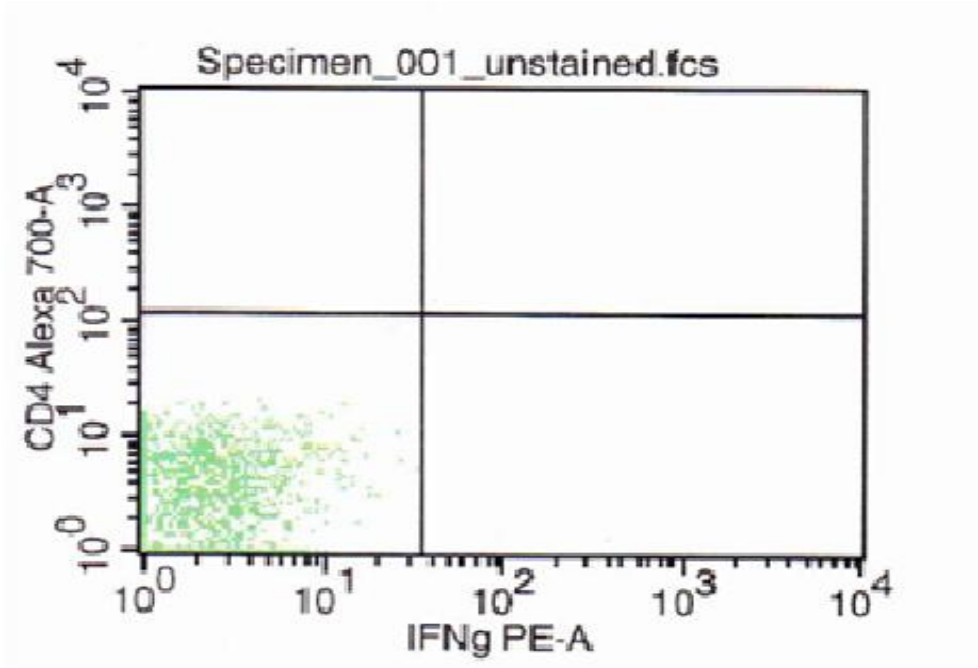

Figure 3-19. IFN-γ^+ $CD4^+$ T cell frequencies in GAA-/- mice using unstained control

Table 3-3. Immune epitope database – T cell epitope prediction tools results
Lowest score = Highest affinity

Allele	Position	Sequence	Consensus Percentile Rank	SMM_align Core	SMM_align Score	SMM_align Percentile Rank	NN_align Core	NN_align Score	NN_align Percentile Rank
H2-IAb	1:196-210	ETPHVHSRAPSPLYS	7.74	VHSRAPSPL	479.0	4.96	SRAPSPLYS	837.7	10.52
H2-IAb	1:197-211	TPHVHSRAPSPLYSV	6.12	VHSRAPSPL	406.0	4.19	SRAPSPLYS	584.4	8.05
H2-IAb	1:198-212	PHVHSRAPSPLYSVE	6.55	VHSRAPSPL	455.0	4.7	SRAPSPLYS	617.7	8.41
H2-IAb	1:316-330	NAMDVVLQPSPALSW	2.71	VVLQPSPAL	215.0	2.15	VLQPSPALS	200.8	3.26
H2-IAb	1:317-331	AMDVVLQPSPALSWR	2.45	VVLQPSPAL	214.0	2.14	VLQPSPALS	168.7	2.75
H2-IAb	1:318-332	MDVVLQPSPALSWRS	2.39	VVLQPSPAL	255.0	2.58	VLQPSPALS	137.1	2.2
H2-IAb	1:336-350	ILDVYIFLGPEPKSV	0.56	YIFLGPEPK	62.0	0.39	FLGPEPKSV	55.5	0.73
H2-IAb	1:337-351	LDVYIFLGPEPKSVV	0.45	YIFLGPEPK	59.0	0.36	FLGPEPKSV	44.5	0.53
H2-IAb	1:338-352	DVYIFLGPEPKSVVQ	0.42	YIFLGPEPK	60.0	0.37	FLGPEPKSV	40.8	0.46

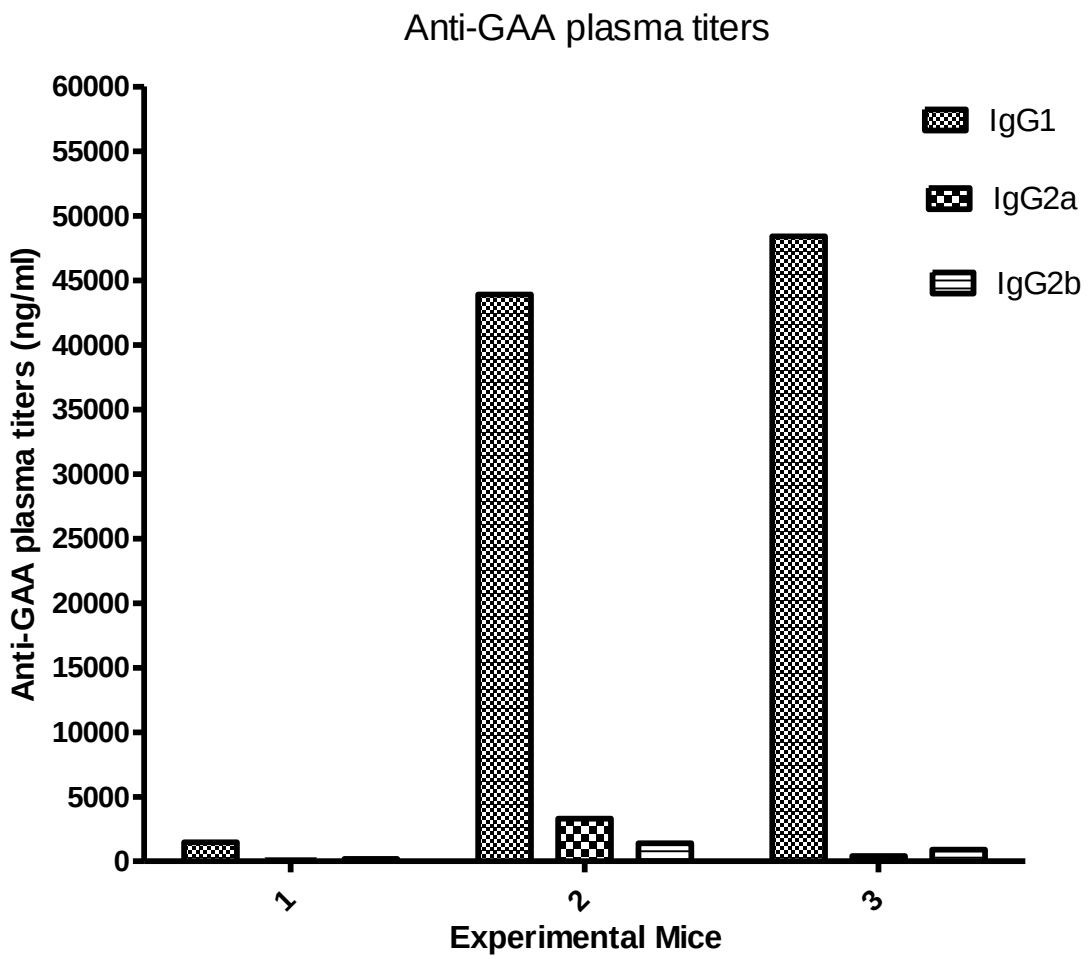

Figure 3-20. Anti- GAA plasma titer levels from GAA-/- mice immunized with rhGAA

CHAPTER 4
DISCUSSION

Immunodominant epitopes are essential for mounting effective immune responses in the body. In the past decade, more attention has been given towards mapping and characterization of T cell epitopes from protein antigens. $CD4^+$ T cells have been garnering additional importance due to their significant roles in the development and functioning of both the cell mediated and humoral reponses of the adaptive immune system. (19)

Although significant improvements have been made in the field of epitope mapping, researchers have been concentrating more towards the humoral immune response of the patients in Pompe. (2)As the functioning and differentiation of B cells depends on the help of $CD4^+$ T cells, determining the $CD4^+$ immunodominant epitope against the rhGAA protein in GAA-/- mice will significantly improve the understanding of the relationship between T and B cell activation and also help in uncovering the peptide and MHC binding motifs for this disorder.

We chose a combination of methods such as ELISpot, in-vitro cytokine staining and in-silico prediction algorithms, for mapping the immundominant $CD4^+$ epitope against the rhGAA protein in 6-8 weeks old GAA-/- (GAA knockout) 129Sv mice. These mice had been created by others through targeted disruption in exon 6 using a neomycin resistance gene ($6^{neo}/6^{neo}$), (20)and their MHC class II haplotypes were identified as $I\text{-}A^{bc}$. (21, 22) Consequently, the immunodominant epitopes mapped using the above techniques were specific for this haplotype.

Of all the 95 peptides spanning the rhGAA protein that were tested by building simple and matrix pools of peptides, peptide 19, 29 and 83 were identified as

candidates for dominant epitopes. Amongst those, peptide 83 emerged as containing the dominant epitopes in all the 3 methods of mapping, clearly showing the prevalence for this portion of the protein during the antigen presentation to $CD4^+$ T cells in the GAA-/- 129 Sv murine model of severe pompe disease.

As the immune responses against the rhGAA protein have in preliminary studies been pivoted on the increased levels of IgG1 antibody, which in other words signifies a Th2 type cellular response, (16)we sought to study the different gene expression levels of Th2 and Th1 cytokines in pompe mice that received ERT similar to humans. The results obtained from running an RT-PCR on cDNA samples from rhGAA immunized GAA-/- mice will help in exploring in detail about the T cell mediated responses for this lysosomal storage disorder. Others have reported formation of IgE and fatal anaphylactic reactions in pompe mice during ERT studies. We hypothesize that the combination of any subclass mapping and the cytokine response from $CD4^+$ T cells to GAA will allow us to propose a mechanism that drives the immune response in ERT.

Identifying the helper pathway chosen by $CD4^+$ effector cells will aid in analyzing the different classes of antibodies which can be secreted by these antigen specific cells and a better understanding of the B cell mediated immunity. The epitope can serve as a tool for designing and developing immunomodulatory and immunotherapeutic protocols for this disorder in the future. Use of this epitope will accelerate subsequent mechanistic studies. For example peptide antigens can be used for stimulation in ICS techniques. These peptides can also be used for inducing tolerance against the protein and for improving their activities in enzyme replacement therapies.

The results from this mapping can be utilized for tolerance studies similar to the ones which were previously done in Dr. Herzog's lab such as the Induction of nasal tolerance against FIX, (13)and for Induction of tolerance to rhGAA using combinations of the immunosuppressant drug rapamycin. (14)

While we may not have a prior knowledge of dominant $CD4^+$ T cell epitopes in pompe patients, our approach should be useful to map dominant epitopes in humans with immune responses against the protein. Peptides encoding known epitopes could then be used in immune tolerance protocols. This is of particular importance for situations where use of protein antigen causes anaphylactic reactions.

The future is encouraging for this field with the developments occurring in the areas of enzyme replacement and gene therapy. (4, 9, 11)The inhibitory antibodies which are hindering the effectiveness of these therapies can be effectively countered by using protocols implementing these immunodominant epitopes.

Thus the mapping of dominant CD4 T cell epitopes for the deficient enzyme of lysosomal storage disorder in GAA-/- 129 Sv mice using in-vitro T cell assays will help in opening more avenues for research in this field and eventually help in unlocking several answers related to T and B cell mediated immunities of the system.

APPENDIX A
GAA PEPTIDE LIBRARY

locatio	peptide name	sequence	c-term	M+H	Mass found	purity
A1	I-1	MGVRHPPCSHRL	NH2	1389.7	1388.8	70%
A2	I-2	RLLAVCALVSLA	NH2	1228.6	1228.9	n/a
A3	I-3	LATAALLGHILL	NH2	1205.5	1205.6	70%
A4	I-4	LLHDFLLVPREL	NH2	1464.8	1464	70%
A5	I-5	ELSGSSPVLEET	NH2	1247.3	1244(M-H)	70%
A6	I-6	ETHPAHQQGASR	NH2	1318.4	1318.1	70%
A7	I-7	SRPGPRDAQAHP	NH2	1288.4	1289	70%
A8	I-8	HPGRPRAVPTQC	NH2	1318.5	1319.5	70%
A9	I-9	QCDVPPNSRFDC	NH2	1380.5	1381.4	n/a
A10	I-10	DCAPDKAITQEQ	NH2	1318.4	1319	70%
A11	I-11	EQCEARGCCYIP	NH2	1371.6	1372.5	70%
A12	I-12	IPAKQGLQGAQM	NH2	1241.5	1241.2	70%
B1	I-13	QMGQPWCFFPPS	NH2	1424.7	1425.4	n/a
B2	I-14	PSYPSYKLENLS	NH2	1397.5	1396.3	70%
B3	I-15	LSSSEMGYTATL	NH2	1259.4	1256.9(M-H)	70%
B4	I-16	TLTRTTPTFFPK	NH2	1409.6	1409.7	70%
B5	I-17	PKDILTLRLDVM	NH2	1413.7	1413.4	70%
B6	I-18	VMMETENRLHFT	NH2	1507.8	1508.7	70%
B7	I-19	FTIKDPANRRYE	NH2	1509.7	1509.4	70%
B8	I-20	YEVPLETPRVHS	NH2	1426.6	1423.6(M-H)	70%
B9	I-21	HSRAPSPLYSVE	NH2	1342.5	1342.1	70%
B10	I-22	VEFSEEPFGVIV	NH2	1351.5	1350.7	70%
B11	I-23	IVHRQLDGRVLL	NH2	1418.7	1417.7	70%
B12	I-24	LLNTTVAPLFFA	NH2	1306.6	1306.5	70%
C1	I-25	FADQFLQLSTSL	NH2	1369.5	1370	n/a
C2	I-26	SLPSQYITGLAE	NH2	1278.4	1279.4	70%
C3	I-27	AEHLSPLMLSTS	NH2	1285.5	1285.2	70%
C4	I-28	TSWTRITLWNRD	NH2	1548.7	1549.2	n/a
C5	I-29	RDLAPTPGANLY	NH2	1287.4	1286.7	70%
C6	I-30	LYGSHPFYLALE	NH2	1409.6	1410	70%
C7	I-31	LEDGGSAHGVFL	NH2	1201.3	1199.6(M-H)	70%
C8	I-32	FLLNSNAMDVVL	NH2	1335.6	1333.6(M-H)	n/a
C9	I-33	VLQPSPALSWRS	NH2	1340.5	1340	70%
C10	I-34	RSTGGILDVYIF	NH2	1340.5	1341.2	70%
C11	I-35	IFLGPEPKSVVQ	NH2	1313.6	1313.3	70%
C12	I-36	VQQYLDVVGYPF	NH2	1427.6	1427	70%
D1	I-37	PFMPPYWGLGFH	NH2	1448.7	1448	70%
D2	I-38	FHLCRWGYSSTA	NH2	1427.6	1427.4	70%
D3	I-39	TAITRQVVENMT	NH2	1362.6	1362.5	70%

D4	I-40	MTRAHFPLDVQW	NH2	1500.7	1500.6	70%
D5	I-41	QWNDLDYMDSRR	NH2	1598.7	1599.6	70%
D6	I-42	RRDFTFNKDGFR	NH2	1558.7	1558.6	70%
D7	I-43	FRDFPAMVQELH	NH2	1489.7	1489.7	70%
D8	I-44	LHQGGRRYMMIV	NH2	1460.8	1461	70%
D9	I-45	IVDPAISSSGPA	NH2	1113.2	1113.1	70%
D10	I-46	PAGSYRPYDEGL	NH2	1324.4	1324.1	70%
D11	I-47	GLRRGVFITNET	NH2	1362.5	1362	70%
D12	I-48	ETGQPLIGKVWP	NH2	1324.5	1324.7	70%
E1	I-49	WPGSTAFPDFTN	NH2	1339.4	1339.6	70%
E2	I-50	TNPTALAWWEDM	NH2	1434.6	1435.5	70%
E3	I-51	DMVAEFHDQVPF	NH2	1434.6	1435	70%
E4	I-52	PFDGMWIDMNEP	NH2	1451.6	1451.2	70%
E5	I-53	EPSNFIRGSEDG	NH2	1307.3	1307.4	70%
E6	I-54	DGCPNNELEN	NH2	1104.1	1104.9	70%
E7	II-1	PPYVPGVVGGTL	NH2	1155.4	1154.9	70%
E8	II-2	TLQAATICASSH	NH2	1202.3	1203	70%
E9	II-3	SHQFLSTHYNLH	NH2	1483.6	1484.7	70%
E10	II-4	LHNLYGLTEAIA	NH2	1314.5	1315.1	70%
E11	II-5	TASHRALVKARG	NH2	1278.5	1279.6	70%
E12	II-6	RGTRPFVISRST	NH2	1376.6	1376	70%
F1	II-7	STFAGHGRYAGH	NH2	1260.3	1260.1	70%
F2	II-8	GHWTGDVWSSWE	NH2	1446.5	1447	70%
F3	II-9	WEQLASSVPEIL	NH2	1371.5	1372.1	70%
F4	II-10	ILQFNLLGVPLV	NH2	1325.7	1349.8(M+Na)	70%
F5	II-11	LVGADVCGFLGN	NH2	1164.3	1163.5	70%
F6	II-12	GNTSEELCVRWT	NH2	1394.5	1394.1	70%
F7	II-13	WTQLGAFYPFMR	NH2	1516.8	1516	70%
F8	II-14	MRNHNSLLSLPQ	NH2	1409.6	1408.9	70%
F9	II-15	PQEPYSFSEPAQ	NH2	1379.4	1379.3	70%
F10	II-16	AQQAMRKALTLR	NH2	1386.7	1386.4	70%
F11	II-17	LRYALLPHLYTL	NH2	1472.8	1471.8	70%
F12	II-18	TLFHQAHVAGET	NH2	1310.4	1309.5	70%
G1	II-19	ETVARPLFLEFP	NH2	1418.7	1420	70%
G2	II-20	FPKDSSTWTVDH	NH2	1419.5	1418.9	70%
G3	II-21	DHQLLWGEALLI	NH2	1407.6	1404.7(M-H)	70%
G4	II-22	LITPVLQAGKAE	NH2	1239.5	1238.6	70%
G5	II-23	AEVTGYFPLGTW	NH2	1340.5	1341	n/a
G6	II-24	TWYDLQTVPIEA	NH2	1435.6	1433.5(M-H)	70%
G7	II-25	EALGSLPPPPAA	NH2	1119.3	1120	70%

G8	II-26	AAPREPAIHSEG	NH2	1234.3	1234.8	70%
G9	II-27	EGQWVTLPAPLD	NH2	1325.5	1326.3	70%
G10	II-28	LDTINVHLRAGY	NH2	1371.6	1371	70%
G11	II-29	GYIIPLQGPGLT	NH2	1228.4	1228.5	70%
G12	II-30	LTTTESRQQP	NH2	1160.2	1159.7	70%
H1	III-1	MALAVALTKGGE	NH2	1160.4	1159.7	n/a
H2	III-2	GEARGELFWDDG	NH2	1351.4	1351.5	70%
H3	III-3	DGESLEVLERGA	NH2	1274.3	1274.2	n/a
H4	III-4	GAYTQVIFLARN	NH2	1352.6	1352.5	70%
H5	III-5	RNNTIVNELVRV	NH2	1426.6	1425.6	70%
H6	III-6	RVTSEGAGLQLQ	NH2	1258.4	1258.8	70%
H7	III-7	LQKVTVLGVATA	NH2	1199.5	1200	n/a
H8	III-8	TAPQQVLSNGVP	NH2	1210.3	1209.6	70%
H9	III-9	VPVSNFTYSPDT	NH2	1326.4	1325.7	70%
H10	III-10	DTKVLDICVSLL	NH2	1318.6	1318.2	n/a
H11	III-11	LLMGEQFLVSWC	NH2	1425.7	1426.3	n/a

APPENDIX B
T CELL EPITOPE PREDICTION RESULTS

NetMHCII 2.2 Server - prediction results

Technical University of Denmark

```
# Input is in FSA format

# Peptide length 15
       NetMHCII version 2.2.

Strong binder threshold  50.00. Weak binder threshold 500.00.

-------------------------------------------------------------------------------
Allele   pos  peptide           core   1-log50k(aff) affinity(nM)    Bind
Level     ------------------------------------------------------------------
------------------------------
H-2-IAb  338  VYIFLGPEPKSVVQQ  FLGPEPKSV   0.6606     39.4           SB
H-2-IAb  337  DVYIFLGPEPKSVVQ  FLGPEPKSV   0.6573     40.8           SB
H-2-IAb  336  LDVYIFLGPEPKSVV  FLGPEPKSV   0.6492     44.5           SB
H-2-IAb  335  ILDVYIFLGPEPKSV  FLGPEPKSV   0.6288     55.5           WB
H-2-IAb  475  LIGKVWPGSTAFPDF  KVWPGSTAF   0.6185     62.1           WB
H-2-IAb  476  IGKVWPGSTAFPDFT  KVWPGSTAF   0.6138     65.3           WB
H-2-IAb  339  YIFLGPEPKSVVQQY  FLGPEPKSV   0.6115     66.9           WB
H-2-IAb  781  ALGSLPPPPAAPREP  SLPPPPAAP   0.6094     68.5           WB
H-2-IAb  474  PLIGKVWPGSTAFPD  KVWPGSTAF   0.6084     69.2           WB
H-2-IAb  142  SSEMGYTATLTRTTP  YTATLTRTT   0.6072     70.1           WB
H-2-IAb  780  EALGSLPPPPAAPRE  SLPPPPAAP   0.6065     70.7           WB
H-2-IAb  143  SEMGYTATLTRTTPT  YTATLTRTT   0.6056     71.4           WB
H-2-IAb  779  VEALGSLPPPPAAPR  SLPPPPAAP   0.6033     73.1           WB
H-2-IAb  141  SSSEMGYTATLTRTT  YTATLTRTT   0.6025     73.8           WB
H-2-IAb  477  GKVWPGSTAFPDFTN  KVWPGSTAF   0.6004     75.5           WB
H-2-IAb  778  PVEALGSLPPPPAAP  SLPPPPAAP   0.5931     81.7           WB
H-2-IAb  144  EMGYTATLTRTTPTF  YTATLTRTT   0.5927     82.0           WB
```

APPENDIX C
SOURCE - FIGURE 1-1.

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AUTHORIZATION to Use or Disclose De-identified Health Information for Publication and Educational Purposes

Patient's Name	Date of Birth	Verification of Identity (Driver's License, ID Card, Passport, etc.)
Phoenix Fox	07-18-2002	
Patient's Address	Medical Record Number	

** Complete the following only if the person authorizing the use or disclosure is not the patient:

Representative's Name	Relationship to Patient	Legal Authority
Gina Fox	Mother	
Representative's Address	Verification of Identity	Verification of Authority

By signing this form, I authorize the following:

Disclosure of the patient's information **from**:		Disclosure of the patient's information **to**:	
Entity or organization where the images were made: University of Florida, Dr. Byrne		*Person or organization who will use/disclose the images:* Ramaya Sivakumar	
Address 1600 SW Archer Rd		*Address* 1600 SW Archer Road	
Gainesville FL 32610		Gainesville FL 32610	
Attn: LeeAnn	*Phone* 352 273 7262	Attn: Ramya	*Phone* 970-443-0269

- **The following health information may be disclosed:** (*Check all that apply*)
  - ☐ Video taken during surgery or other health care encounter and related health data.
  - ☑ Photographs taken during surgery or other health care encounter and related health data.
  - ☐ Other images recorded during surgery or other health care encounter *(describe)*_____

  No identifiable information (name, address, etc.) or images (face or other uniquely identifiable body parts) will be included with or in the videos, photographs, or medical data.
- **I further authorize** the disclosure of the following information which may be included in the health information listed above. (*Check all that are approved.*)
  ☐ Mental Health ☐ Substance Abuse ☐ HIV/AIDS
- **This health information will be used or disclosed only for educational purposes,** which may include publication in books or journals, or classroom instruction and/or medical training at the University of Florida, other educational institutions, and/or national and international conferences.

I understand that, by federal law, the University of Florida may not use or disclose *protected health information* (health information which identifies or could reasonably be expected to identify, a person) without authorization except as provided in the University's Notice of Privacy Practices. By signing this Authorization, I am giving permission for the uses and disclosures of the health information described above. I hereby release the University of Florida and its employees from any and all liability that may arise from the release of information as I have directed.

I understand that I have the right to revoke this Authorization at any time, if I do so in writing, and address it to the person or institution named above. I understand that the revocation will not apply to any actions already taken as a result of this authorization.

I understand that I may refuse to sign this Authorization, and that the institutions or individuals named above cannot deny or refuse to provide treatment, payment, enrollment in a health plan, or eligibility for benefits if I refuse to sign.

I understand that information disclosed pursuant to this Authorization may no longer be protected by any medical privacy laws and could be redisclosed by the person or agency that receives it.

I have the right to inspect and/or receive a copy of the Health Information released.

This authorization expires automatically after: ☑ 1 Year ☐ 2 Years ☐ 3 Years
I understand that the expiration will not apply to any actions already taken as a result of this authorization.

I have read and understand the information in this authorization form.

Signature of Patient or Legal Representative:	Date
	3/15/2011

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BIOGRAPHICAL SKETCH

Ramya Sivakumar was born in India. She obtained her Bachelor's degree in Biotechnology from Anna University,India. She chose to pursue her Masters in the United States of America, and entered the Biomedical Engineering program at the University of Florida during the year 2009. She was working as a Graduate Research Assistant in the Cellular and Molecular therapy division during her course of study. Upon completion of her graduation, she will be joining the PhD program in Biomedical Sciences at UF. Her research interests encompass several areas such as Immunology and genetic and tissue engineering. She intends to continue working in the fields of Immunology in an Industrial environment in the future. Apart from studies, she loves to travel, read books, and enjoys adventurous sports