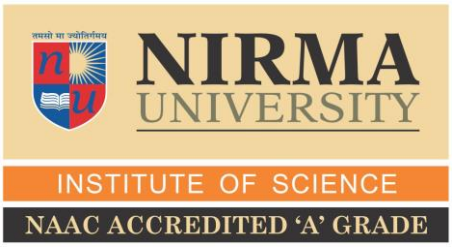
"DEVELOPING A MATHEMATICAL MODEL FOR UNDERSTANDING GENERATION OF MEMORY T CELLS"

A Thesis submitted to



INSTITUTE OF SCIENCE
NIRMA UNIVERSITY

In partial fulfillment for the award of the Degree of

MASTER OF SCIENCE

IN BIOTECHNOLOGY

By

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Under the guidance of
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Krima Sheth

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ABBREVATION

Ag	Antigen
ANN	Artificial Neural Network
APC	Antigen Presenting Cell
BCR	B cell Receptor
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CTL	Cytotoxic T Lymphocytes
EES	Engineering Equation Solver
FACS	Fluorescence Activated Cell Sorter
GA	Genetic Algorithm
HIV	Human Immunodeficiency Virus
IFNγ	Interferon γ
IL	Interleukins
MHC	Major Histocompatibility Complex
NK	Natural killer cells
OVA	OVAlbumin
PAD4	Protective Antigen Domain 4
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
Poly I:C	PolyinosinePolycytidylic acid

PRRs	Pattern-Recognition Receptors
R848	Resiquimod 848
T_{CM}	Central Memory T-cells
T_{EM}	Effector Memory cells
T_H	T helper cells
TLRs	Toll like Receptors
TNF α	Tumor Necrosis Factor

INTRODUCTION

INTRODUCTION

Natural infection induces protective immunity which is required to generate an effective response for elimination of disease causing pathogen as well as subsequent re-infection. The main goal of vaccination is to generate and maintain a heterogeneous pool of long lived memory cells. But still we do not have effective vaccines against disease such as malaria, HIV, and tuberculosis (amanna et al, 2008). This shows that there are some defects in designing conventional vaccine strategies. The possible reason could be that conventional vaccines fail to induce immune response same as natural infection (Rappuoli, 2007).

The innate and adaptive immunity plays an important role for the elimination of any pathogen or any infection. Innate immunity includes a non-antigen specific host defense, Whereas, adaptive immunity includes B-cells and T-cells. Adaptive immunity exhibits memory characteristics (Zloza et al, 2012). Innate immune system recognizes the PAMPS (Pathogen associated molecular patterns) present on pathogen. Toll like receptors (TLRs) are expressed by cells of both innate and adaptive immune system like T-cells, B-cells and NK cells DCs, granulocytes, macrophages.

TLR triggers the intracellular signaling cascades and it activates the production of various pro-inflammatory molecules. It activates the APCs such as dendritic cells and macrophages, thus it activates the adaptive T cell immune response. They do so by the activation of gene expression and by synthesizing cytokines, chemokines and cell adhesion molecules.

Memory cells have mainly two classes: Memory T cells and Memory B cells. In most of the viral infection, we require a vaccine that targets the T cell response to create T cell memory. The features of true memory are known but induction of such type of memory through immunization strategies or vaccination is a thing which is yet to be achieved. Memory T-cell response generated is depends on the dosage of antigen as well as cytokine produced during antigenic stimulation (Sedar et al., 2008).

T cell differentiation during acute viral infection

When pathogen encounters the cell, the antigen is processed and presented by MHC-II in the APC (antigen presenting cells). Naïve T cells recognise the MHC-peptide complex. Activation of naïve cells required 3 signal. Signal 1 includes the presentation of antigen peptide by MHC class II and it is recognized by the antigen-specific TCR. Signal 2 generate the signal via co-stimulatory molecules present on the surface of APCs and T cells. That includes CD40 on APC and CD40L on T cell or b7 on APC and CD28 on TCR. Signal 3 is produced by the secretion of cytokines like IL-12 and Type I IFN (IFN α/β) by antigen presenting cells, which signal via cytokine receptors on T cells. (J Clin Invest., 2007). Studies shows that the T cell activation is also regulated by some parameters including strength and duration of T cell antigen receptor (TCR) and co-stimulatory receptor signaling.

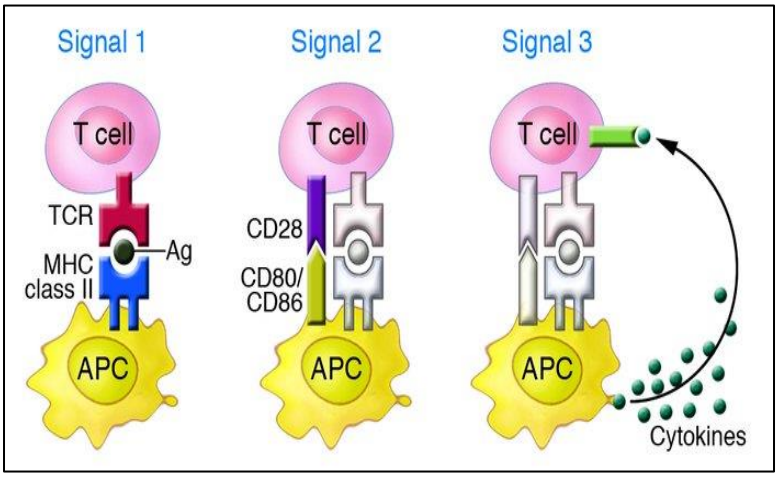

Figure 1 - T cells Activation by three signals

(Julie M. Curtsinger and Matthew F. Mescher 2010)

The differentiation process of CD8 T-cell in response to viral infection is complex. During initial encounter with antigen, naïve T cell transition occurs through three different phases of an antiviral response, initial activation and expansion, the death phase or contraction, and the memory T cells formation.

Once a naive Tcell recognize an antigen it activates, proliferates and differentiated into the effector T cells. Effector T cells moves to peripheral tissues where they produce antiviral cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), and cytotoxic molecules, such as perforin and granzymes, and it mediate killing of infected cells as part of their effector functions. 90-95% of the effector T cell die due to apoptosis while other 5-10% of them are converted into long lived memory cells.

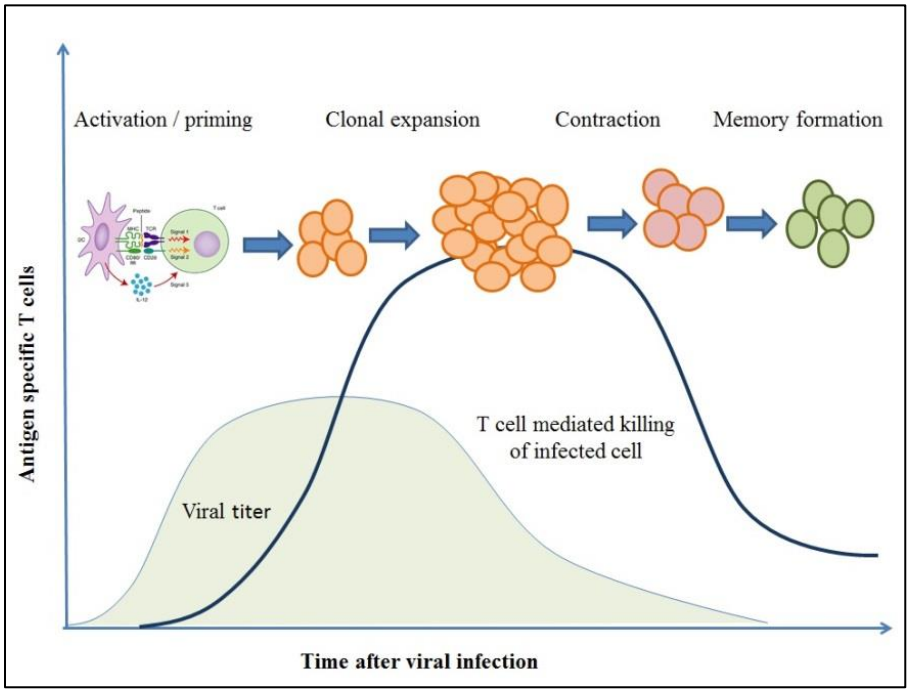

Figure 2 - Antigen specific T cell proliferation

Effector cells are generated in the presence of antigen while Memory cells are assumed to be generated during the phase of viral clearence.

One study reported that after antigenic stimulation naïve T cells continuously divide at least seven times without further antigen stimulation and it differentiate into effector and memory cells (Susan M. Kaech and Rafi Ahmed, 2001). Naïve T cells required at least five cell divisions for CD8+ memory cell formation. CD8+ naïve cells divided less than five times do not produced memory cells (Opferman JT et al, 1999 and Susan M. Kaech and Rafi Ahmed, 2001).

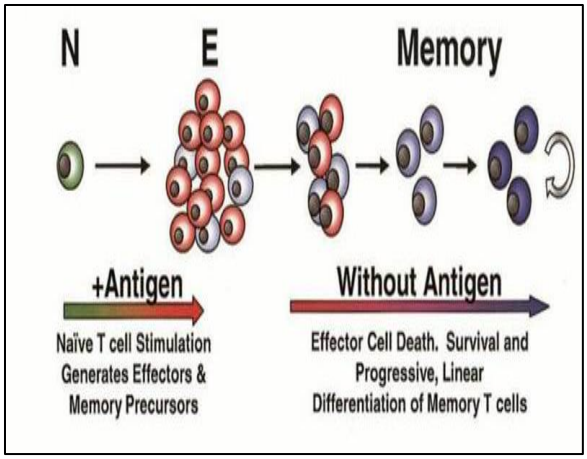

Figure 3 - Effect of Antigen on T Cells Differentiation

(E. John Wherry and Rafi Ahmed, 2004)

Adaptive immune system can remember the pathogen by formation of the memory cells. Memory T cells are long lived cells and they have the potential to rapidly develop into effector cells upon re-encounter with a pathogen and provide protective immunity against infections.

Memory cells are maintained in inactive form for a long term in absence of the antigen but it activates when the antigen re-encounters. Generation of these memory cells is the goal of all vaccines. For the development of effective T cell-based vaccines we should know the mechanisms that regulate the differentiation and maintenance of CD8+ memory. Memory cell differentiation is influenced by the cytokines, inflammation, antigen amount, the history of previous antigen encounters.

Types of Memory T cells:

Memory T cell populations comprise of central memory T cells (T_{CM}), and effector memory T cells (T_{EM}). Central memory cells are restricted to the secondary lymphoid tissues and effector memory cells migrate between peripheral tissues, the blood, and the spleen (Mueller et al., 2013).

T_{CM} (Central memory T cells)

Memory cells that express CCR7 and L- selectin (CD62L) are termed as central memory (T_{CM}). They have high expression of CD44. This type of memory cells found in the lymph nodes and in the peripheral circulation. Central memory cells are long lived cells compared to effector memory cells and it have high proliferation rate. Activation time of central memory cells is more than the effector memory cells. Central memory T (T_{CM}) cells have a longer lifespan and it differentiate into T_{EM} cells on antigenic encounter.

T_{EM} (Effector memory T cells)

Memory cells that do not express CCR7 and CD62L are termed as effector memory cells (T_{EM}). They also have high expression of CD44. Effector cells are excluded from lymph nodes and it found in spleen. They are relatively short lived. They immediately activated when the antigen re-encounters and they produce cytokines, perforin and granzyme B to eliminate the viral infection.

Models for Memory Cell formation

Generation of memory cells T cells follow two different pathways, linear or divergent.

1. **Divergent pathway**

   In this pathway the naive T cell activates and differentiated into memory or effector cells depends on the amount of antigen stimulation. High antigen stimulation will convert naïve T cells to effector cells. But the low antigen stimulation will convert naïve T cells to memory cells. In this pathway memory cells phase may bypass the effector cell phase and directly converted to memory T cells.

2. **Linear differentiation**

   In this pathway the naïve T cells differentiated into effector cells in the presence of an antigen and after the antigen encounters, some of them will be converted into memory cells. Memory cells are assumed to be generated during the phase of low antigen and inflammation.

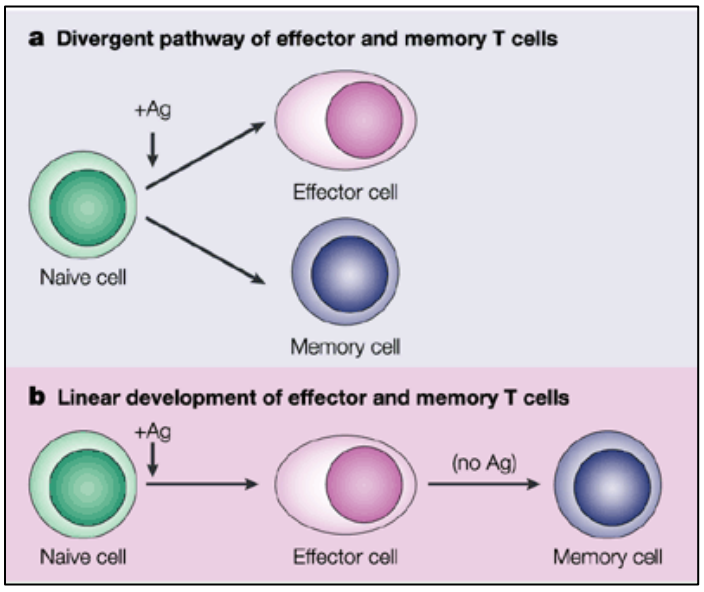

Figure 4 - Pathways for Memory T Cell Differentiation

Factors Affecting Memory T Cell Generation:

Antigen stimulation

In the divergent pathway, the naive T cell activates and differentiated into memory or effector cell that depends on the amount of antigen stimulation. Low antigen stimulation will leads to memory cell formation while high antigen stimulation will form the effector T cells. Researchers suggest that the Low antigen and low inflammation condition will give heterogeneous pool of memory cells. High antigen and high inflammation cause the activation induces cell death (AICD).

Inflammatory cues

Inflammatory environmental affect the memory cell formation. IL-12 and IFN-α/β provides signal-3 and promote development of effector and memory cell formation. Stimulated signals during infection can increase the effector cell population but it may delay the generation of memory cells.

Cytokines TNF-α, IL-2, and IFN-γ increases the memory cell formation (Sara E. Hamilton and Stephen C. Jameson, 2012). IL-15 and IL-7 enhance the memory cell formation while the IL-12 and IFN-γ limits the memory cell formation by generation of effector cells. (Schluns, Kimberly S., and Leo Lefrançois. (2003)

Antigen dose and the adjuvant dose is the variable of my study. Researchers suggested that low antigen and low inflammation condition will provide heterogeneous pool of memory cells. High antigen and high inflammation condition would lead to activation induced cell death. Goal of vaccination is to create heterogeneous pool of memory cells.

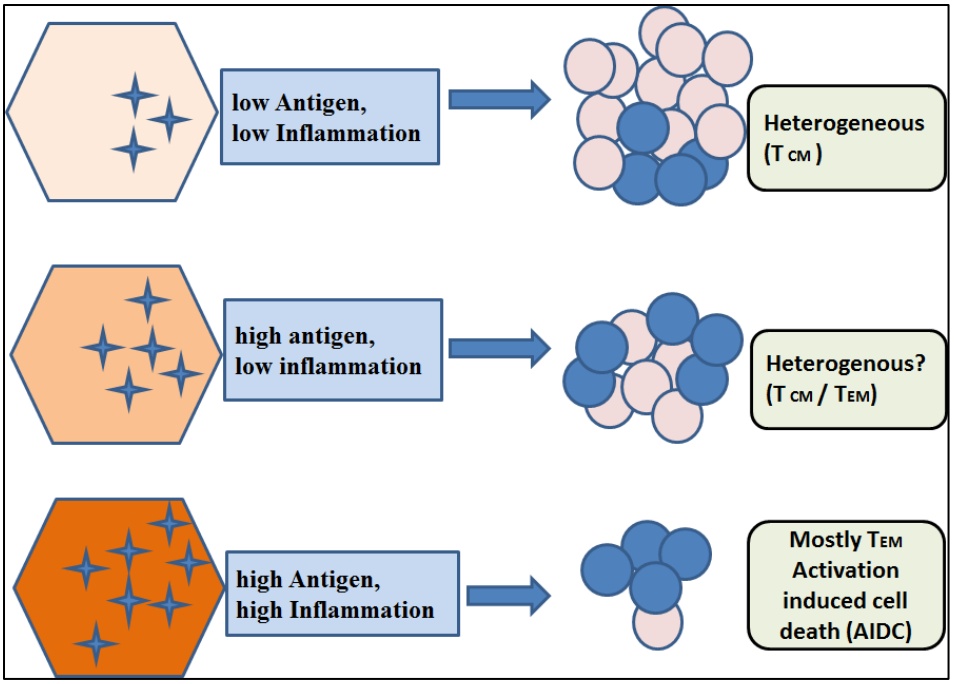

Figure 5 - Effect of Antigen and Inflammation On Memory Cell Formation

[Swain. S., *et al* (2005), Bush. D., *et al* (2000)]

HYPOTHESIS

&

OBJECTIVES

HYPOTHESIS

We hypothesize to quantify the amount of antigen that should be given for development of required amount of memory cells.

This can be done by assuming the number of naive T cells activated by antigen attack with the use of mathematical model formulated.

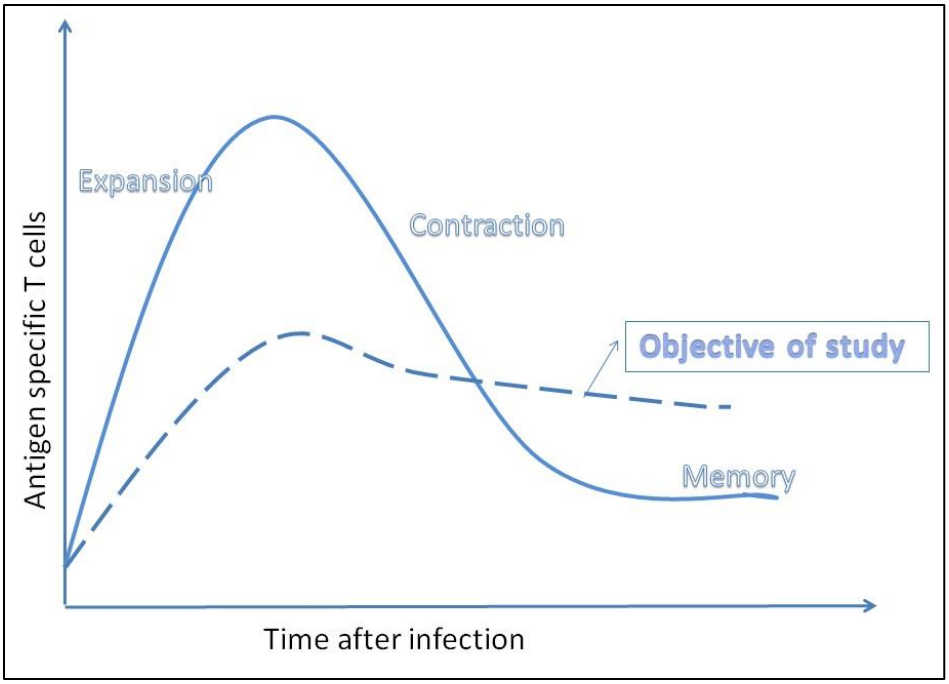

Figure 6 - Objective of study

OBJECTIVES

1. **To develop a mathematical model for T cell activation and memory generation using various engineering software (Engineering Equation Solver, Statistica)**

2. **To generate memory T cells by mimicking the natural course of infection using antigen along with TLR ligands**

OBJECTIVE

I.

I. Developing a mathematical model using various Engineering Software tools

- **Literature studies and procurement of raw data.**

Effect of Antigen and Inflammation on number of the effector cells

Here in four different conditions the number of effector cells that are formed is given. Papers suggested that the both inflammation and antigen would give rise to higher number of effector cells. In presence of both antigen and inflammation there would be >3 log expansion of naïve cells to effector cells. It reported that there would not be higher expansion in conditions of Inflammation only, Antigen only, the absence of both antigen and inflammation.

Table 1 Effect of Antigen and Inflammation on number of the effector cells

	No Antigen, No Inflammation (Uninfected)	**Inflammation only (LCMV)**	**Antigen only (ova)**	**Antigen, Inflammation (LCMV + ova)**
Number of CD8+ T cells0 **DAY 0**	5×10^3	5×10^3	5×10^3	5×10^3
Number of CD8+ T cells **DAY 7**	2×10^4	10^4	10^5	10^7
	<1 log expansion	Not enhance expansion	~ 1 log expansion	> 3 log expansion

[Schaulov, A., & Murali-Krishna, K. (2008).]

Effect of antigen and inflammation on number of memory cells

Here in three different conditions the number of memory cells that are formed is given. We assumed the number of memory cells that are formed in different condition by the graphical data. Paper suggested that the memory cell formation required both inflammation and antigen. (Schaulov, A., & Murali-Krishna, K. (2008)).

Uninfected (No Antigen, No Inflammation) --› 10^4 Memory Cells

Inflammation --› 3×10^4 Memory Cells

Inflammation + Antigen --› 5×10^5 Memory Cells

Effect of Antigen Dose on number of Effector cells

From the literature survey we got some data about the effect of antigen dose on the number of cells recruited and the number of effector cells that are formed.

Naive T cells - 1×10^5

Mice- C57BL/6 (B6)
Antigen dose – A recombinant Listeria monocytogenes bacterial strain (LM-GP33)

Table 2 Effect of Antigen Dose on number of Effector cells

Dose of antigen	Number of bacteria	Number of naïve cells recruited into immune system	Number of CD8+ T cells (effector cells)
High dose 3×10^4 CFU	4×10^6 - 8×10^6CFU/spleen (undetectable by day 7)	1×10^5 (100% of cells recruited)	20×10^6 (200 fold increase)
Intermediate dose 3×10^3 CFU	-	5×10^4 (50% of cells recruited)	7×10^6 (70 fold increase)
Low dose 100 CFU	1×10^3 - 2×10^3 CFU/spleen (undetectable by day 3)	3×10^4 (30% of cells recruited)	1×10^6 (10 fold increase)

(Susan M. Kaech and Rafi Ahmed, 2001)

Naïve cells are exposed to three different conditions of antigen stimulation, High Dose, Intermediate Dose and Low Dose. In high antigen dose all naïve cells are recruited to the immune system which increased effector cell population by 200 fold (20×10^6 effector cells). In intermediate dose of antigen 50% of cells are recruited, which give rise to 7×10^6 effector cells. In low dose of antigen 30% of cells are recruited, which give rise to almost 1×10^6 effector cells.

It shows that varying the antigen dose affects the number of naive CD8+ T cells recruited into the immune response. Papers suggested that after antigen stimulation all the recruited naïve CD8 T cells continue to divide at least seven times and it differentiated into the effector and memory cells. Once naïve cells are activated, it continues to divide without further antigen stimulation.
Here, it is suggested that varying the antigen dose would affect the percentage of naïve cells recruitment. The number of effector cell increased with the higher antigen dose. The number of APCs is less in Low and Intermediate dose of antigen which reduces the MHC-peptide complex. Thus, the number of CD8+ T cells that are activated and recruited to the immune system would be less. (Susan M. Kaech and Rafi Ahmed, 2001)

- **Feeding the raw data in software to create Artificial Neural Networks**

There are some online available software that includes an array of data analysis, data management, data mining and data visualization. We required more data to create Artificial Neural Network using this software. Therefore we created more values by taking mean values of available data. Then added these values in the software and created a neural network.

Table 3 Effect of antigen dose on percentage of cells recruited and number of effector cells formed

Antigen Dose (cfu)	Naïve Cells	% Of Cells Recruited	Number Of Cells Recruited	Effector Cells
100	**100000**	**30**	**30000**	**1000000**
463	100000	32.5	32500	1750000
825	100000	35	35000	2500000
1188	100000	37.5	37500	3250000
1550	100000	40	40000	4000000
1913	100000	42.5	42500	4750000
2275	100000	45	45000	5500000
2638	100000	47.5	47500	6250000
3000	**100000**	**50**	**50000**	**7000000**
6375	100000	56.25	56250	8650000
9750	100000	62.5	62500	10300000
13125	100000	68.75	68750	11900000
16500	100000	75	75000	13500000
19875	100000	81.25	81250	15125000
23250	100000	87.5	87500	16750000
26625	100000	93.75	93750	18370000
30 000	**100000**	**100**	**100000**	**20000000**

(Susan M. Kaech and Rafi Ahmed, 2001)

Antigen dose and the percentage of the cells recruited to the immune system are the inputs/ variables, while Number of the effector cells formed is the output. The number of naïve cells is constant (10^5). If we take the combination of different antigen dose values and the percentage of naïve cells that recruited to the immune system, so this artificial neural network would give the value of output or the number of effector cells that are formed.

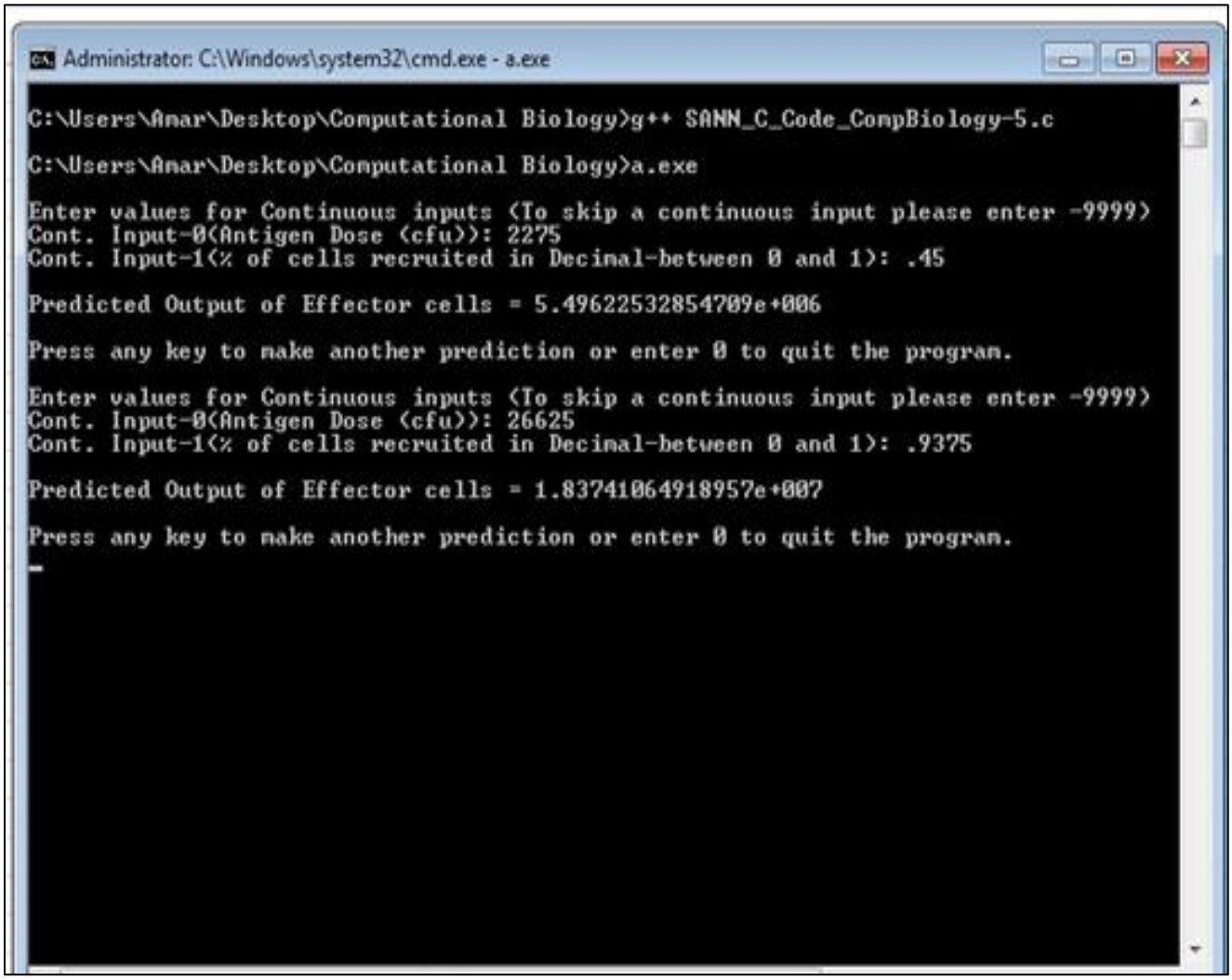

Figure 7 - Artificial Neural Network (screenshot of software)

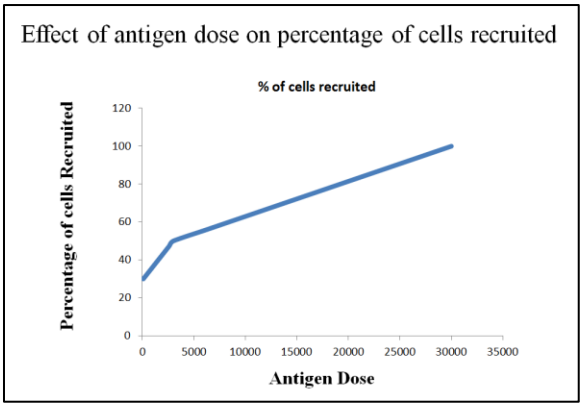

Figure 8 - Percentage of cells recruited vs antigen dose

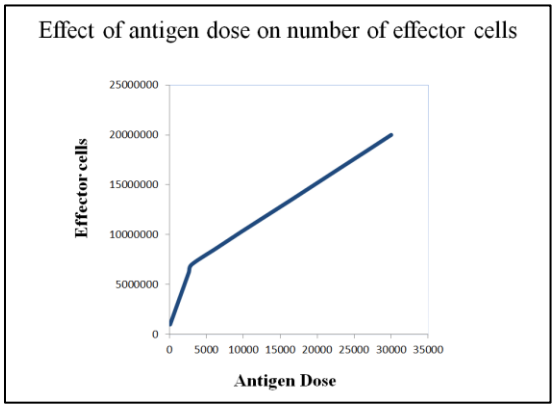

Figure 9 Effector T cells vs Antigen dose

Table 4- Influenced of antigen dose on division of T cells

Antigen dose	Naïve cells recruited	Number of Cell division	Effector T cells generated
100	30000	**5**	1×10^6
3000	50000	**7**	7×10^6
30000	100000	**8**	20×10^6

Cell division is an important parameter that regulates formation of memory CD8+ T cells. Naïve cells are exposed to three different conditions of antigen stimulation, high dose, intermediate dose and low dose. It is already reported that varying the antigen dose affected the number of naive CD8+ T cells recruited into the immune response.

From the given data, I can conclude that in high antigen dose all naïve cells (10^5) are recruited to the immune system and it give rise to 20×10^6 effector cells. From this we assumed that each cell divided at least 8 times.
In intermediate dose of antigen 50% of cells (5×10^4) are recruited to the immune system which divided at least 7-8 times to give rise to 7×10^6 effector cells.
In low dose of antigen 30% of cells (3×10^4) are recruited, which give rise to 1×10^6 effector cells. From this we assumed that each cell divided at least 5 times.

The generation of heterogeneous pool of memory T cells following vaccination seems empirical when it comes to characterizing the immune response. Developing a model would thus help us

delineate the factors favoring the generation of memory T cells, such as antigen dose and inflammatory milieu. Memory T cells are an important component of protective immunity against viral infections, and understanding their development will aid in the design of optimal vaccines. Our idea is to increase memory cell phase by decreasing the effector cell phase. For the vaccination strategy we do not require highly activated effector cells. In high dose of antigen the cell division is higher which gives highly activated effector T cells. Highly activated effector cells have undergone more cell division which results into the activation induced cell death. When low antigen dose is given, it is assumed that the effector cells divide less time and are not highly activated. Therefore, most of the effector cells may differentiates into the memory cells. [Lefrançois, L., & Masopust, D. (2009).]

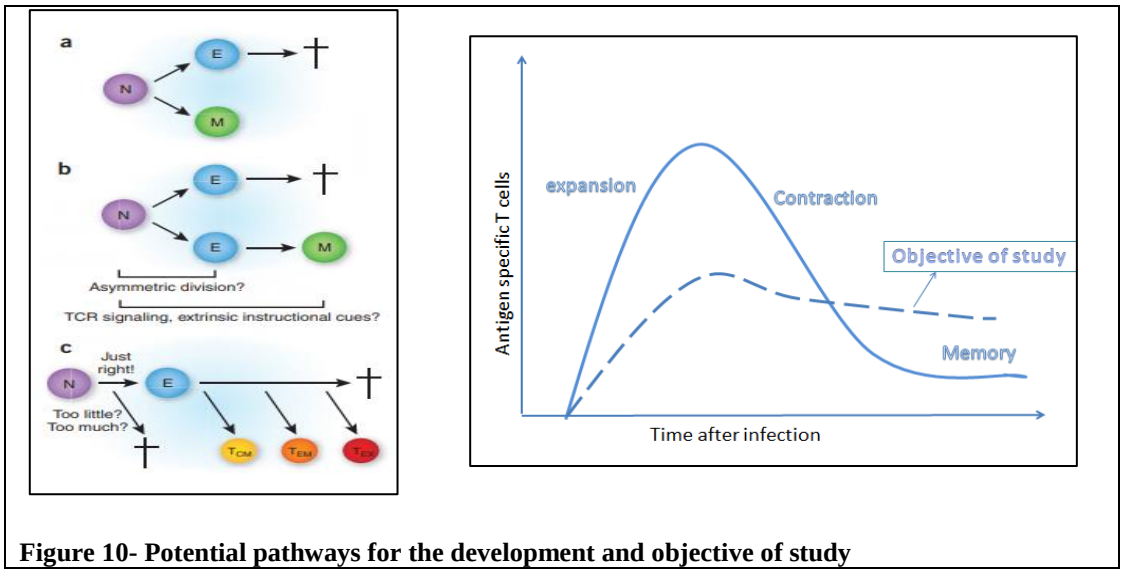

Figure 10- Potential pathways for the development and objective of study

- **Solve the equations in the software EES**

In one of the review paper we found two different equations about the generation of memory T cells. (Antia, Rustom et al,2005)

This equation is solved using EES (Engineering Equation Solver) software and got the graph and table of number of cells generated at each time point. (EES software used from Mechanical Department, Institute of Technology, Nirma University)

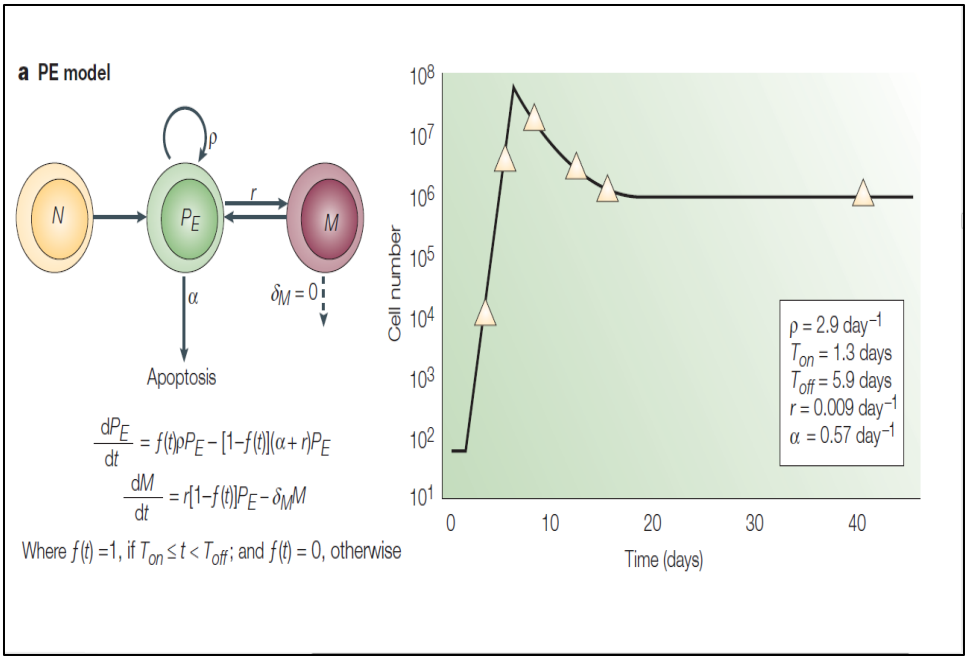

Figure 11 – Mathematical model for linear pathway of T cell differentiation

(Antia, Rustom et al,2005)

Naïve T cells are activated and give rise to effector cells and which differentiated into Memory cells. In this model, naive cells (*N)*, are recruited into the immune response at time (*t*). T_{on} *is* a time after the naïve cells are recruited and give rise to proliferating effector cells (P_E). The P_E-cell population grows at the rate ρ until time T_{off}. After this, some of the effector cells undergo apoptosis at the rate α or some of them are differentiated at the rate *r* into memory cells (M).
Here, N= number of naïve cells
P_E = number of effector cells
ρ= growth rate of effector cell population= 2.9 day^{-1}
T^{on} = time after naïve cells are recruited to immune system = 1.3 days
T^{off} = time untill P_E cell population grows = 5.9 days
r = differentiation rate of effector to memory = 0.009 day^{-1}
α = apoptosis rate of effector cells = 0.57 day^{-1}

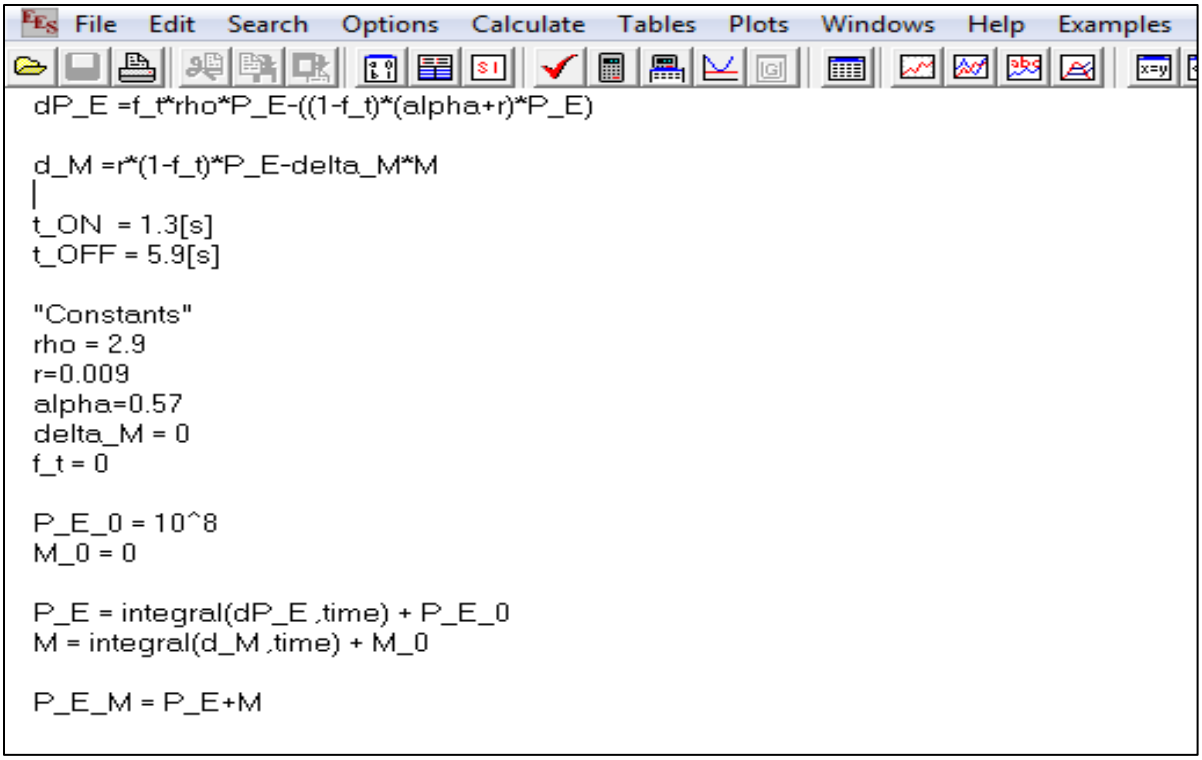

Figure 12 - Equation solved in EES

This equation is solved in the EES software. After solving the equation, we obtained the number of effector cells and memory cells at each seconds and we retrieved the graph which shows conversion of effector to memory cells. But these results did not provide information about the differentiation of naïve cell into effector cells.

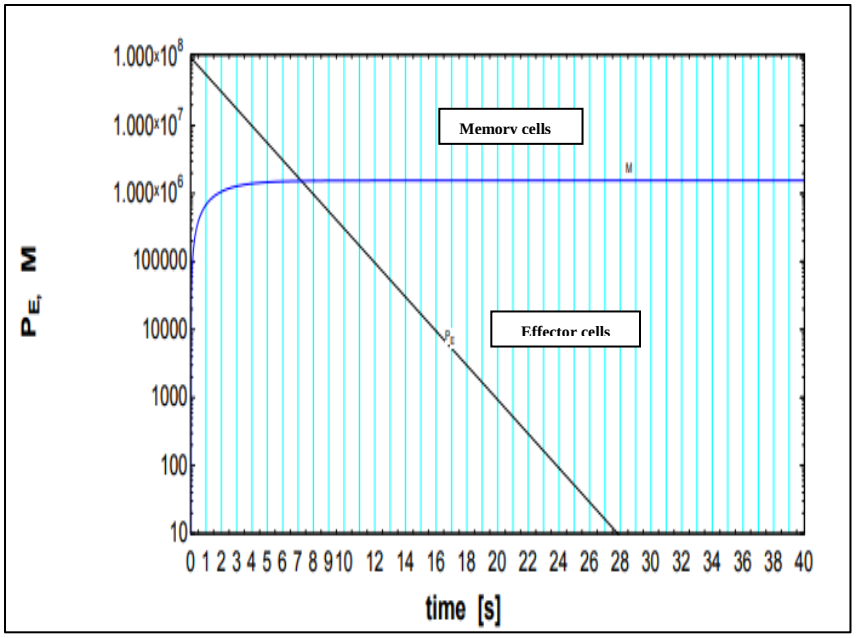

Figure 13 - Number of effector and memory cell vs time (EES)

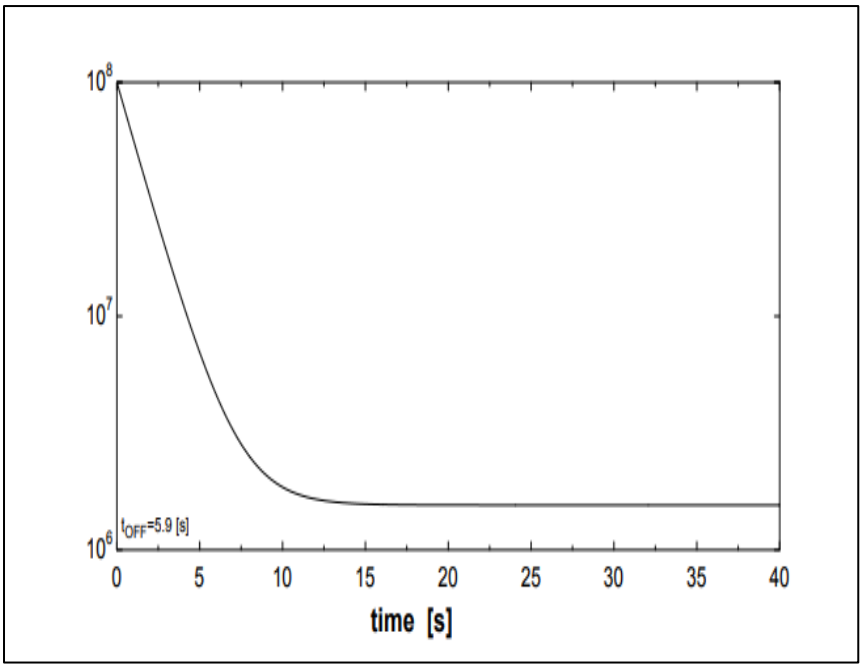

Figure 14 - Number of effector + memory cell vs time (EES)

Table 5 – number of effector and memory T cells (EES data)

1..1000	1 time [s]	2 P_E	3 M	4 $P_{E,M}$
Run 1	0	1.000E+08	0	1.000E+08
Run 2	0.04004	9.771E+07	35623	9.774E+07
Run 3	0.08008	9.547E+07	70430	9.554E+07
Run 4	0.1201	9.328E+07	104439	9.339E+07
Run 5	0.1602	9.114E+07	137668	9.128E+07
Run 6	0.2002	8.905E+07	170137	8.922E+07
Run 7	0.2402	8.701E+07	201861	8.722E+07
Run 8	0.2803	8.502E+07	232858	8.525E+07
Run 9	0.3203	8.307E+07	263144	8.333E+07
Run 10	0.3604	8.117E+07	292737	8.146E+07
Run 11	0.4004	7.931E+07	321651	7.963E+07
Run 12	0.4404	7.749E+07	349903	7.784E+07
Run 13	0.4805	7.571E+07	377507	7.609E+07
Run 14	0.5205	7.398E+07	404478	7.438E+07
Run 15	0.5606	7.228E+07	430832	7.271E+07
Run 16	0.6006	7.063E+07	456581	7.108E+07
Run 17	0.6406	6.901E+07	481741	6.949E+07
Run 18	0.6807	6.743E+07	506324	6.793E+07
Run 19	0.7207	6.588E+07	530343	6.641E+07
Run 20	0.7608	6.437E+07	553812	6.493E+07
Run 21	0.8008	6.290E+07	576743	6.347E+07
Run 22	0.8408	6.145E+07	599149	6.205E+07
Run 23	0.8809	6.005E+07	621041	6.067E+07
Run 24	0.9209	5.867E+07	642431	5.931E+07
Run 25	0.961	5.733E+07	663331	5.799E+07

1..1000	1 time [s]	2 P_E	3 M	4 $P_{E,M}$
Run 975	39	0.0156	1.554E+06	1.554E+06
Run 976	39.04	0.01524	1.554E+06	1.554E+06
Run 977	39.08	0.01489	1.554E+06	1.554E+06
Run 978	39.12	0.01455	1.554E+06	1.554E+06
Run 979	39.16	0.01421	1.554E+06	1.554E+06
Run 980	39.2	0.01389	1.554E+06	1.554E+06
Run 981	39.24	0.01357	1.554E+06	1.554E+06
Run 982	39.28	0.01326	1.554E+06	1.554E+06
Run 983	39.32	0.01296	1.554E+06	1.554E+06
Run 984	39.36	0.01266	1.554E+06	1.554E+06
Run 985	39.4	0.01237	1.554E+06	1.554E+06
Run 986	39.44	0.01209	1.554E+06	1.554E+06
Run 987	39.48	0.01181	1.554E+06	1.554E+06
Run 988	39.52	0.01154	1.554E+06	1.554E+06
Run 989	39.56	0.01127	1.554E+06	1.554E+06
Run 990	39.6	0.01101	1.554E+06	1.554E+06
Run 991	39.64	0.01076	1.554E+06	1.554E+06
Run 992	39.68	0.01052	1.554E+06	1.554E+06
Run 993	39.72	0.01027	1.554E+06	1.554E+06
Run 994	39.76	0.01004	1.554E+06	1.554E+06
Run 995	39.8	0.009809	1.554E+06	1.554E+06
Run 996	39.84	0.009584	1.554E+06	1.554E+06
Run 997	39.88	0.009365	1.554E+06	1.554E+06
Run 998	39.92	0.00915	1.554E+06	1.554E+06
Run 999	39.96	0.00894	1.554E+06	1.554E+06
Run 1000	40	0.008736	1.554E+06	1.554E+06

We had 1000 runs or data values from the software. Only few of them are displayed over here. (1-25 run, and 975-1000 run)

We retrieved the graph which shows conversion of effector to memory cells. But these results did not provide information about the differentiation of naïve cell into effector cells. We found more papers, to understand the conversion of naïve cells to effector cells.

From one of the paper, we found some more equation about the conversion of naïve to effector and memory cells. They had shown the equations for infunced of antigen dose and the viral load on T cell differentiation.

$$F_V = \frac{V}{k + V}$$

$$dAdt = F_V \cdot (a \cdot (M + N) + \rho \cdot A_a) - ((1 - F_V) \cdot (r + \alpha)) \cdot A_a$$

$$dNdt = -F_V \cdot a \cdot N$$

$$dMdt = r \cdot (1 - F_V) \cdot A_a - a \cdot F_V \cdot M - \delta_m \cdot M$$

Figure 15 Mathematical Models for viral load, naïve cells, effector cells, memory cells

(De Boer at.al (2001).)

Here, F_v = function of viral load
V= viral load(pfu)
K= saturation constant(pfu)
A = effector cell population
N= naïve cells = 60 cells
M = memory cells
a=activation rate=1 day^{-1}
ϱ = proliferation rate=2.92
α = apoptosis rate= 0.41
r = memory cell formation rate=0.015
delta_M = 10^{-5}

The parameters a=activation rate=1 day^{-1}, delta_M =10^{-5}, N = naïve cells= 60 are the fixed parameter.

We could not have information about, change in viral load according to time. For that we assumed the change in viral load from the graphical data.

Table 6 viral load changing with time

time(day)	viral load
0	100
1	1000
2	1000000
3	5000000
4	900000
5	200000
7	100
15	60
17	0

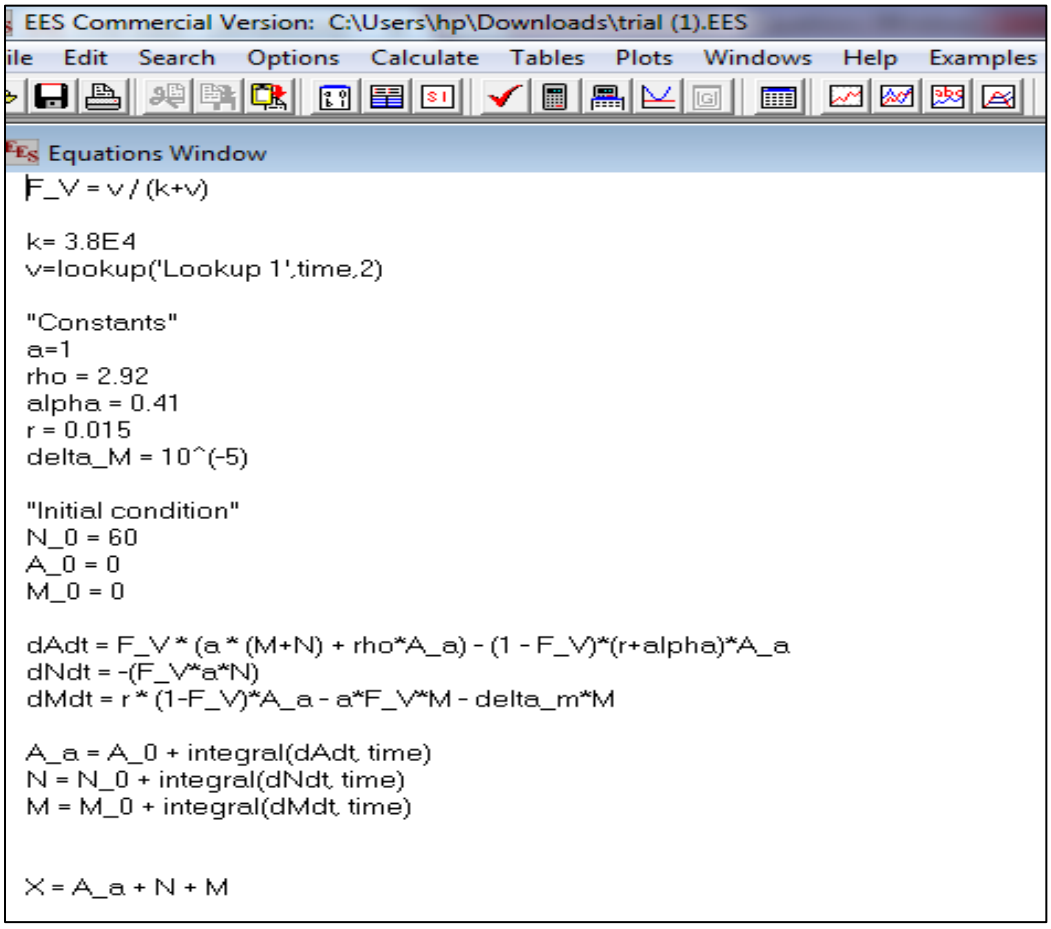

Figure 16 Equation solved in EES

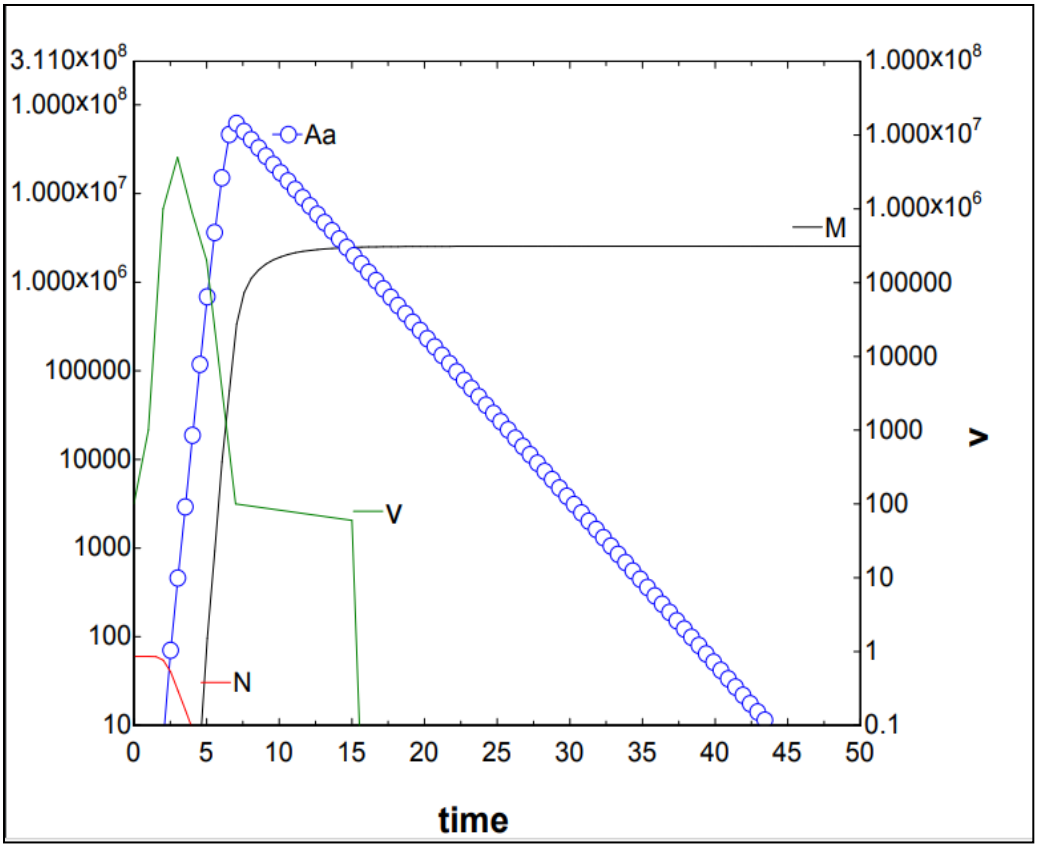

Figure 17 naïve cell, effector cells, memory cells vs time (EES data)

The graph represents the naïve cells, effector cells and memory cells changing with time. By solving this equation, we got the data table about number of each cells at each time point.

We had 100 runs or data values from the software. Only few of them are displayed over here. (1-25 run, and 75-100 run)

Table 7 – Number of naïve cells, effector cells and memory T cells (EES data)

EES Commercial Version: C:\Users\hp\Documents\trial.EES - [Parametric Table]

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Table 1

1..100	time	A_a	X	M	N	dAdt	dMdt	dNdt
Run 1	0	0	60	0	60	1.538	0	-1.538
Run 2	0.5051	0.7111	59.94	0.002608	59.23	1.278	0.01033	-1.519
Run 3	1.01	1.301	59.78	0.009952	58.47	1.058	0.01876	-1.499
Run 4	1.515	1.789	59.52	0.02115	57.71	0.8736	0.0256	-1.48
Run 5	2.02	2.191	59.2	0.03547	56.97	0.7185	0.03111	-1.461
Run 6	2.525	2.521	58.81	0.05229	56.24	0.5882	0.0355	-1.442
Run 7	3.03	2.79	58.38	0.0711	55.51	0.4788	0.03896	-1.423
Run 8	3.535	3.009	57.9	0.09145	54.8	0.3868	0.04163	-1.405
Run 9	4.04	3.185	57.39	0.113	54.1	0.3096	0.04365	-1.387
Run 10	4.545	3.325	56.86	0.1354	53.4	0.2449	0.04512	-1.369
Run 11	5.051	3.435	56.31	0.1584	52.71	0.1905	0.04613	-1.352
Run 12	5.556	3.519	55.73	0.1819	52.03	0.145	0.04677	-1.334
Run 13	6.061	3.583	55.15	0.2056	51.36	0.1069	0.04709	-1.317
Run 14	6.566	3.629	54.56	0.2294	50.7	0.07495	0.04715	-1.3
Run 15	7.071	3.66	53.96	0.2532	50.05	0.04828	0.047	-1.283
Run 16	7.576	3.679	53.36	0.2768	49.41	0.02601	0.04666	-1.267
Run 17	8.081	3.687	52.76	0.3003	48.77	0.007444	0.04619	-1.251
Run 18	8.586	3.687	52.15	0.3235	48.14	-0.008004	0.04559	-1.234
Run 19	9.091	3.68	51.55	0.3463	47.52	-0.02083	0.0449	-1.219
Run 20	9.596	3.667	50.95	0.3688	46.91	-0.03145	0.04413	-1.203
Run 21	10.1	3.648	50.35	0.3909	46.31	-0.04023	0.0433	-1.187
Run 22	10.61	3.626	49.75	0.4125	45.71	-0.04744	0.04242	-1.172
Run 23	11.11	3.601	49.16	0.4337	45.13	-0.05334	0.0415	-1.157
Run 24	11.62	3.573	48.57	0.4544	44.54	-0.05815	0.04056	-1.142
Run 25	12.12	3.542	47.99	0.4747	43.97	-0.06203	0.0396	-1.127

EES Commercial Version: C:\Users\hp\Documents\trial.EES - [Parametric Table]

File Edit Search Options Calculate Tables Plots Windows Help Examples

Table 1

1..100	time	A_a	X	M	N	dAdt	dMdt	dNdt
Run 75	37.37	1.952	25.92	0.9563	23.01	-0.04773	0.004006	-0.5901
Run 76	37.88	1.928	25.6	0.9582	22.72	-0.04715	0.003606	-0.5825
Run 77	38.38	1.905	25.29	0.9599	22.42	-0.04657	0.003216	-0.575
Run 78	38.89	1.881	24.98	0.9614	22.14	-0.046	0.002835	-0.5676
Run 79	39.39	1.858	24.67	0.9628	21.85	-0.04543	0.002464	-0.5603
Run 80	39.9	1.835	24.37	0.9639	21.57	-0.04488	0.002101	-0.5531
Run 81	40.4	1.813	24.07	0.9649	21.29	-0.04433	0.001747	-0.5459
Run 82	40.91	1.791	23.77	0.9657	21.02	-0.04378	0.001401	-0.5389
Run 83	41.41	1.769	23.48	0.9663	20.75	-0.04325	0.001064	-0.532
Run 84	41.92	1.747	23.19	0.9668	20.48	-0.04272	0.0007349	-0.5251
Run 85	42.42	1.726	22.91	0.9671	20.22	-0.04219	0.0004141	-0.5184
Run 86	42.93	1.704	22.63	0.9672	19.96	-0.04167	0.0001012	-0.5117
Run 87	43.43	1.683	22.35	0.9672	19.7	-0.04116	-0.0002038	-0.5051
Run 88	43.94	1.663	22.08	0.967	19.45	-0.04066	-0.0005012	-0.4986
Run 89	44.44	1.642	21.81	0.9667	19.2	-0.04016	-0.0007911	-0.4922
Run 90	44.95	1.622	21.54	0.9662	18.95	-0.03967	-0.001074	-0.4859
Run 91	45.45	1.602	21.27	0.9656	18.71	-0.03918	-0.001349	-0.4796
Run 92	45.96	1.583	21.01	0.9648	18.46	-0.0387	-0.001617	-0.4735
Run 93	46.46	1.563	20.75	0.9639	18.23	-0.03822	-0.001878	-0.4674
Run 94	46.97	1.544	20.5	0.9629	17.99	-0.03775	-0.002133	-0.4614
Run 95	47.47	1.525	20.25	0.9618	17.76	-0.03729	-0.002381	-0.4554
Run 96	47.98	1.506	20	0.9605	17.53	-0.03683	-0.002622	-0.4496
Run 97	48.48	1.488	19.75	0.9591	17.31	-0.03638	-0.002857	-0.4438
Run 98	48.99	1.47	19.51	0.9576	17.08	-0.03593	-0.003085	-0.4381
Run 99	49.49	1.452	19.27	0.956	16.86	-0.03549	-0.003307	-0.4324
Run 100	50	1.434	19.04	0.9543	16.65	-0.03506	-0.003523	-0.4269

We had solved the equation of T cell differentiation during acute viral infection in 2 separate parts. First part represents the naïve cell activation in presence of viral load and differentiation into effector T cells. Second part represents the conversion of effector cells to memory cells. We would combine these equation and EES data in GA (Genetic Algorithm) software. Thus we would have the model for both part of T cell differentiation.

Summarizing, we have developed a model that accounts for the T cell differentiation. Fitting data to this model would help us to assume the memory cell formation. Memory T cells are an important component of protective immunity against viral infections, and understanding their development will aid in the design of optimal vaccines. Thus this model would help us to decide the Antigen dose and Adjuvant dose optimal vaccine development.

OBJECTIVE

II.

II. To generate memory T cells by mimicking the natural course of infection using antigen along with TLR ligands

Course of viral infection:

Adjuvants enhance immune response towards a particular antigen. Recent researches suggested that TLR synthetic analogs can help in mimicking natural conditions. Many TLRs when immunized with model antigens have shown to generate T cell protective immune responses (Akira and Takeda).

Natural course of infection means the way in which infection progresses when a pathogen encounters the host. The load of antigen is very low when the virus has just infected the host. This is the onset of viral infection. In the initial days of infection Ag load, inflammation, effector cell numbers is very low. The cytokines like IL-12, TNF-α secreted which will inhibit the viral replication. With the progression of infection, the virus begins to replicates so the Ag load will be increased. This condition will lead to the activation of natural killer cells (NK cells), which kills virus-infected cells and it activates the T cells. After 2-3 days of infection the Ag loads increases. By this time naïve T cells activated and proliferated into large number of effector cell pool. This effector cells encounters the Ag load, and after 6-7 days Ag load goes down which indicated the clearance of pathogen from the host. After that most of the effector cells enters the contraction phase in which infected cells are eliminated, while some of them survive and generate long lived memory T cells. This memory cells rapidly activated to protect the host from re-encounter of pathogens (Park et al.).

Protective Antigen Domain 4 (PAD4):

The anthrax bacteria (*Bacillus anthracis*) produces a toxin which consists of the three proteins, the Protective Antigen (PA), Lethal factor (LF) and the Edema Factor (EF). Protective Antigen (PA) is a cell binding moiety and it acts as carrier to translocate lethal factor and edema factor. Protective Antigen (PA) binds to the host cell receptors. (Baillie, L. W., Huwar et al, 2010.) after binding to receptor, PA is cleaved into two fragments by cell surface protease, (a) 20kDa fragment, (b) 63kDa fragment. 20kDa fragment dissociates within extracellular milieu while 63kDa fragment is oligomerized into heptameric structure called prepore. This prepore creates binding sites for Lethal Factor (LF) and Edema Factor (EF), two enzymatic moiety which along with PA constitutes anthrax toxin (Papatriantafyllou et al.).

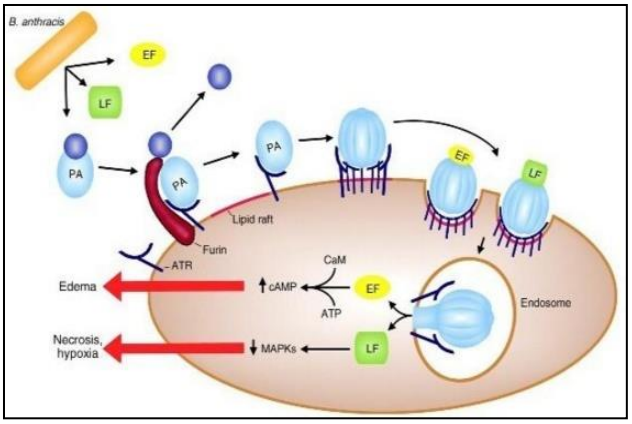

Figure 18 - Mechanism of Protective Antigen Domain

Protective antigen (PA) is a monomer and PA has four main domains.

Domain 1 - N-terminal Domain containing 2 Ca^{+2} ions and cleavage site for activating proteases. Domain 2 - a heptamerization domain containing a large flexible loop implicated in membrane insertion.

Domain 3 - a small domain and the function is unknown.

Domain 4 - a Carboxy terminal receptor binding domain. It plays an important role in the cellular receptor recognization and a pH dependent pore formation. It comprises of 596 to 735 amino acids.

Domain 4 is Immunodominant domain of PA. I want to mimic the natural course of infection using pathogenic Ag, So I am using PAD4 as an antigen. Administration of PAD4 with the adjuvant dose generates better Th1/Th2 response and it also generates PAD4 specific antibodies (Manish et al.).

Based upon this, I would like to inject PAD 4 protein with the adjuvants in manner that mimic the natural course infection and may generate memory CD4 T cells which leads to generate of long term protective immunity.

Immunization strategy:

To mimic natural course of viral infection mice are immunized with the combination of TLR ligands (Poly I: C and R848) and the PAD4. The mice are sacrificed on day 60 post immunization.

Previous studies have shown the positive result of mimicking the natural course of infection using ova as an antigen. To mimic the viral infection we are using the PAD4 antigen as it generates a protective CD4 T cells immune response.

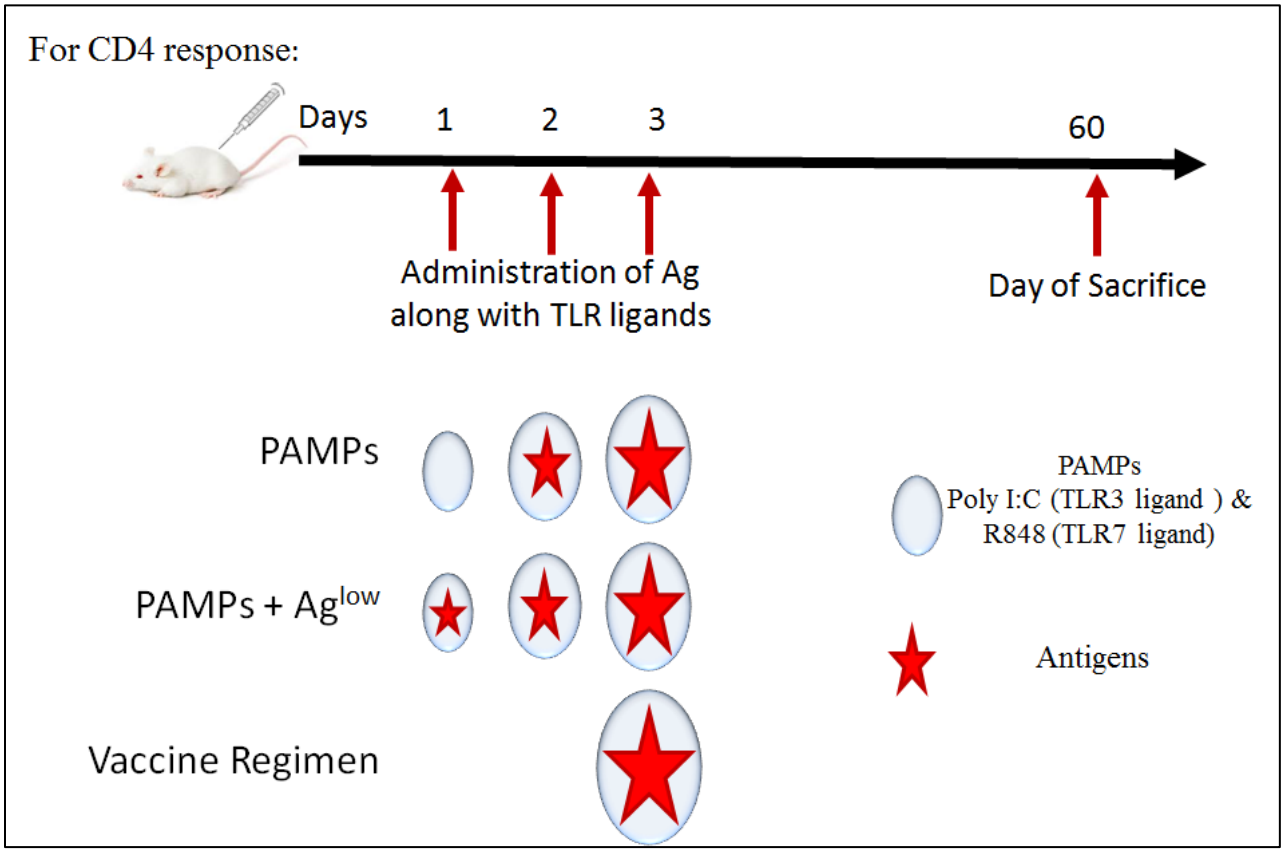

Figure 19- Immunization strategy for PAD4 study

To mimic natural course of viral infection mice are immunized with the PAD4 antigen and TLR ligands (Poly I: C and R848). The concentration of antigen and inflammation in three different groups is shown in table. The antigen and adjuvant dose is increasing day by day to mimic natural course of infection.

Group	Day 1	Day 2	Day 3	No. of mice sacrificed on Day 60
1	Poly I:C + R848 **(15** μg **+15** μg **)**	Poly I:C + R848 + PAD4 **(20** μg **+ 20** μg + **10** μg **)**	Poly I:C + R848 + PAD4 **(30** μg **+ 30** μg + **50** μg **)**	**3**
2	Poly I:C + R848 + PAD4 **(15** μg **+15** μg +**3** μg **)**	Poly I:C + R848 + PAD4 **(20** μg **+20** μg + **7**μg **)**	Poly I:C + R848 + PAD4 **(30** μg **+ 30** μg + **50** μg **)**	**3**
3	**-**	**-**	Poly I:C + R848 + PAD4 **(65** μg **+ 65** μg + **60** μg **)**	**3**

Figure 20 antigen and adjuvant dose in PAD4 study

Route of immunization: Subcutaneous

Mice: BALB/c

The immunization strategy includes use of PAD4 Ag along with TLR ligands to mimic natural course of infection and vaccine regimen. First two groups (RP, RPD) mimic natural course of infection. In RP group, the host was immunized with only TLR ligands without antigen on day 1 and increasing doses of antigen and TLR ligands on subsequent two days. In RPD group, host was immunized with increasing concentration of Ag and TLR ligands for 3 days. In Vaccine group, all the components (TLR ligands and Ag) were administered at one shot.

Mice were sacrificed on day 60 post infection to examine memory CD4 T cell response, IFN-γ production.

MATERIALS

1. Mice

   BALB/c mice (6-8 weeks old)) from Zydus Research Center, Ahmedabad.

   These animals were housed in Animal house facility, ISNU.

   All experiment were performed in accordance with protocol approved by Animal care and use committee of Nirma University.

2. Immunization:
   - Model Ag : PAD4 antigen (Stock: 800µg/ml)
   - TLR Ligands :
     - Poly I:C (Invivogen) (Stock: 20 mg/ml)

       25mg of Poly I:C from stock was dissolved in 1.25ml of endotoxin free physiological buffer
     - R848: Sigma (cat# SML-0196) (Stock:- 10 mg/ml)

       4mg of R848 from stock was dissolved in 1ml of endotoxin free water

3. Dissection of mice:
   - Autoclaved dissection tools
   - Wax tray
   - 1% complete RPMI media

4. Cell Surface Staining
   - 1% Complete RPMI Media
   - PAD4 antigen
   - FACS Buffer – 1% FCS serum in PBS
   - Fc Block
   - 1% Formaldehyde (1% HCHO)

5. Intracellular Cytokine Staining
   - Brefeldin A

- FACS Buffer – 1% FCS in PBS
- Fc Block
- Ic Fix
- Perm Wash buffer
- 1% Formaldehyde
- Fluorescently Labeled Antibodies (Appendix)

6. Petri Plates
7. Autoclaved Frosted Slides
8. 96 Well Micro titer Plates
9. CO_2 incubator
10. Centrifuge
11. Flow Cytometer
12. Data Analysis Software : Flow –Jo

METHODS

Cell Surface Staining:

1. **Harvesting of cells :** Inguinal lymph nodes and spleen harvested from BALB/c Mice.
2. Inguinal Lymph nodes and spleen were harvested and minced in sterile 1% RPMI media. To pellet down the cells, spin the cells at 1000 RPM for 10 min at 10ºC.
3. The cells were resuspended and then spleen cells were treated with 2ml RBS lysis solution. Kept it for 3 minutes. Repeated the washing step using 1X PBS (or 1% RPMI).
4. Dissolved the pellet in complete RPMI media.
5. Cells were counted under cell counting chamber and after that make appropriate dilutions to add almost 0.5 million cells.
6. **In vitro Antigen stimulation:** 0.5 million cells per well was added in each well of 96-well plate. Desired number of wells were prepared according to the need.

   Note: For in-vitro antigen stimulation, antigen dilutions were made in complete media and make up 200µl volume in each well).
7. Plates were incubated at 5% CO_2 and 37ºC in CO_2 incubator for 18 hours.
8. After incubation, centrifuged the plates at 300-400 x g for 3-4 minutes and then discarded the media directly by inverting the plate without disturbing the pellet.

   Note: At the base of each well, clear the pellets were seen.
9. 200µl staining buffer was added in any one well and then the cells were pulled in one well. Centrifuged the plates at 300-400 x g for 3-4 min, the media was discarded directly by inverting the plate without disturbing the pellet.
10. The pellets were resuspended in minimum volume (approx. 10µl). After that Fc-block was added. Mixed it by gentle tapping, and incubated the plates on ice for almost 10 minutes.

    Note: Fc-block used to block the immunoglobulin of Fc-receptors.
11. 10µl of antibody cocktail was added and mixed it by gentle tapping on the plate. Incubated the plates on ice for 30 to 40 minutes in dark.

    (Dilutions are made in such a way that Fc-block is eventually diluted 1:100 times and antibodies are diluted 1:150 times in the suspension of cells.)

12. After incubation 200µl of staining buffer (FACS buffer) (1% FCS in PBS) was added. Centrifuged the plates at 300-400 x g for 5 minutes. Pellet obtained was washed once again with staining buffer (FACS buffer) and spin it same as above.
13. After centrifugation, pellets were re-suspended in 400µl of staining buffer with 1 % Formaldehyde.

    (1 % Formaldehyde used for the fixation of the cells.)
14. Acquired samples using flow-cytometer, then the results were analyzed using FlowJo software

Intracellular Cytokine Staining:

1. **Harvesting of cells:** lymph nodes/ spleen harvested from BALB/c mice.
2. Inguinal lymph nodes were minced in sterile 1% RPMI media in 15 ml tube. The cells were pellet down by spinning at 1000 RPM for 10 min at 10ºC.
3. The cells were resuspended in minimum volume and then spleen cells were treated with 2ml of RBS lysis solution and kept for 3 min. Repeated the washing step using 1X PBS (or 1% RPMI).
4. The pellet were dissolved in chilled complete RPMI media and cells were counted using thermo cell auto counter after making appropriate dilutions.
5. **Antigen stimulation:** 0.5 million cells per well was added in each well of 96-well plate, in 200 µl complete media and the desired number of wells were prepared according to the need.

   (**Note:** For in-vitro antigen stimulation, antigen dilutions were made in complete media and make up 200µl in each well).
6. Plates were incubated at 37ºC and 5% CO_2 for 15 hours for intracellular cytokine staining.
7. **Brefeldin A** 1000X solution (5.0mg/ml stock): BFA was added, Incubated the 96-well plate at 37ºC in 5% CO_2 for 3 hours.
8. After final wash of cell surface staining, aspirated the supernatant and agitated the 96-well plate to disrupt cell pellets (Only gentle tapping of plate is enough).
9. 100 µl of **Cytofix/Cytoperm** Buffer was added to each sample well. The plates were incubated at room-temperature for 20 minutes. This step will fix the mouse cell morphology and permeabilized the activated cells for subsequent intracellular staining.
10. 100 µl of **1X Perm/Wash** buffer added to each well and centrifuged the plate at 400-500 x g for 5 min at 10°C.
11. After centrifugation, aspirated supernatant from each well and agitated plate to disrupt cell pellets.
12. Repeat step 10-11 above.
13. Added Purified Blocking Antibody Cocktail (**Fc-Block**) to the desired sample wells in 20µl aliquots. 30 µl of Perm/Wash buffer was added to the same sample wells, each well should now have a total volume of 50µl.

14. Conjugated **Isotype Control Cocktail** and conjugated **anti-cytokine antibodies** were added to the desired sample wells in 20µl aliquots. Additionally 30µl of Perm/Wash buffer was added to the same sample wells, total of 50µl of volume is added to each well.
15. 50µl of BD Perm/Wash Buffer was added to the sample wells designated as auto-fluorescence controls.
16. Incubated the 96-well plates for 30 minutes at 4ºC or on ice.
17. 100µl of Perm Wash Buffer was added to each sample well and centrifuged the plate at 400-500 x g for 5 min at 10°C.
18. Aspirated the supernatant and agitated plate to disrupt the cell pellets.
19. Repeat step 17-18 as above.
20. Transferred the contents of each well into tubes using 200µl of FACS Buffer (**1% FCS in PBS**). Bring the final volume in each tube to 400 µl using FACS Buffer with 1% formaldehyde to fixed the cells.
21. Acquired samples using flow-cytometer, then the results were analyzed using FlowJo software.

    (Lamoreaux, Roederer, and Koup,2006)

RESULTS

&

DISCUSSION

RESULTS AND DISCUSSION

Generation of CD4 T-cell response by mimicking natural course of viral infection

In the present study, mice were immunized with combinations TLR ligands along with model Ag PAD4. TLR ligands used in our study are R848 and Poly I:C. Here, we had compared 3 different groups as shown in the immunization strategy. The first two groups are a mimic of viral infection and the third group represents vaccine regimen. The mice were sacrificed on day 60 post immunization and CD4 T cell response was measured.

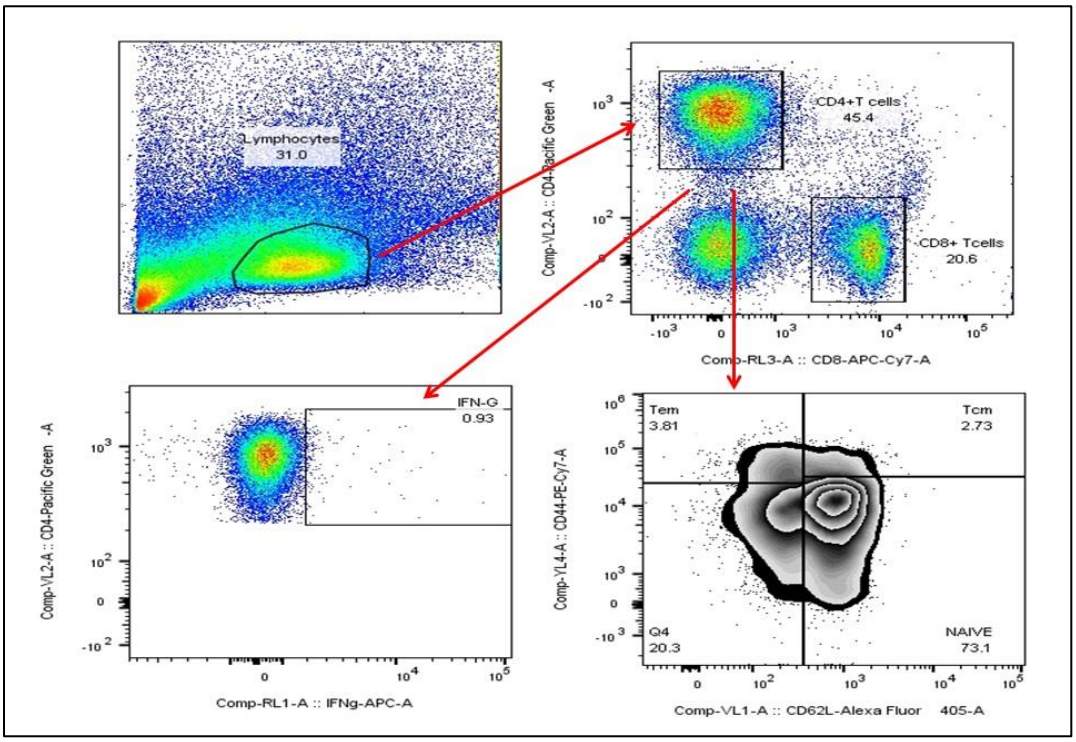

Fig : Schematics for CD4+ Memory T cell Response

Analysis of T cell assay using Fluorochrome labeled antibodies is shown in the figure. Side scatter describes about granularity of the cells and forward scatter describes about the size of cells. Lymphocytes have small size and relatively less granularity thus it shows low side scatter and an intermediate forward scatter. Lymphocyte population had been gated from whole cell

population. Further CD4 and CD8 T cell population had been gated from the lymphocytes population. PAD4 antigen generates CD4 specific response. So CD4 population was further gated to see the central memory cells and effector memory cells by using Pecy7 (for CD44) and V_{450} (CD62L) conjugated antibodies. CD4 population was further gated to see the IFN-γ production.

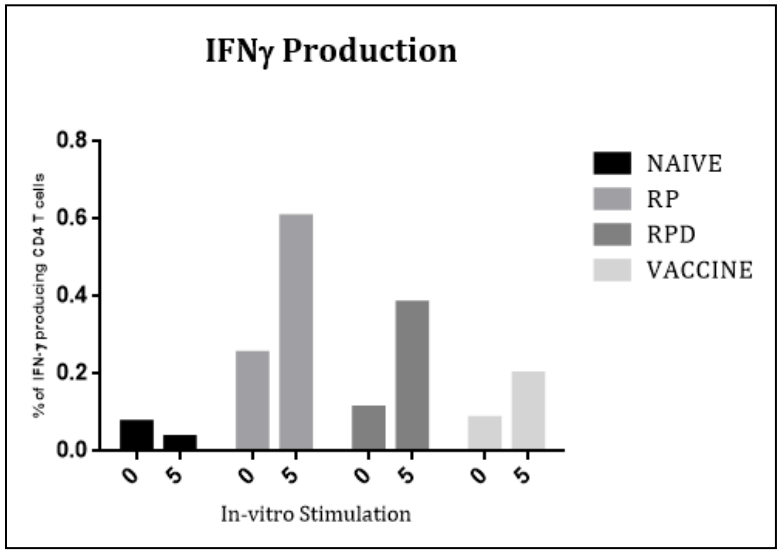

Figure 21 - IFN γ production in CD4 T cells

Fig : To measure CD4+ T cell response, lymphocytes from lymph nodes (Inguinal LNs) were harvested on Days 60 post immunization and stimulated in-vitro with PAD4 Ag (0.5µg/ml) for 15 hours and 3 hours with golgi stop (Brefeldin A). Cells surface staining and intracellular staining was done with flourochrome conjugated antibodies for cell surface markers CD4, CD8, CD44 and CD62L and for IFN γ production and acquired on FACS Caliber. Graph represents frequencies of IFN γ production in stimulated and unstimulated samples .

In this experiments day 60 represents the actual memory phase. Lymphocyte population had been gated from whole cell population. Further CD4 and CD8 T cell population had been gated from the lymphocytes population. This would generate higher number of PAD4 specific CD4 T cell compared to CD8 T cells.

IFN γ production in CD4 T cells:

Here, we had compared four different groups, naïve, RP, RPD, Vaccine. To measure CD4+ T cell response, lymphocytes from lymph nodes (Inguinal LNs) were harvested on Day 60 post immunization and stimulated in-vitro with PAD4 Ag for 18 hours. Cells surface staining and intracellular staining was done with flourochrome conjugated antibodies for cell surface markers

CD4, CD8, CD44 and CD62L and for IFN γ production and acquired on FACS Caliber. Graph represents frequencies of IFN γ production in stimulated and unstimulated samples.

IFN γ production is compared in four different groups for both stimulated and unstimulated conditions. The above graph indicates that there would be high IFN γ production in in-vitro stimulated samples compared to unstimulated samples. There would be non specific IFN γ production in unstimulated samples.

IFN γ production in CD4 T cells (in-vitro stimulated)

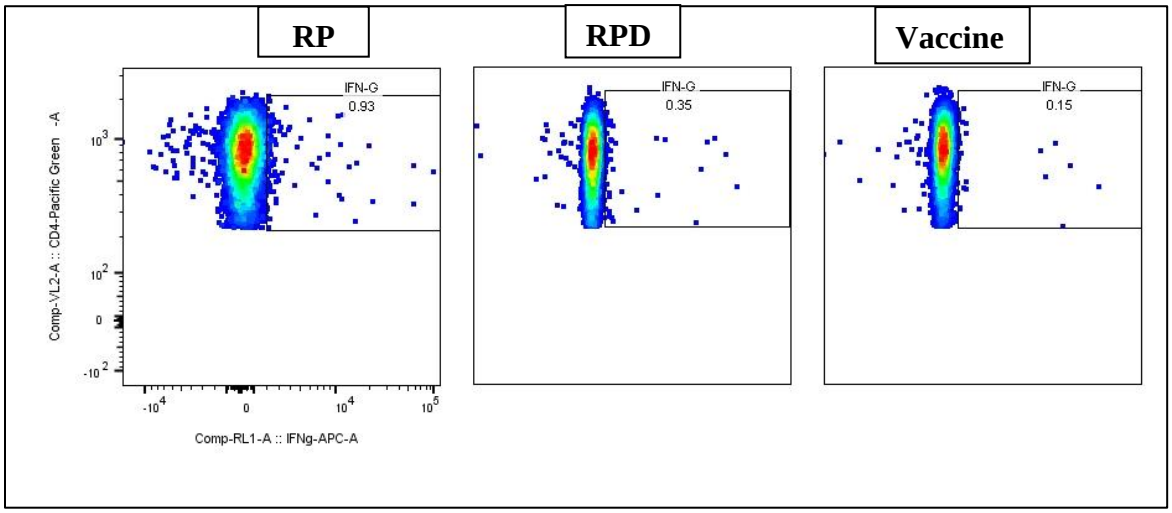

Figure 22 - Schematics of IFN γ production in CD4 T cells (in-vitro stimulated)

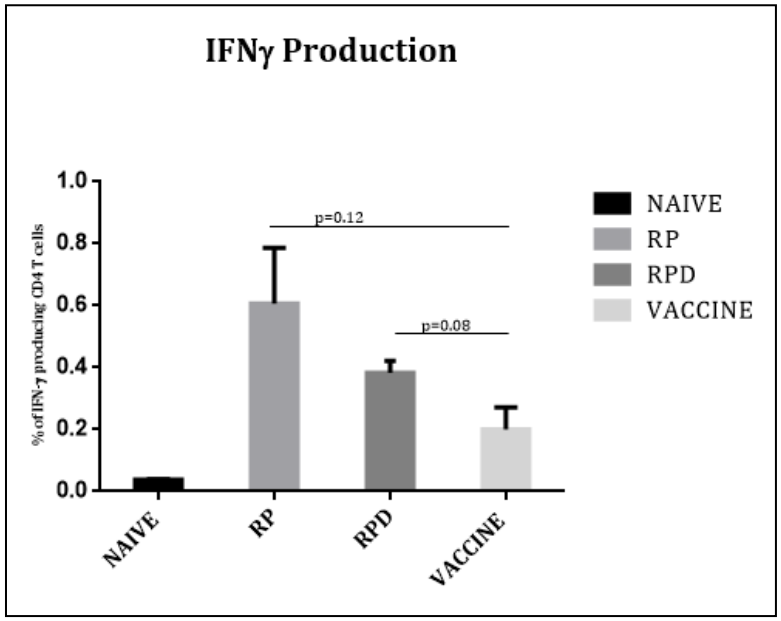

Figure 23 IFN γ production in CD4 T cells (in-vitro stimulated)

Graph represents frequencies of IFN γ production for in-vitro stimulated samples. The above graph indicates that there would be high IFN γ production in natural infection compared to vaccine group. RP group shows maximum IFN γ production. Hence, in a way we can correlate that after antigenic stimulation effector and central memory cells are reactivated, which would lead to generation of good number of effector cells. This effector cells might have been activated which were reflected in the IFN- γ response (sedar et al, 2008).

SUMMARY

The generation of heterogeneous pool of memory T cells following vaccination seems empirical when it comes to characterizing the immune response. Developing a model would thus help us delineate the factors favoring the generation of memory T cells, such as antigen dose and inflammatory milieu.

We have attempted to develop an Artificial Neural Network (ANN) using available published data. If we have combination of antigen dose and naïve cells recruited, this ANN would give the effector cell population. From the literature survey we found some equations. We had solved these equations of T cell differentiation during acute viral infection in two distinct parts. First part represents the naïve cell activation in presence of viral load and differentiation into effector T cells. Second part represents the conversion of effector cells to memory cells. We can potential combine these equation and EES data in GA (Genetic Algorithm) software. Thus we would have a model for both parts of T cell differentiation.

Summarizing, we have developed a model that accounts for the T cell differentiation. Fitting data to this model would help us to assume the memory cell formation. Memory T cells are an important component of protective immunity against viral infections, and understanding their development will aid in the design of optimal vaccines. Thus this model would help us to decide the optimal antigen and adjuvant dose for vaccine development strategies.

The main goal of vaccination is to generate and maintain a heterogeneous pool of long lived memory cells. But still we do not have effective vaccines against disease such as malaria, HIV, and tuberculosis. This shows that there are some defects in designing conventional vaccine strategies. The possible reason could be that conventional vaccines fail to induce immune response same as natural infection. Thus, we had proposed a strategy in which we injected Ag and adjuvants in a similar manner to the course of natural infection. We believe that injecting host with increasing concentration of antigen along with TLR ligands will mimic a condition in host which is similar to replicating pathogen as natural infection and increasing the overall antigen exposure to the immune system.

Mice were sacrificed on day 60 post infection. Cells from Inguinal lymph nodes were harvested and stimulated in-vitro to reactivate antigen specific (PAD4 specific) cells. Cells surface staining and intra-cellular staining was performed. In RP and RPD group we observed more memory cells and IFN-γ producing cells. Thus, Creating such conditions would lead to more CD4 memory cells and IFN-γ producing cells compared to vaccine regimen.

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