Elucidating the role of Gpr114 in immunological memory: Attempts at ligand identification

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A dissertation submitted for the partial fulfilment of
BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali
April 2016

Certificate of Examination

This is to certify that the dissertation titled "***Elucidating the role of Gpr114 in immunological memory: Attempts at ligand identification***" submitted by **Mr. Shubham Chauhan** (Reg. No. MS11032) for the partial full-filment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Mahak Sharma Dr. Kausik Chattopadhyay Dr. Sharvan Sehrawat

(Supervisor)

Dated: 22th April, 2016

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Sharvan Sehrawat at the Indian Institute of Science Education and Research, Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Mr. Shubham Chauhan

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In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Sharvan Sehrawat

(Supervisor)

Acknowledgement

I would like to take this opportunity to express my profound gratitude and deep regard to my (Project Guide Dr. Sharvan Sehrawat), for his exemplary guidance, valuable feedback and constant encouragement throughout the duration of this research project. His valuable suggestions were of immense help throughout my project work. Working under him was an extremely knowledgeable experience for me.

In addition, I would like to thank my committee members, Dr. Mahak Sharma and Dr Kausik Chattopadhyay as well as Dr A.K. Bachhawat and Shiven Dhiman for his valuable suggestion during the period when I have to choose between what is easy and what is right. I also thank specially to Dhanesh, Prerna, Manpreet, Nitin Dhamu, Jasreen, Ankit, Nirdosh, Nitin Kishor, Pallavi, Praveen sir and other lab members for their constant encouragement, guidance, and valuable supervision at every stage of my work.

Finally, I sincerely thank to my parents and my brother, without them none of this would indeed be possible.

Mr. Shubham Chauhan

(Reg.No. MS11032)

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Abbreviations

PCR	Polymerase Chain Reaction
CD	Circular Dichroism
GuCl	Guanidinium chloride
SDS	Sodium Dodecyle Sulphate
PAGE	Poly-acrylamide Gel Electrophoresis
DNA	Deoxyribose nucleic acid
TAE	Tris-Acetate-EDTA Buffer
EtBr	Ethidium Bromide
ECD	Extra-cellular Domain
FL	Full Length Domain
PMSF	Phenylmethylsulfonyl Fluoride
PBS	Phosphate Buffer Saline
AGE	Agarose Gel Electrophoresis

INTRODUCTION

Immunity is the protective mechanism of our body. Unlike Innate, adaptive immunity is highly specific and can adapt itself to a variety of antigens. Adaptive immunity is effected by predominantly two branches of immune system: humoral wherein B cells expressing an antigen-specific receptor (BCR) and respond to exogenous unprocessed antigens and cell mediated immunity wherein T cells expressing specific receptor recognize and respond to processed antigen in context with appropriate class of MHC molecule. Adaptive immunity is acquired overtime after exposure to inducing agents or foreign substances. Immunological memory is the hallmark of the acquired immunity and is required for long lasting protective immunity against infectious disease. In fact the one of the crucial aims of vaccination is to increase the quality and quantity of memory cell population. CD8 T cell constitute a critical component of the host defense against cancers and intracellular infections caused by the viruses. Immunogenic peptides are generated within the infected cells predominantly via a proteasome pathway and are then loaded onto appropriate Class I MHC molecule to be displayed onto the surface of infected cells. By sensing these peptides, CD8 T cells get activated and eventually try to kill the infected cells. In so doing the infectious agent is also removed from the system.

In order to activate, CD8 T cell required at least three signals -Signal 1 comes from interaction between T cell receptor and peptide, which are loaded, on the MHC molecule. Signal 2 comes from the pairing of the appropriate co- stimulatory molecule B7.1, B7.2 on antigen presenting cells and CD28 molecule on naïve T cell. Signal 3 comes from the cytokines, which are secreted from Antigen presenting cells. CD8 T cells get activated after receiving these 3 signals and expand enormously. The activated CD8 T cells then kill Target cells to eliminate the intracellular pathogens. After the primary response, most of the effector cells die by apoptosis. The remaining T cell population, which is specific for that particular antigen, will remain and become memory cells. Till now, it is not unambiguously known that out of this cell population that has responded to antigen, which cell population will die or which will live to become long term memory CD8T cells. Therefore, it is important to understand what molecular cues help to determine the cell fate decision. It is necessary to understand the memory T cell differentiation for limiting T cell response against some

disease and in future it will provide a path for designing vaccines that can provide long-term immunity against various infections.

It is important to understand downstream signaling events during T cell activation as these events are responsible for differentiation of effector and memory cells. These initial events are known to control long-term cell fate determination. Many transcription factors such as T-bet, Eomes, Blimp1, Bcl6 and ID2 are induced in CD8+ T cell, which have a role in the differentiation of effector and memory population.

One of the key questions in cellular immunology is which cells are chosen to become long term memory cells out of the cells that responded initially. Several models have been proposed to explain the memory T cell differentiation or developmental fates of CD8 T cells. Model one shows a divergent pathway in which stimulation of naïve T cell with antigen give rise to daughter cells that will develop into either effector cells which die after pathogen is cleared or effector memory T cell which survive to stay in the system. This effector memory T cells (T_{EM} cells) which reside mainly in non-Lymphoid tissues, do not express CCR7 and express very low level of CD62L (L-selectin) and have immediate effector function. This model suggests that naïve T cells can bypass an effector cell stage and can develop directly into memory T cells. Second model states a linear differentiation pathway where effector cells give rise to memory T cells. In other words, memory T cells are direct descendants of effector cells. Another proposition of this model is that transition to memory CD8 T cells does not occur until antigen is removed or greatly decreased in concentration. The third model suggests that a short duration of antigenic stimulation favors the development of central memory T cells, which reside in the lymphoid organs, whereas a longer duration of stimulation favors the differentiation of effector and effector memory T cells. Another model which is probably an extension of third model is known as decreasing potential hypothesis and it suggests that if antigen persist for a longer period of time such as during chronic infections then the effector functions of T cells decrease with many encounter with antigen and eventually effector cells become more susceptible to the apoptosis. Increased apoptosis of effector cells lead to a reduced number of memory T cells.

Recent studies shows that the memory T cells can also develop without passing an effector cell stage and those memory T cells are called central memory T cells. Different priming

condition for example the duration of antigen stimulation and the type of cytokine present can also affect the formation of these subsets. Presently, the idea of memory T cell development is based on the finding that Memory T Cells can be divided into subsets, which can be distinguishable, by the expression of homing molecule and chemokine receptors as well as cytokine receptors. Central memory T cells (Tcm) reside in lymphoid organ and express CCR7 and CD62L whereas Effector memory T cell (TEM) cells reside mainly in non-lymphoid tissue, and they do not express CCR7 and CD62L and have immediate effector function. These central and effector memory T cells have different functional roles and abilities in conferring protection against different antigen encounters. It has also been shown that, as the time passes after immunization, the population of cells, which are CD62L high, increases within the memory population because of the higher Naïve CD8+ T cell precursor frequency as more cells emerge from thymus.

Antigen persistence and its localization have a huge impact on the phenotype, functionality and survival/maintenance of the antigen specific CD8+ T cell. Our efforts in lab focus on understanding the mechanisms that is crucial for the cell fate decision. By understanding this, we can develop strategies to increase the number of memory T cells by vaccination. As outlined earlier, this is an active area of research and has many clinical implication/application.

It has been shown that after the contraction phase, whatever the memory T cell are left express a high level of interleukin 7 receptor than terminally differentiated effector T cell.(Susan.et.al) .This group also established that IL-7R could serve as a marker of memory CD8 T cells at least during acute viral infections. We performed a transcriptional profiling of virus-specific Rag1 deficient naïve and virus-stimulated CD8+ T cells using RNA sequencing. The results are shown in figure 1. Based on the analyses of our RNA Seq data set, we plan to investigate the role of Gpr114 during differentiation of CD8+ T cells as Gpr114 showed a massive down regulation upon T cell activation (Figure 1) and the kinetics of its expression exhibited a specific pattern similar to that of IL-7R, a known marker of memory CD8+ T cells (www.immgen.org).

GPCR is the largest superfamily of receptors in the mammalian genome. These receptors are present on many cells and can recognize a variety of extracellular signals. For some of the GPCRs, receptors have been identified yet a large numbers of them still remain as orphan. Today more than 50% newly discovered drugs target these receptors.

Many member of adhesion GPCR (ad-GPCR) family are involved in developmental process and in many human diseases. Ad-GPCRs have ubiquitous expression and are present in a wide variety of tissues. Ad-GPCR are one of the class of GPCR which is poorly understood and it is a second class of GPCR. Ad-GPCRs have seven transmembrane alpha helices like other GPCRs and are characterized by large extracellular N-termini which have different structural domain like EGF like domain, cadherin, thrombospondin, immunoglobulin, leucine rich repeats etc. This N terminal is linked to the trans membrane region via GPCR- Auto proteolysis Inducing (GAIN) domain which is a 320 residue domain. Within this GAIN domain there is a GPS motif which is a part of GAIN domain and this is around 40 residue long. The GPS site induce the cleavage of the receptor during its maturation leading to the generation of N terminal which is extracellular alpha subunit and a C terminal which is transmembrane β subunit. Subsequently, NTF and CTF remain non -covalently associated as heterodimers at the plasma membrane. The adhesion like motifs on the N terminal makes contact with the cellular or extracellular matrix associated molecule. Gpr114 is also a member of the adhesion Gpcr family. The expression of Gpr114 can be seen in tumor and several type of cancer at transcript level .The protein is composed of the 524 aa and extracellular domain has 222 aa. Recent studies shows that, if we truncate the N-Terminus, surprisingly they found that the receptor will become functional and downstream signaling will happen and it can lead to the high ubiquitination and binding of arrestin can take place which shows the enhanced constitutive activity of these GPCRs. It could also mean that N-termini region is providing an inhibitory effect on the signaling of these receptors. Till Date, most of the adhesion GPCRs are the orphan receptor and Gpr114 is one of them.

Identification of the ligand of Gpr114 could provide insight into the function of the receptor during homeostasis conditions as well as during the course of an infection. We designed studies to identify the endogenous ligand for gpr114 and to decipher the downstream signaling events as a result of result of ligand receptor interaction. By deorphanizing this receptor, we can understand the signal transduction pathway in a much better way so that we

can modulate the signaling and can boost the magnitude and functional superiority of memory CD8 T cells. Furthermore this will also help understand of the cell fate decision. In future it is important to understand about the epigenetic changes, chromatin modification, Intracellular molecule, transcription factors and the role of the niche in which the T cell resides, signaling mechanism which is happening in the Naïve T cell. We can also increase the effectiveness of vaccine by using the fact that the burst size determine the number of memory T cell.So,we can target these stages of the T cell response to increase the vaccine efficiency.

We can target the contraction phase in order to increase the number of memory T cell by decreasing the death of the effector cells. But for that we need to understand the signaling mechanism which is one of the crucial factor which regulates the T cell differentiation. By understanding the Gpr114 signalling which can be a marker of memory T cells, we can target the contraction phase in order to increase the number of memory T cell. By understanding this it will enhance the efficacy of vaccine and can provide long term immunological protection. It is important to understand the mechanism of this cell fate of these memory T cell to make a better vaccine.

2. Materials and Methods:

A. Transcriptomic profiling of naïve and activated viral specific CD8 T cells.

$5x10^4$ MHV68 specific $CD8^+$ T cells (ORF8 TN cells) from CD45.2 mice were adoptively transferred to CD45.1 congenic mice. After one day of cell transfer these mice were infected with MHV68 with $5x10^{5\ pfu}$ in footpad. On day 5 post infection the antigen specific $CD8^+$ T cells from CD45.2 background were analyzed by Flow cytometry to check their activation state, whereas sorted naïve and activated cells were analyzed by RNA sequencing. Specifically we focused on those transcripts that were down regulated in upon activation and then exhibited a pattern of expression similar to that of IL-7R, a known marker of memory CD8 T cells during some viral infections. This was done using data set available at www.immgen.org. The one of the transcript Gpr114 was chosen for further deciphering its role in differentiating CD8 T cells.

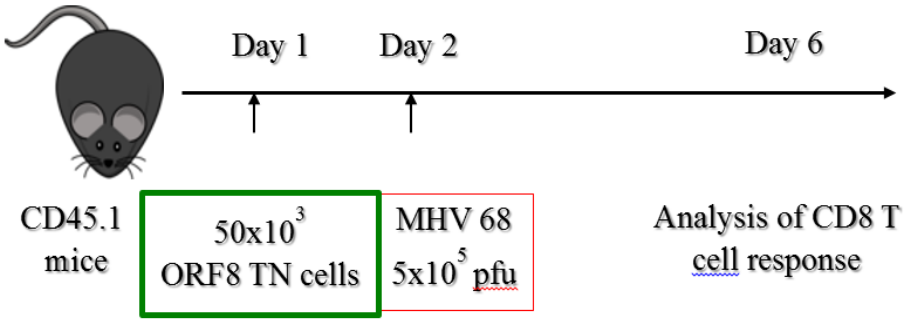


Fig 1- Schematic for obtaining RNA seq data

B. Cloning of Gpr114

Full length and extracellular domain of Gpr114 were cloned into pET22b (+) for expression of protein, its purification and ligand identification. Extracellular domain with 6xHIS residues at C terminal was used for ligand identification. Simultaneously Full length of gpr114 were cloned in to pcDNA3.1 (+) plasmid to study Gpr114 mediated signaling mechanism upon its transfection in eukaryotic cells such as Hela cells and 3T3 cells.

For cloning, total RNA from lymph node of naïve C57BL/6 mice was isolated. Then cDNA was synthesized using a first strand synthesis kit from Takara. cDNA was then used for amplification of full length as well as extracellular domain of gpr114 gene. This PCR amplicon for ECD was cloned into pET 22b (+) vector and full length amplicon as cloned in pcDNA3.1 (+) plasmid. After confirming sequencing results for any mutation that may lead to stop codon formation amplified full length and extracellular domain were cloned into pET22b (+) vector. The steps that were followed for cloning include as follows:

a) RNA Isolation

Lymph nodes isolated from naïve mice were stored in RNA Later to avoid the RNA degradation during tissue transportation and subsequent steps. Total RNA was isolated using TRIZOL method using following steps.

1) Homogenize the tissue sample (50 to 100mg) in TRIZOL reagent by passing it through insulin syringe needle.
2) Mixed the solution properly by repeated pipetting.
3) Then, 0.2 ml of chloroform per 1 ml of TRIZOL reagent was added. The tube was shaken vigorously by hand for 20 second and then incubated for 2 or 3 minutes.
4) The samples were centrifuged at 12,000xg for 15 minutes at 4 ^{o}C.
5) After centrifugation, the mixture got separated into lower red phase consisting of phenol-chloroform, an interface and a colorless upper aqueous phase. As RNA remains exclusively in the aqueous phase, upper aqueous phase was carefully removed without disturbing the interphase and transferred into a fresh tube. The volume of the aqueous phase was measured.
6) According to the volume of aqueous phase, isopropanol (0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent used for the initial homogenization) was added.
7) The tube was kept at -20^{o}C for 1 hr for RNA precipitation.

8) Then the tube with the content was centrifuged at 12,000xg for 15 min at 4 ^{o}C. A pellet at the side bottom of the tube was observed.

9) The supernatant was removed completely and carefully leaving pellet intact.

10) The pellet was then resuspended in 75% ethanol per 1 ml of TRIZOL Reagent that was used for the initial homogenization. The content in the tube was then centrifuged at 7500xg for 5 minute at 4 ^{o}C.

11) Ethanol was removed and the pellet was air dried for 5-10 minutes.

12) The RNA pellet was dissolved in DEPC treated water.

13) Quantification of RNA was done using spectrophotometer and the integrity of RNA was ascertained by running on agarose gel.

b) Preparation of cDNA

1) A reaction mixture with the following composition was prepared in a microcentrifuge tube.

Reagent	Volume
OligodT Primer (50µM) or	0.5ul
Random hexamers (50 µM)	0.5ul
dNTP Mixture (10 mM each)	1ul
Template RNA	5µg
RNAase free dH2O	
Total	10 µl

2) The mixture was incubated at 65ºC for 5 min., then cooled immediately on ice which served as a template for cDNA synthesis as described in point 3.

3) The reaction mixture was prepared in in a total volume of 20 µl.

Composition:

Template RNA Primer Mixture from step 2)	10µl
5x Prime Script Buffer	4 µl
RNAse Inhibitor (40U/µl)	0.5 µl (20 units)
PrimeScript RTase (200U/µl)	1.0 µl (200 units)
RNAse free dH2O	x µl
Total	20 µl

4) The content was mixed gently and incubated at following conditions.

30 ºC	10 min (required when using Random6mers)
42 ºC	30-60 min

5) The enzyme was incubated at 95ºC for 5 minutes and then cooled on ice.

c) Polymerase chain reaction (PCR) to amplify Gpr114

Extracellular domain (ecd) and full length (fl) of gpr114 DNA were amplified by PCR using high fidelity *fusion* DNA polymerase enzyme. After scaling up the reaction for 150µl the PCR products were run separately by Agar gel electrophoresis (AGE) and specific product was extracted and purified from the gel. The primers for amplification of different inserts are mentioned as follows.

1) For ecd into pET22b (+)

i. Forward Primer

5' GAT AAC TCC ATA TGG AAA CCC TGT CAG ACC TAC TTG TCT TGA TGA AGC 3’

ii. Reverse Primer

5' TAT ACT CGA GCT CAA GAG GCA CCT GCA GCT CAG CGG 3'

Sequence (5’ to 3’):

5’GATAACTC**CATATG**GAAACCCTGTCAGACCTACTTGTCTTGATGAAGCGCCTT
GAGCAGCCCGTAGGCCGGGGCTTGTCTTCCAGAGCGAGGCATATCCACAGCCTG
GAGCAGAAGCTGCTCAATGCCAGCTTCGGCGGGCACAACTTGACCTTGCAGACA
AACTCCATCCAGTCTCTTGTCTTCAAGCTGAGCTGCGACTTTCCTGGTCTTTCCCT
GTCCAGCACCACACTGACAAATGTCTCCCAGGTGCGAGCCCCACATGCCATGCA
ATTCCCTGCTGAGCTGACCAAGGGTGCCTGTGTGACCTCCCGGCCTGCTGAGCTT
CGACTCATCTGCATCTATTTCTTCACTGCACACCTCTTTCAGGATGACCGGAACT
CATCACTGCTCAATAACTATGTCCTGGGGGCCCAGCTGGATCACAGGCCTGTGA
ACAACCTTCAGAAGCCAGTCAACATCAGCTTCTGGCATAATCGGAGTCTGGAAG
GGTATACAGTATCCTGTGTTTTCTGGAAGGAGGGAGCCAGCAAGAGCAGCTGGG
GGGCCTGGAGCCCTGAGGGCTGTTATACAGAGCAGCCCTCAGCCACCCAGGTTC
TCTGCCATTGCAACCATCTCACCTACTTCGCTGTGCTTATGCAGCTCTCTGGAGA
CCCCGTGCCCGCTGAGCTGCAGGTGCCTCTTGAG**CTCGAG**CGG 3’

-Restriction enzyme used were: 5’:- **NdeI**, 3’**:-XhoI**

-Optimized amplification was achieved at: Tm-57.6 ^{o}C

2) For full length into pET22b (+) for recombinant protein purification.

i. Forward

5'GATAACTCCATATGGATCCACACGGCGCCCTTTTCTTCTACCTGTGC 3'

ii. Reverse

5' TATACTCGAGGTGTGTCATCTGGGAGGAGCTGACTGC 3'

Sequence (5’ to 3’) :

TAACaagcttATGGATCCACACGGCGCCCTTTTCTTCTACCTGTGCCTTCTGGCTGCT
CAAGTTGTCCTAGTAGAAACCCTGTCAGACCTACTTGTCTTGATGAAGCGCCTTG
AGCAGCCCGTAGGCCGGGGCTTGTCTTCCAGAGCGAGGCATATCCACAGCCTGG
AGCAGAAGCTGCTCAATGCCAGCTTCGGCGGGCACAACTTGACCTTGCAGACAA
ACTCCATCCAGTCTCTTGTCTTCAAGCTGAGCTGCGACTTTCCTGGTCTTTCCCTG
TCCAGCACCACACTGACAAATGTCTCCCAGGTGCGAGCCCCACATGCCATGCAA
TTCCCTGCTGAGCTGACCAAGGGTGCCTGTGTGACCTCCCGGCCTGCTGAGCTTC
GACTCATCTGCATCTATTTCTTCACTGCACACCTCTTTCAGGATGACCGGAACTC
ATCACTGCTCAATAACTATGTCCTGGGGGCCCAGCTGGATCACAGGCCTGTGAA
CAACCTTCAGAAGCCAGTCAACATCAGCTTCTGGCATAATCGGAGTCTGGAAGG
GTATACAGTATCCTGTGTTTTCTGGAAGGAGGGAGCCAGCAAGAGCAGCTGGGG
GGCCTGGAGCCCTGAGGGCTGTTATACAGAGCAGCCCTCAGCCACCCAGGTTCT
CTGCCATTGCAACCATCTCACCTACTTCGCTGTGCTTATGCAGCTCTCTGGAGAC
CCCGTGCCCGCTGAGCTGCAGGTGCCTCTTGAGTACATCTCCTTTGTGGGTTGCA
GCATCTCCATCGTGGCCTCGCTGCTCACCATACTGCTGTACGCTCAGTCCAGGAA
GCAAAGCGATTCCACCACACGTATCCACATGAACCTGAATGGCTCTGTTCTGCTC
CTGAACGTCACCTTCCTTCTGAGCTCCCAGATGACCCTGCCCACCATGCCCAGGC
CCGTCTGCAAGGTGCTGGCTGCTGTCCTACACTACGCACTGCTCAGCAGCCTTAC
CTGGATGGCCATCGAAGGCTTCAACCTCTACCTTTTCCTGGGGCGTGTCTACAAC
GCCTACATTCGCCGATACTTGCTCAAGCTCTGCATGCTGGGCTGGGGGTTTCCAG
CCCTCTTGGTGCTGCTGCTTCTGATGATCAAGAGCTCAGTGTATGGACCCTGTGT
GACCTCACTCTCCAAAAGCCAGGAAAATGGCACAGGCTTCCAGAATGTGTCCAT
GTGCTGGATCCGAAGTCCCATGGTACACAGCATCCTGGTCATGGGCTATGGTGGC
TTCACATCTCTGTTCAACCTGGTGGTGCTGGCCTGGGCTCTGTGGATCTTGTGCA
GGCTTCGGGCACGGGAGAAGGCACTGAGTCCCTGGGCCTACCGGGACACTGCCA
TGGTGTTGGGTCTCACTGTGCTGCTTGGCACCACCTGGACCCTAGCCTTCTTCTCC
TTCGGTGTGTTCCTGCTGCCTCAGCTCTTCCTCTTTACCATCTTCAACTCGCTCTAT
GGTTTCTTCCTCTTCCTGTGGTTCTGCTCACAAAAGCGTTACTCGGATGCCGAAG

CCAAGGCGGAGATGGAGGCAGTCAGCTCCTCCCAGATGACACACTGAggtaccCCG
A

- Restriction enzyme used were: 5':- NdeI 3':- XhoI
- Optimal amplification was achieved at: Tm-61.2 ^{o}C

3. For full length into pcDNA3.1(+) for transfection in eukaryotic cells and deciphering signaling events

i. Forward

5' TAA CAA GCT TAT GGA TCC ACA CGG CGC CCT TTT CTT CTA CC 3'

ii. Reverse

5' TCG GGG TAC CTC AGT GTG TCA TCT GGG AGG AGC TGA CTG 3'

- Sequence (5' to 3'): Same as above
- Restriction enzyme used were: 5':- HindIII, 3':- KpnI
- Optimal amplification was achieved at: Tm-61.2 ^{o}C

d) Gel extraction and PCR purification

The gel extraction kit from Invitrogen was used, and PCR product was purified according to the manufacturer's protocol.

e) Plasmid isolation

Following plasmids was used as a vector for cloning.

- pET 22b for extracellular and full length gpr114
- pcDNA3.1 (+) for full length gpr114

f) Restriction digestion for cloning

After digestion the restriction enzymes were inactivated by incubating the reaction

1. Mixtures (insert and vector) at 65 ^{o}C for 20 minutes.

2. CIP treatment to the digested vector for 1 hour at 37 ^{o}C.

3. Heat inactivation at 65 ^{o}C for 20 minutes.

4. Digested inserts and vectors were run on agarose gel and then purified from the gel.

g) Ligation

The conditions for extracellular domain (ecd) of GPR114 for ligation into pET22b (+) vector were as follows:

- At the ratio of 1:3 and 1:5 (vector :Insert)
- The size of vector and insert were 5367 bp and 678 bp respectively.

pET22b (+) vector were as follows:

- At the ratio of 1:3 and 1:5
- The size of vector and insert were 5367 bp and 1574 bp respectively.

The conditions for full length of GPR114 for ligation into pcDNA3.1 (+) vector were as follows:

- At the ratio of 1:3 (vector :Insert)
- The size of vector and insert were 5426 bp and 1582 bp respectively.

The ligation mixtures were kept at 25 ^{o}C for 3 hour.

h) Transformation

The whole ligation mixtures transformed into *E. coli* DH5-**α** strain, and Kept the transformation plates at 37 ^{o}C for 12-16 hrs.

i) Colony PCR

Colony PCR was performed to ascertain the proper ligation. Colony PCR was performed on transformants by resuspending the diluted colony in the PCR reaction mixture.

j) Confirmation of clones by Restriction Digestion

Plasmids isolated from colony PCR positive clones, were digested with respective restriction enzymes that were used for cloning.

(C) Purification of Gpr114 extracellular domain

Gpr114 extracellular domain protein purified from SDS PAGE by Electroelution. Protein of interest was excised and placed in an appropriate cutoff dialysis membrane. The membrane with the content was then kept into an electroelution chamber of horizontal electrophoresis and an electric field was applied to remove other small molecules from the gel.

Requirements: 1M KCl, gel cutter, Dialysis bag [fill it with electrode buffer], Laemmli electrode buffer [Tris glycine ph 8.3], Horizontal electrophoresis unit, Buffer [10mM Tris-HCl, ph 8, 5M urea, 1% BME], Dialysis buffer [20mM Tris-HCl, 50mM NaCl].

1) Gpr114 protein from stock was treated in 1:1 ratio with 5x Laemmli's sample buffer (LSB).
2) The samples were then boiled at 95 ^{o}C for 10 mins using a thermo-mixer. After cooling samples were loaded and resolved on a 15% SDS PAGE.

3) After completion of the run, the gel was kept in 1M KCL solution for about five minutes. (The polypeptide were visible as white band in a slightly pale white background).
4) Then, the prominent band corresponding to 26 kda was excised with gel cutter and kept at -20 ^{o}C until electro-elution.
5) For electro-elution, the strips were cut into smaller pieces and kept in a dialysis bag containing appropriate amount of electrode buffer.
6) The bag was submerged in Lammeli's electrode buffer in a horizontal gel electrophoresis unit] and was run at constant 3W at room temperature overnight.
7) The bags were then washed with distilled water and again kept in electrophoresis unit containing freshly prepared buffer [10mMTris-HCL, 5M Urea, and 1% 2ME] for removal of SDS from the sample.
8) The electrophoresis was performed at constant 1W for 12-14 hrs at 4^{o}C. Finally the dialysis was done in dialysis buffer [Tris buffer pH 8] for the removal of urea and 2ME.
9) For confirmation of eluted band, the 20ul sample was electrophoresed on 15% SDS PAGE. The remaining sample was stored at -80^{0}C

(D) Characterization of Gpr114 ecd protein

CD (Circular Dichroism):

When the differential absorption of right and left circularly polarized light by a molecule is measured and plotted over a range of wavelengths, the result is called a circular dichroism (or CD) spectrum. This characterisation technique can be used to estimate the secondary structure of a protein with different conformations such as α helix, β sheet, random coil etc. The Gpr 114 belong to the adhesion GPCR family. Since it is a GPCR and it contains mostly α helices which can be seen in CD plot at 208 nm and 222 nm.

This CD was done to ensure that weather the protein has its native conformation or not.

(E) Pull Down Experiment

This experiment is performed to determine the interaction between two or more proteins. The steps for doing this experiments included:

1) Lysate was prepared from the mouse spleen.
2) Lysate and Gpr114 ecd protein (which had 6XHIS at C terminal end) at 1:1 ratio were incubated in the presence of binding buffer at 4^{o}C for 2 hours.
3) Ni-NTA beads were added and the mixture was kept at 4^{o}C for 2 hours.
4) The samples were then centrifuged at 500 rpm at 4^{o}C.
5) The pellets were then washed 3 times with binding buffer and 2µl Triton was added. In addition PMSF (1mM final concentration) was added and stored it along with supernatant.
6) Lammeli's sample buffer was added to the pellets, supernatant and different controls and these were then boiled at 95^{o}C for 10 minutes. After cooling all samples loaded on 12% SDS gel.
7) A reducing SDS PAGE was then run.
8) Commasie and silver staining was done to detect the low nanogram range of proteins.

(F) Silver Staining

Composition of solution

1) Fixing Solution -- 40% ethanol + 7% Acetic Acid
2) Densitizing Solution -- 0.2% Sodium thiosulphate Pentahydrate
3**)** Silver Nitrate Solution -- 0.1% AgNo3 + 0.02% HCHO
4) Developing Solution --3% Sodium Carbonate +0.05% HCHO
5) Stop Solution --5% Acetic Acid

The procedure that was followed is given below:-

1. After resolving polypeptides in the sample on SDS PAGE, the gel was washed with water 2 times for thirty minute each.

Fixation

2. Polypeptides were fixed in the gel using a fixative solution for 90 minutes at ice.

3. The gel was washed with water two times at room temperature for 1 hour, 30 minute each.

Sensitization

4. The sensitizing solution was added in gel for one minute at room temperature.

5. The gel washed two times with water for five minute each.

Silver Reaction

6. The gel was stained with freshly prepared ice cold silver nitrate solution and kept for 20 minutes.

7. The gel was washed with water for 2 minutes.

Developing Solution

8. The bands were developed in developer.

9. The gel was then washed in water for 20 seconds.

Stopping

10. The reaction was stopped when protein bands were visible.

Washing

11. Then, the stained gel was washed in water for 5 minute.

After silver staining, along with Gpr114 protein specific band, there was a huge band which may be a potential ligand for the Gpr114 .The appropriate band extracted from the gel which came along with Gpr114, which can be processed. Washing was done with water multiple times in order to completely remove the acetic acid.

(G) In Gel Digation Protocol

1. The band of interest was cut from the gel and dehydrated in 100µl of 1M ACN (expand it) three times.

2. Destaining was achieved using 100 µl 30% ACN containing 25 mM ABC (expand it) by incubating samples while shaking at 30 ^{o}C for 30 minutes.This step was repeated three times.

3. Further dehydrated was achieved by incubating with 40 µl CAN. The samples were then allowed to dry.

4. 50 µl of 25 mM DTT in ABC was added and samples were incubated at 56 ^{o}C for 20 minutes. 5. The samples were then cooled at room temperature and any residual liquid was pipetted off.

6. Then 50 µl of freshly prepared 55mM idoactetamide in 25mM ABC was added for alkylation for 20 minute at room temperature in dark.

7. Liquid was pipetted out and gel pieces were washed off with autoclaved dH_2O for 20 sec to remove residual IDA.

8. The gel pieces were dehydrated for 5 minutes with 200 µl of 50% ACN in 25mM ABC and then with 100% CAN for 30 seconds.

9. Thereafter the gel pieces were dried.

(H) Trypsinization

Trypsin is a highly specific protease that cleaves proteins or peptides on the carboxyl side of arginine and lysine. For trypsinization following steps were followed:-

1). 50 µl of trypsin was added to the reaction buffer to be added to the gel pieces and incubated at 37^{o}C.

2) After one hour of incubation at 37 ^{o}C the samples were incubated overnight and supernatant was collected after centrifugation at 9500 rpm for 20 seconds.

3) The samples subjected to mass spectrometry thereafter.

(I) Mass spectroscopy

The parameter used for mass spectroscopy and data analysis include:

- Nano LC coupled with MS QTOF
- Raw Data was analyzed in PLGS(protein lynx global server)
- Databank was used was Swissprot
- Lock mass correction value is 785.8426 Da/e
- MS^{e} Mode was used

a) Workflow Parameters

Peptide Tolerance	100
Fragment Tolerance	50

Min Fragment Ion Matches	3
Min peptide Matches	1
Maximum Hits to return	20
Maximum Protein Mass	250000
Primary Digest Reagent	Trypsin
Missed Cleavages	1
Fixed Modifier Reagents	Carbamidomethyl C
Variable Modifier Reagents	Oxidation M

b) Processing Parameters

Sample Run Injection Parameter

Injection Volume	10 µl
M/z Range	50-2000 in the positive mode

C) **Nano LC programming**

	Time	Flow rate (µl/min)
1. Initial	**0.2**	**97.0**
2. 5.0	**0.2**	**90.0**
3. 6.0	**0.2**	**85.035**
4. 10.0	**0.2**	**80.0**
5. 11.0	**0.2**	**75**
6. 15.0	**0.2**	**70**

7. 20	**0.2**	**60**
8. 30	**0.2**	**50**
9. 40	**0.2**	**40**
10. 45	**0.2**	**30**
11. 50	**0.2**	**20**
12. 60.0	**0.2**	**10**
13. 70.0	**0.2**	**5**
14. 80.0	**0.2**	**20**
15. 90.0	**0.2**	**50**
16. 100	**0.2**	**80**
17. 105	**0.2**	**95**

d) Column used for Separation – 1.7 µm

Results and Discussion

We analyzed the activation of antigen (MHV68) specific CD8 T cells 5 days post infection by transferring $50x10^3$ cells in congenic mice. At 5dpi surface staining was followed by flow cytometric analysis (Figure 1.2). About 40 percent cells were those that expanded due to viral infection in CD45.1 mice as can be recorded for CD45.2 positivity. Almost all of the transferred cells exhibited activation markers of CD44 and PD1. Thereafter, activated cells and the population of naïve cells from the same animal used for adoptively transfer were processed similarly for isolation using FACS sorting. RNA isolated from such cells was subjected to RNA sequencing. Selected transcripts that showed down regulation in activated cells but again got up regulated in CD8 T cells as they make a transition to memory population are shown in Figure -1.3 A similar expression pattern was shown for IL-7r, which was demonstrated to be marker whose expression in acute phase of a viral infection could mark those cells that specifically go on to become memory cells. We chose Gpr114 for further investigations, as this is a surface expressed molecule and therefore would be valuable in settings where no knock-in mice are available. Furthermore, Gpr114 belong to a family of GPCRs whose role is developmental and immunological studies have been elucidated.

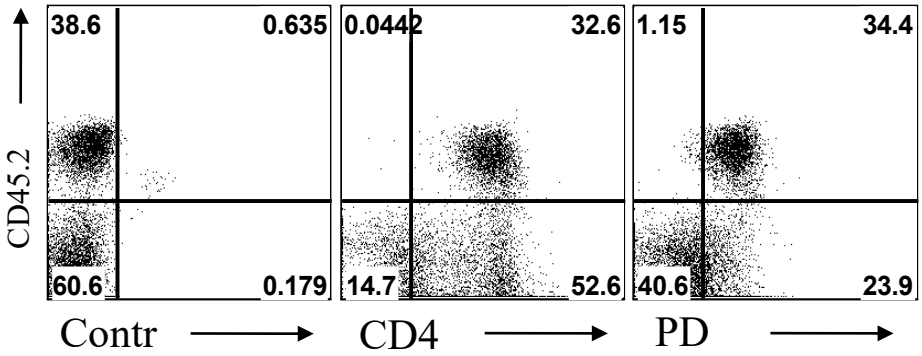


Fig 1.2- FACS plot showing the activation of antigen specific CD8 T cells

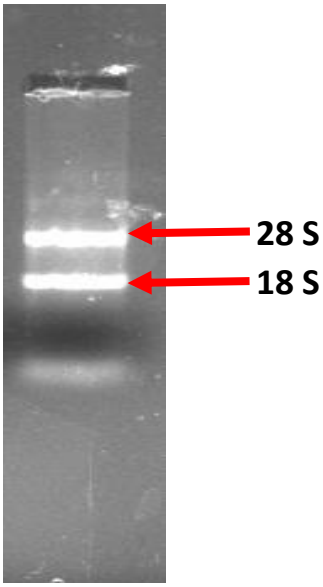
	D6/naïve (fold down regulated)
Gpr114	**20**
Tnfsf8	**50**
Vipr1	**30**
Slfn5	**25**
Rab6b	20
Fam101b	100
Nr1D1	20
Il7r	***30***

Fig 1.3- Some hits to pursue as possible markers of memory cells

Cloning of full length and Extracellular domains of GPR114:-

In order to start Cloning of Gpr114, total RNA from mouse lymph nodes was isolated. Figure:-1.4 shows the quality of isolated RNA. A predominance of two bands corresponding to 28S and 16S RNA was visible when analysed by AGE. Thereafter, cDNA was synthesized using RNA as a template. The cDNA thus synthesised was used as a template for PCR amplification of ecd and full length Gpr114. The sequence of primers that were used for two amplification reaction is given in materials and method section. Extracellular domain of Gpr114 corresponded to 685 bp and the full length amplicon of Gpr114 corresponded to 1588 bp (Fig 1.5 and 1.6).

Fig 1.4-RNA from mouse Lymph Node



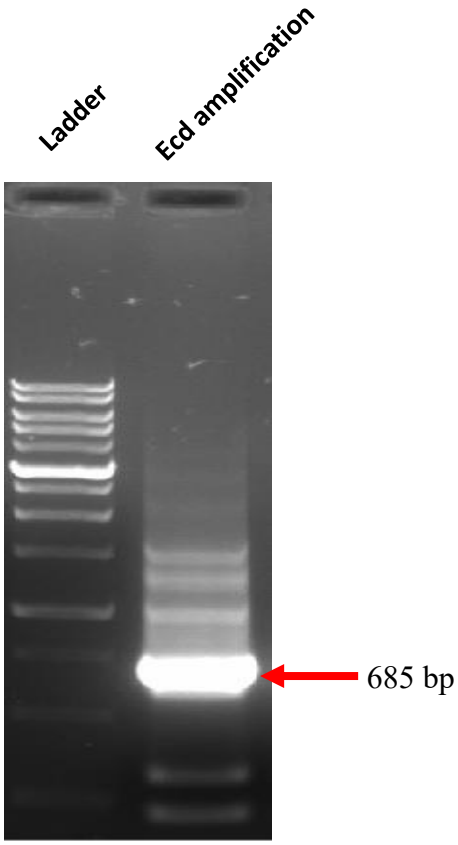


Fig1.5- PCR amplification of extracellular domain of Gpr114

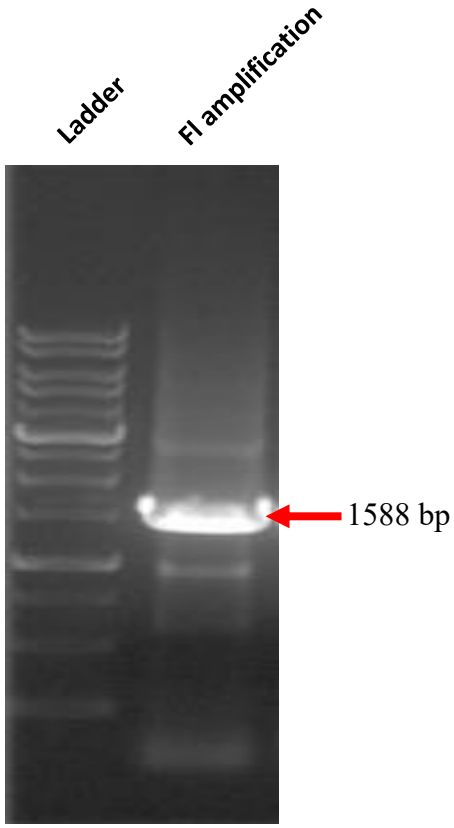


Fig1.6- PCR amplification of Full length domain of Gpr114

The PCR product obtained were gel purified and a digestion was set up using (restriction enzymes as described in materials and methods). Similarly, the vector was digested using same restriction enzymes. The gel purified and digested insert was ligated as described in materials and methods. The ligated vector was then transformed in competent E. coli DH5 alpha strain. As a control, self-ligated vector was also transformed similarly. As there were some colonies in self-ligated vector as well therefore, to confirm the presence of insert under test conditions, a colony PCR was performed (Fig 1.7, 1.8, 1.9). Those colonies that showed positive reaction in colony PCR were further propagated. Minipreps were prepared to obtain positive clones that were confirmed using restriction digestion. As shown in figure 1.10.The desired insert corresponding to 678bp was obtained after restriction digestion confirming a successful cloning of the ecd of Gpr114.

In a similar fashion, full length was cloned in pET 22b and also in pcDNA3.1 vectors. For these two vectors however the restriction sites and the enzymes used were different. The results are shown in figure 1.11

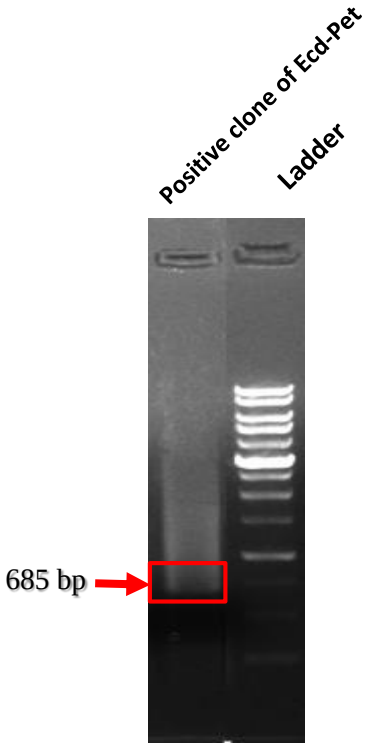


Fig1.7- Colony PCR of ecd-pet

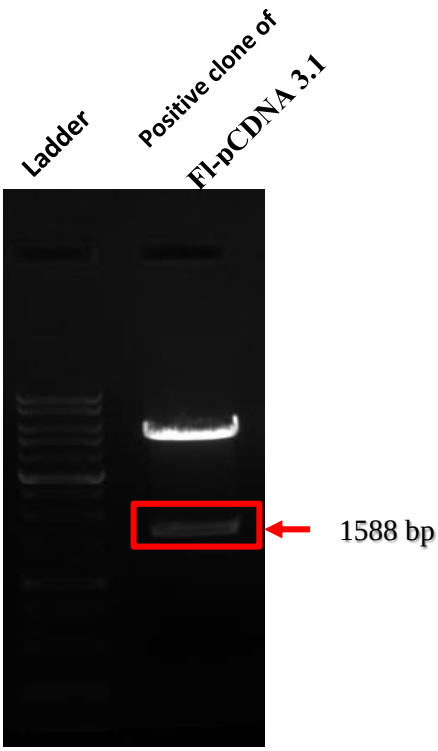


Fig1.8 –Colony PCR of Fl-pCDNA 3.1

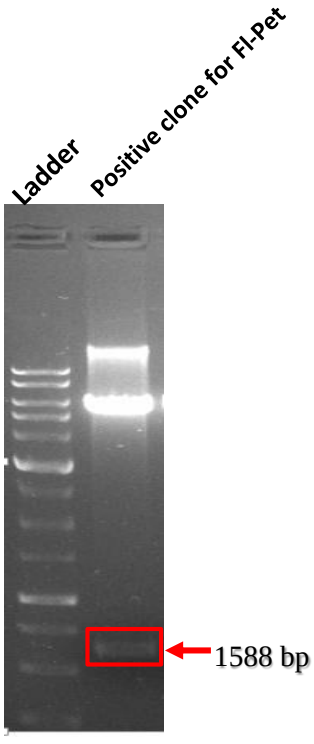


Fig 1.9 –Colony PCR of Fl-pET

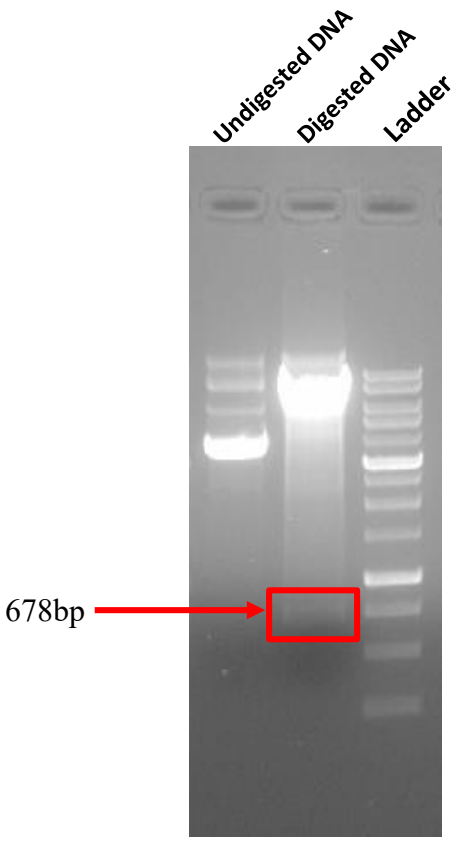


Fig 1.10- Cloning confirmation by restriction digestion of ecd

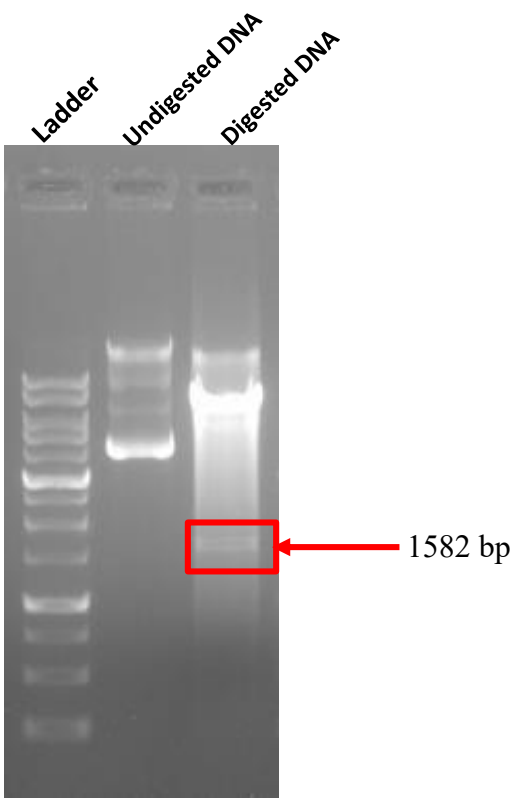


Fig 1.11- Cloning confirmation by restriction digestion of fl

Gpr114 specific band got at

- 678 bp which represented the extracellular domain of Gr114 for Ecd-pet22b(+)
- 1582 bp which shows the full length domain of Gpr114 for Fl-Pet(+)
- 1582 bp which shows the full length domain of Gpr114 for Fl-pCDNA 3.1(+)

Expression of ECD of Gpr114:

In order to identify a CD spectrum of the protein was performed to ascertain whether any alpha helices were present in the structure. After base line correction, the protein exhibited predominance of alpha helices over beta sheet structures. The presence of alpha helical structures was considered that the protein was refolded and thus could be used for further

experiments to identify ligand in pull down experiments.a ligand for Gpr114, the ECD of Gpr114 was expressed as a C terminal 6xHis protein. The induction of Gpr114 was achieved using IPTG induction in transformed BL21 strain of *E. coli.* The protein was expressed in inclusion bodies which were purified and stored in 6M Urea+ DTT. As we faced problems with purification of this protein using NiNTA beads we resorted to purification by electroelution followed by dialysis. The electroeluted gpr114 exhibited a reasonable purity when analysed by SDS PAGE (Fig1.12). An intense band of 26kDa was visible and the yield was 170ug/ml.

Attempts at Identification of putative ligand/s for Gpr114:

Our hypothesis was that if expression of Gpr114 on CD8 T cells is responsible for inducing downstream signalling events which would then might have an impact for differentiation of CD8 T cells in either effector or a memory precursor cells, it may need to interact with other biomolecules either constitutively expressed or induced after an infection in lymphoid organs. Therefore, pull down experiments were designed to fish out a specific ligand. Purified ECD of Gpr114 was coincubated with spleen lysate in different ratios as described in materials and methods. After incubation, NiNTA beads were added as Gpr114 had 6xHis tag that is responsible for a high affinity interaction with NiNTA beads. The expectation was that if a potential interactor was present in spleen lysates, it would interact with the Gpr114 which can then be pulled out using NiNTA beads. Different fractions collected during pull down experiments were resolved on SDS-PAGE. Silver staining was performed to detect any specific interaction even if present at a lower level. From pull down experiment, a protein corresponding to (12 Kda) which might be a potential ligand of Gpr114 was observed. Furthermore, we electroeluted protein from spleen lysate this range of molecular masses and performed dialysis. After that, this relatively cleaner preparation was used for pull down experiments. As shown in Fig-1.14, the electroeluted putative ligand interacted with Gpr114.

In order to identify what is the nature of this potential ligand, the electroeluted proteins were analysed by Mass Spec-Nano LC –MS QTF. This was done because it can detect even if there is small number of peptides are generated. For this an in gel digestion was performed as

described in materials and methods and mass spec analysis was performed. Hits obtained after doing mass spectroscopy analysis are shown in table A.

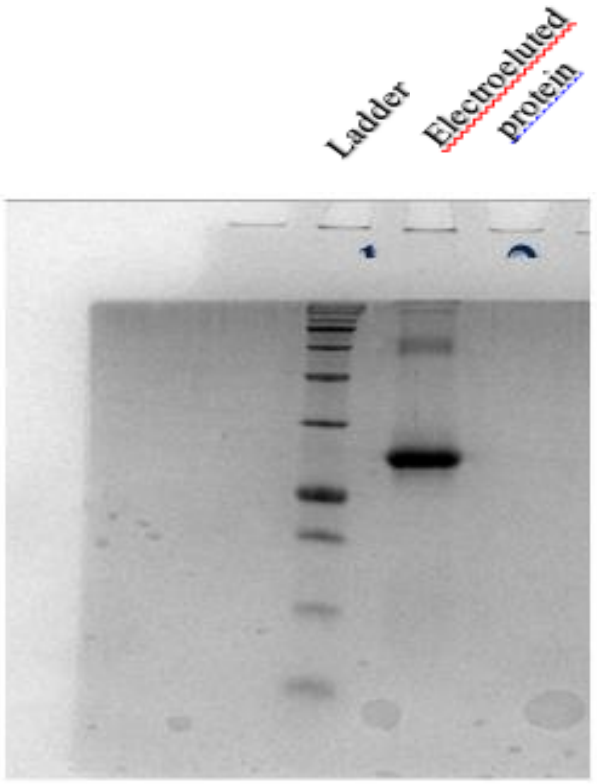


Fig 1.12 – Purified ecd Gpr 114

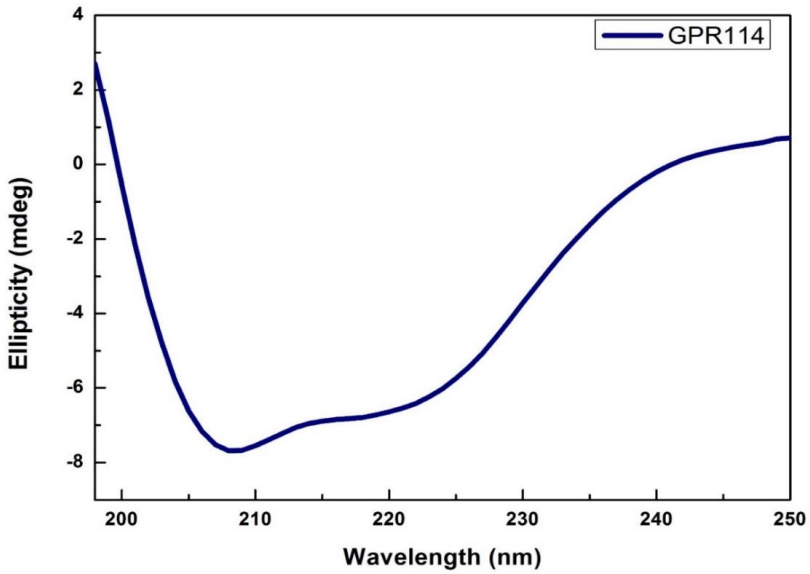


Fig 1.13- CD Plot of ECD Gpr114

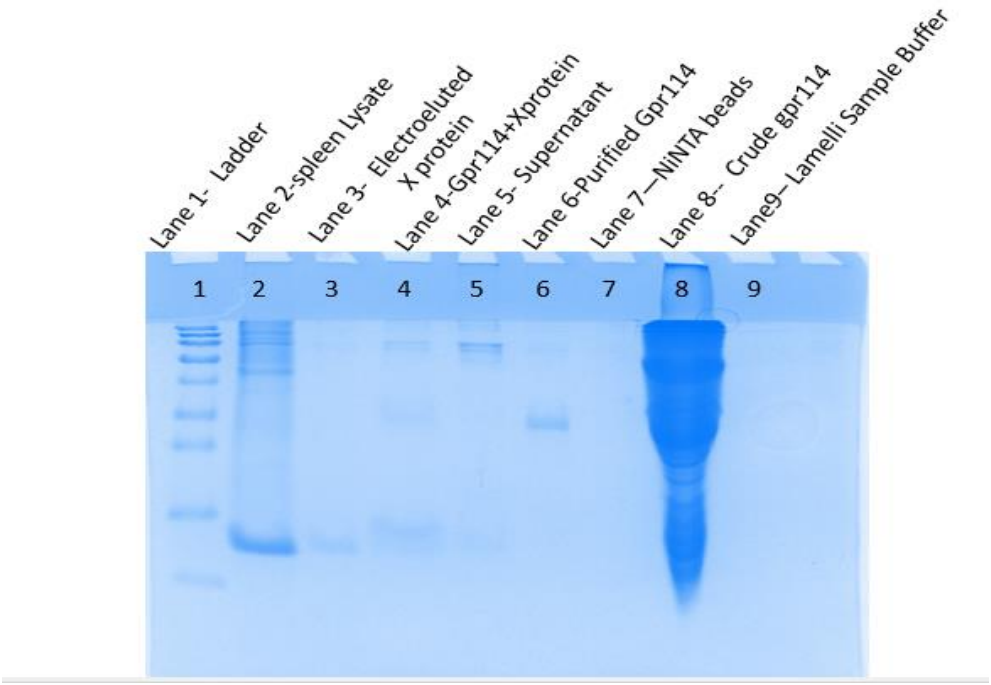


Fig 1.14- Commasie stained SDS-PAGE gel

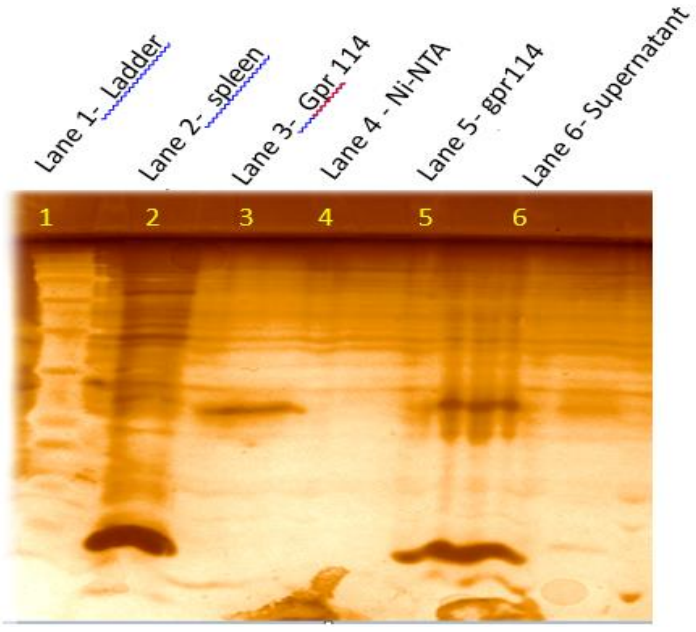


Fig 1.15-Silver stained SDS-PAGE gel

Mass Spec Data—The List of the proteins which were identified from mass spectrometry are as follows.

The PLGS(protein linked global server) score are high for Hemoglobin beta 1 chain which might be a potential ligand of Gpr114. Keratin is a contaminant which comes from the hands during the processing of the sample.

Table -A

Sr No	Accession	Entry	Description	mW (Da)	pI (pH)	PLGS Score	Peptides
1.	P02088	HBB1_MOUSE	Hemoglobin beta-1 chain (B1) (Major).	15699	7.4971	3006.142	9
2.	P11758	HBB_MYOVE	Hemoglobin beta chain.	15786	9.5259	2908.304	11
3.	P01942	HBA_MOUSE	Hemoglobin alpha chain.	14944	8.6382	2820.407	5
4.	P02064	HBB1_TAPTE	Hemoglobin beta-1 chain (Major).	15963	6.249	1683.869	7
5.	P37815	ATPL_BACSU	ATP synthase C chain (EC 3.6.3.14) (Lipid-binding	7088	7.2407	1655.385	2
6.	P02091	HBB1_RAT	Hemoglobin beta chain, major-form.	15838	8.2471	1571.416	8
7.	P04264	K2C1_HUMAN	Keratin, type II cytoskeletal 1 (Cytokeratin 1) (K	65846	8.2749	1536.537	19
8.	P15523	CSMB_CHLLT	Chlorosome envelope protein B (Chlorosome 7.5 kDa	7483	4.7183	1510.876	4
9.	P02049	HBB_NYCCO	Hemoglobin beta chain.	15890	8.625	1467.842	7
10.	P00761	TRYP_PIG	Trypsin precursor (EC 3.4.21.4).	24393	6.9141	978.8437	6
11.	P02535	K1CJ_MOUSE	Keratin, type I cytoskeletal 10 (Cytokeratin 10) (	57675	4.8164	894.4575	11
12.	P06394	K1CJ_BOVIN	Keratin, type I cytoskeletal VIB (Cytokeratin VIB)	54815	4.8589	841.0033	14

13.	P55479	Y4HL_RHISN	Hypothetical 9.3 kDa protein Y4HL.	9320	9.3809	629.8323	2
14.	Q9ZCJ1	TATA_RICPR	Sec-independent protein translocase protein tatA/E	5880	7.7124	558.5765	2
15.	P06483	UL45_HSV23	Protein UL45 homolog (18 kDa protein).	18136	8.0156	502.5957	1
16.	Q9RXX0	CRCB_DEIRA	Protein crcB homolog.	12776	11.6396	320.8061	1
17.	Q57685	Y233_METJA	Hypothetical protein MJ0233.	31286	9.145	315.5726	2
18.	O63796	NU2M_ANACA	NADH-ubiquinone oxidoreductase chain 2 (EC 1.6.5.3	37747	10.3096	311.8387	1
19.	P08727	K1CS_HUMAN	Keratin, type I cytoskeletal 19 (Cytokeratin 19)	44079	4.8604	276.6638	7
20.	P22376	YPA3_ASCIM	Hypothetical 9.8 kDa protein (ORF3).	9833	10.0313	276.2758	1
21.	O67049	AROE_AQUAE	Shikimate 5-dehydrogenase (EC 1.1.1.25).	30087	9.4307	275.4298	2
22.	P75554	OPPB_MYCPN	Oligopeptide transport system permease protein opp	43510	10.1177	225.2808	2
23.	O66698	Y376_AQUAE	Hypothetical protein AQ_376.	35682	9.2314	223.7817	2
24.	P05960	POL_HV1C4	POL polyprotein [Contains: Protease (Retropepsin)	13188	5.3145	216.5416	1
25.	P00756	KLK3_MOUSE	Glandular kallikrein K3 precursor (EC 3.4.21.35)	28979	6.3984	210.0464	2
26.	P27180	ATPD_SYNY3	ATP synthase delta chain (EC 3.6.3.14).	20080	6.8262	200.1223	2
27.	O29417	PURA_ARCFU	Adenylosuccinate synthetase (EC 6.3.4.4) (IMP—asp)	36540	6.019	197.8486	2
28.	Q08648	HE2_HUMAN	Sperm antigen HE2 precursor.	11309	11.7803	182.9405	3

29.	P13184	COXJ_BOVIN	Cytochrome c oxidase polypeptide VIIa-liver/heart,	9299	10.377	177.3031	2
30.	O78714	NU1M_TACAC	NADH-ubiquinone oxidoreductase chain 1 (EC 1.6.5.3	35907	7.3667	168.2017	1
31.	P26822	CH10_CLOPE	10 kDa chaperonin (Protein Cpn10) (groES protein).	10301	4.7065	166.1673	1
32.	P06897	H2A1_XENLA	Histone H2A.1.	13826	11.3115	161.984	1
33.	P22479	ATPD_BACFI	ATP synthase delta chain (EC 3.6.3.14).	20522	6.1992	151.5202	1
34.	P14147	PHOQ_SALTY	Virulence sensor protein phoQ (EC 2.7.3.-).	55431	5.9297	137.1794	2

Conclusion and Future Direction

First attempts were made in identifying the ligand, however the future studies will focus on confirming weather one or more of these are putative ligand/s, Cloning and expression of that putative ligand, Co-IP Experiments, by using the Ligand. Then will decipher the downstream signaling events in $CD8^+$ T cells mediated by the interaction of gpr114 and its receptor.

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