IMMUNOLOGICAL STUDIES ON THE ROLE OF GPR114 AND RUSSELL'S VIPER VENOM

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Certificate of Examination

This is to certify that the dissertation titled “Immunological studies on the role of gpr114 and Russell’s viper venom” submitted by Ms. NirdoshDadwal(Reg. No. -MS10111) for the partial fulfilment 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.

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(Supervisor)

Dated: 24th April 2015

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr.SharvanSehrawat 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.

NirdoshDadwal

24th April, 2015

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.SharvanSehrawat(Supervisor)

24th April, 2015

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Nirdosh Dadwal

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NOTATION

Abbreviations	Full form
PCR	Polymerase Chain Reaction
DLS	Dynamic Light Scattering
CD	Circular Dichroism
HIS	Hyper-immune Serum
PIS	Pre-immune Serum
GuCl	Guanidiniumchloride
IMAC	Immobilized Metal Affinity Chromatography
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
PMSF	Phenylmethylsulfonyl Fluoride
FBS	Fetal Bovine Serum
PBS	Phosphate Buffer Saline
BSA	Bovine Serum Albumin

Introduction

CD8 T cells constitute an important arm of adaptive immune response and are critically involved in controlling intracellular pathogens and cancers. After initial expansion of pathogen-specific CD8 T cells, most cells die (the contraction phase), leaving behind a memory population that represents a small fraction of the cells that responded initially to the antigen. But, which cells after activation and expansion are chosen to become long-term memory CD8 T cells is still under investigation. We aimed at identifying novel molecular signatures of differentiating CD8 T cells that can mark the phenotype of memory cells in the acute phase of infection and explored the feasibility of Gpr114 being one such marker. The study was initiated based on our observation that Gpr114 has similar pattern of expression as that of IL-7R, which is one of the established marker of memory CD8 T cells atleast during some viral infections. GPR114 is a member of GPCR (G-protein coupled receptor) 2 family and the members of this family share a common molecular architecture which consists of trans-membrane domains, extracellular and intracellular loops. The protein is composed of 529 amino acids of which 29 amino acids constitute a signal sequence and 222 amino acids form extracellular domain. Establishment of such markers could come handy in gauging the magnitude and efficacy of vaccination strategies. Once established, this would increase our knowledge about pathways required for transition of effector and memory cells from their naïve precursors.

We also focused on the immunomodulation by Russell's Viper venom, as is shown recently that snakebites constitute a major public health problem in tropical and subtropical countries such as India. Therefore, the snakebites have an important implication in halting the growth of economy of countries where they occur. So the number of people affected by snake bite is more than the number of people affected from infectious disease such as HIV/AIDS. Every year people dying from snake bite is around one lakh in India alone. Russell viper is one of the four poisonous snake species responsible for most human snake bite cases in India. It is often found in agricultural regions. Once bitten, people experience a variety of symptoms including pain, swelling, dizziness, blood coagulopathies and kidney failure.

Then depending on the extent of envenoming and management procedures followed either recovery begins or the patient dies. So one of the long terms goal that is still elusive is to develop a cure for snakebite using immunotherapeutics with minimal damage to the host. Recently researchers have described an association between Russell Viper snake bite and development of Herpes labialis. Herpes labialis is caused by Herpes Simplex virus I and is characterized by development of painful blisters on or around lips. The virus remains in dormant state in facial nerves and ganglionic tissues. Depending on the immunological status of host HSV the virus gets reactivated. Some of the factors such as stress, trauma, fever, other systemic concurrent infections etc can trigger the reactivation. Recently a correlation between Russell's viper envenomation and HSV reactivation was demonstrated. Therefore we designed this study, to study the effect of Russell viper venom components on immune cells that could potentially lead to the reactivation of virus causing debilitating disease.

Characterization of Cel CCA protein of *Clostrodium cellulolyticum: Clostridium Cellulolyticum* is an anaerobic, motile, gram-positive bacterium. It's a mesophilic bacterium. Some studies also focused on Cellulase gene of CelCCA consists of 1140 base-pairs. In my project, I focused on the biophysical characterization of this protein using different techniques.

Chapter 1

Exploring the possibility of GPR114, serving as a novel marker of memory CD8 T cells during viral infections

1.1 Introduction

CD8 T cells constitute an important arm of adaptive immune response and are critically involved in controlling intracellular pathogens as well as some cancers. CD8 T cells recognize class I MHC products loaded with the appropriate peptides and can be identified unambiguously by MHC multimers loaded with specific immunogenic peptides. After initial expansion of pathogen-specific CD8 T cells, the host uses multiple mechanisms to control the scope, composition and duration of immune reactions not only to ensure protection, but also to limit possible damage caused by immune cells. Many of these mechanisms are still not identified. After the primary response most cells die (the contraction phase), leaving in place a memory population that represents a small fraction of the cells that responded initially to the antigen. Memory cells rarely account for >10% of the total antigen-reactive repertoire. Accordingly, which cells after activation and expansion are chosen to become long-term memory CD8 T cells is an open field of investigation and is pursued with high interest. We aim to identify novel molecular signatures of differentiating CD8 T cells that can mark the phenotype of memory cells in the acute phase of infection. Such markers could come handy in gauging the magnitude and efficacy of vaccination strategies. Once established, this would increase our knowledge about pathways required for transition of effector and memory cells from their naïve precursors. An understanding of such mechanisms is crucial to develop successful vaccination strategies against intracellular infections and cancers. Accordingly understanding such pathways might provide new insights in devising strategies to boost the magnitudes and superiority of memory CD8 T cell responses. It has been recently appreciated that the fate of differentiating cells is governed by the induction and persistence of a panel of transcription factors. Thus, the role of transcription factor such as Tbet, Blimp1, Id2, Id3, Tcf1, Bcl6 and Eomes has been implicated in the differentiation of CD8 T cells. The

expression of Eomes seems to be an essential feature for the maintenance of memory CD8 T cells. Antigen exposure, appropriate expression of co-stimulatory molecules on antigen presenting cells (APCs) and inflammatory milieu, all constitute key components for the differentiation of effector and memory CD8 T cells. Of the three signals the value of inflammatory environment in determining the fate of memory CD8 T cells has been elucidated recently. Accordingly it was demonstrated that in a highly pro-inflammatory environment that lasts longer during the differentiation of T cells, more of the short-lived effector cells (SLECs as defined by KLRG1 or TIM-3 positivity) are formed at the expense of memory cells (as defined by IL-7R positivity) generation. The transcriptomic profile of naïve and activated cell during a viral infection was performed with an aim to discover novel markers that could serve to detect the cell fate (unpublished data by Sehrawat et al). Such a marker/s could serve to detect memory cells during primary infection or more importantly in gauging the efficacy of immunization in a timely manner. This is because one of the essential aims of immunization is to increase the representation of memory cells that can be recalled quickly in event of an infection. Based on the analyses of the data that was made available to us by Sehrawat et al (unpublished data), this study was planned to explore the feasibility of using gpr114, a marker of memory CD8 T cells.

GPR114 is a member of GPCR (G-protein coupled receptor) 2 family and the members of this family share a common molecular architecture which consists of trans-membrane domains, extracellular and intracellular loops. The protein product is encoded by a gene on chromosome 8 in mouse and is composed of 529 amino acids of which 29 amino acids constitute a signal sequence and 222 amino acids form extracellular domain. This extracellular domain gives surface expression, which is expressed, on Myeloid cells, αβT cells, γδ T cells, NK cells and exhibit a mild to strong expression in tumors of several origins. It also shows 60% homology with humans but the function and ligand/s are not yet known.

As the *gpr114* was getting down regulated upon activation (Fig 1.1) and bioinformatics analyses aiming at finding out the expression pattern of

GPR114 using (online data at www.immgen.org), revealed that naïve and memory cells express it. Therefore, it was hypothesized that this molecule could serve as memory marker on the surface of these cells. This is predominantly because of the fact that its mRNA expression pattern on naïve, activated and memory cells follows a similar pattern of expression as that of IL-7R (which is an established marker of memory CD8 T cells atleast during a viral infections. (*Susan et al.[Nature immunology]*). Thus, it was demonstrated that the expression of IL-7R is moderate on naïve cells and gets down-regulated in a large majority of effector cells during acute infection with a strain of lymphocytic choriomengitis virus (LCMV-Armstrong), that causes an acute infection in mice. What was surprising was that approx 5-8% population of effector cells still retained its expression and those cells then go on to become memory cells. During the transition from effector to memory cells the expression of IL-7R continues to increase in these cells (a,b). Using MHC tetramer specific to one of the immunodominant epitope of the virus and adoptively transfer approach, the fate of IL-7R^{hi} and IL-7R^{lo} cells in congenic mice, it was demonstrated that the cells that retain expression of IL-7R alpha chain (CD127) are preferentially chosen to become memory cells.

Another impetus to explore the feasibility of using GPR114 as a potential memory marker was provided by its surface expression. Thus, for carrying out immunological investigations on live cells using flow cytometric analyses, sorting of positive and negative cells would be necessary to perform further experimentations.. Thus, molecules that are expressed intracellularly, genetic manipulations and transgenesis approach would be mandatory to decipher their function and that has its share of problems.

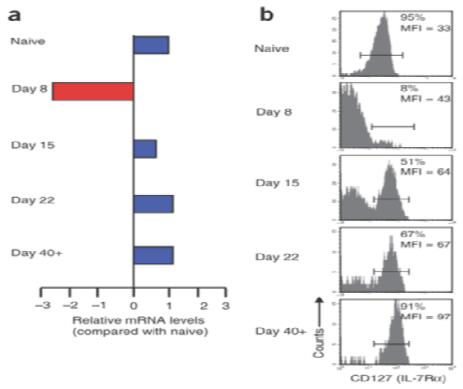

Fig 1.1a) Differential expression of IL-7R mRNA in naïve, effector and memory CD8 T cells. b) The percent and mean fluorescent intensity (MFI) of IL7R^{hi} on CD8 T cells from days 8, 15, 22 and ~40 after infection.

[Kaech, Susan M., et al. "Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells." *Nature immunology* 4.12 (2003): 1191-1198]

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	D6/naïve ORF8 TN cells (fold downregulated)
Gpr114	20
Tnfsf8	50
Vipr1	30
Slfn5	25
Rab6b	20
Fam101b	100
Nr1D1	20
Il7r	*30*

Fig 1.2: Some selected interested hits to pursue as possible markers of memory cells.

Furthermore, the role and the relevance of GPR114 in T cells have not been investigated till date. As mentioned above GPR114 consists of an extracellular, seven transmembrane and intracellular domains, therefore it was imperative to focus on cloning, expression and generation of immunological reagents such as antibodies against its extracellular domain (ECD) that consisted of 229 amino acids. This is because such reagents can be used to detect its expression on cell surface. The nucleotide and amino acid sequence of extracellular domain of GPR114 (shown below)

Nucleotide sequence of gpr114 extracellular domain:

5' CTGAGCGACCTGCTGGTGCTGATGAAGAGGCTGGAGCAGCCCGTGGGCAGGGGCCTGAGC
AGCAGGGCCAGGCACATCCACAGCCTGGAGCAGAAGCTGCTGAACGCCAGCTTCGGCGGC
CACAACCTGACCCTGCAGACCAACAGCATCCAGAGCCTGGTGTTCAAGCTGAGCTGCGAC
TTCCCCGGCCTGAGCCTGAGCAGCACCACCCTGACCAACGTGAGCCAGGTGAGGGCCCCC
CACGCCATGCAGTTCCCCGCCGAGCTGACCAAGGGCGCCTGCGTGACCAGCAGGCCCGCC
GAGCTGAGGCTGATCTGCATCTACTTCTTCACCGCCCACCTGTTCCAGGACGACAGGAAC
AGCAGCCTGCTGAACAACTACGTGCTGGGCGCCCAGCTGGACCACAGGCCCGTGAACAAC
CTGCAGAAGCCCGTGAACATCAGCTTCTGGCACAACAGGAGCCTGGAGGGCTACACCGTG
AGCTGCGTGTTCTGGAAGGAGGGCGCCAGCAAGAGCAGCTGGGGCGCCTGGAGCCCCGAG
GGCTGCTACACCGAGCAGCCCAGCGCCACCCAGGTGCTGTGCCACTGCAACCACCTGACC
TACTTCGCCGTGCTGATGCTGAGCGGCGACCCCGTGCCCGCCGAGCTGCAGGTGCCCCTG
GAGTAC 3'

Amino acid sequence of gpr114 extracellular domain:

LSDLLVLMKR LEQPVGRGLS SRARHIHSLE QKLLNASFGG HNLTLQTNSI QSLVFKLSCD

FPGLSLSSTT LTNVSQVRAP HAMQFPAELT KGACVTSRPA ELRLICIYFF TAHLFQDDRN

SSLLNNYVLG AQLDHRPVNN LQKPVNISFW HNRSLEGYTV SCVFWKEGAS KSSWGAWSPE

GCYTEQPSAT QVLCHCNHLT YFAVLMLSGD PVPAELQVPL EY

Number of amino acids: *222*
Molecular weight: *24733.2 = ~25 Kda*
Theoretical pI: *7.79*

1.2 Materials and Methods:

Polypeptide derived from the ECD of GPR114 was expressed as a C-terminal 6xHIS tagged product using pET 28 expression vector and BL21 *E. coli* cells (Sehrawat et al., unpublished data). The induction of the polypeptide was achieved by 0.5M IPTG at 37 degree C. Inspite of many attempts at expressing the ECD of GPR114 in soluble form, we could only get it expressed as an inclusion body. Therefore we decided to purify inclusion bodies and proceed with biochemical and physical approaches to purify a soluble and refolded product.

a) Purification of ecdGPR114:

- **Using Ni-NTA:**

The inclusion body containing recombinant protein were purified and stored in a buffer (6M Urea + DTT). Therefore, the first aim was to obtain this protein in purified, soluble and properly refolded form so that antibodies could be generated against some of its conformational epitopes. This is for the reason that the hyper immune serum (HIS) obtained could be used for downstream flow cytometric application to measure its expression in differentiating $CD8^+$ T cells. This would also allow it to be used for sorting live cells for deciphering downstream signaling events and fate mapping. As the protein was expressed with a C terminal 6xHIS tag Ni-NTA purification was performed. The buffers and their composition used for the purification are mentioned below:

1. Denaturing Binding Buffer(50ml, ph 7.8)

8M Urea	24.02g
20mM NaH_2PO_4	0.156g
500mM NaCl	1.461g

2. Denaturing Wash Buffer(50ml, ph 6.0)

8M Urea 24.02g

20mM NaH_2PO_4 0.156g

500mM NaCl 1.461g

3. Denaturing Elution Buffer(50ml, ph 4.0)

8M Urea 24.02g

20mM NaH_2PO_4 0.156g

500mM NaCl 1.461g

4. Imidazole (50ml, ph 6.0)

3M Imidozole 24.02g

20mM NaH_2PO_4 0.156g

500mM NaCl 1.461g

5. Wash buffer (50ml, ph 6.0)

20mM NaH_2PO_4 0.156g

500mM NaCl 1.461g

a) A schematic describing the purification using NI-NTA column:

- The column containing Ni-NTA agarose (aka beads) was used.
- Pre-washed with water (one volume) and then with Denaturing binding buffer (to equilibrate the column).
- Passed gpr114 protein (500ul) and collected the flow through.
- The washing was done using Denaturing wash buffer (two –three volume). Collected the wash.
- The starting elutions in 3M imidazole and then in 8M Urea. Collected the elutions in 1.5ml MCTs.
- The samples were run on 12% SDS gel.

NOTE: The washing step should be fast.

The level of purification was measured by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and a subsequent staining with comassie blue brilliant red (CBBR) 250. For resolving protein/s the eluted in the samples were run on a 5% stacking gel and 12% resolving gel. The composition of the respective gels and the buffer used is as follows.

Composition of SDS PAGE:

12% Resolving gel (10ml ph 8.3):	
Water	3.3ml
Acrylamide	4.0ml
Tris –HCl buffer (ph 8.3)	2.5ml
SDS (10%)	0.1ml
APS	0.1ml
TEMED	10ul

5% stacking gel (5ml ph 6.8):	
Water	3.4ml
Acrylamide	830ul
Tris-HCl buffer (ph 6.8)	630ul
SDS (10%)	50ul
APS	50ul
TEMED	5ul

Staining solution (100ml):	
CBB	0.1g
Water	45ml
Methanol	45ml
Acetic acid	10ml

De-staining solution (100ml) :	
Water	45ml
Methanol	45ml
Acetic acid	10ml

10X SDS PAGE Reservoir Buffer (500ml ph 8.3):	
25nM Tris Base	15.15g
192mM Glycine	72.05g
0.1%SDS	5g

b) Batch purification of GPR114 using Ni-NTA:

As the purification achieved using columns was not adequate both qualitatively and quantitatively, attempts were made at purifying the expressed GPR114 in a batch format rather than using a columns. This is also to make sure that the purified protein product is not diluted. The Ni-NTA sepharose beads (1ml) were washed in 2ml microcentrifuge tubes with (water and then binding buffer) multiple times. Centrifugation at 500 rpm for 5 mins was performed to remove the supernatant in a microcentrifuge at 4 degree C. The washing and equilibration of beads

with distilled water and denaturing binding buffer respectively. We used 200-300ul of protein preparation to purify GPR114. Flow through was collected after centrifugation. In order to remove unbound residual protein, beads were washed several times and final elution of bound proteins was performed by using 8M urea and 3M imidazole. All the samples were then electrophoresed on a 12% SDS-PAGE. The eluted samples were dialysed in a buffer without imidazole and decreasing concentrations of urea for 30-40 hrs by changing the buffer atleast three times. At 4M concentration of urea, the proteins were soluble but denatured and reducing further the concentration of urea had adverse effect in terms of its solubility.

We tried to refold the protein in the following refolding buffer:

Dialysis buffer (500ml, ph 7.8)

4M Urea	180.18g
20mM NaH_2PO_4	1.56g
300mM NaCl	8.76g

Refolding Buffer (250ml, ph 8.0)

100mM Tris-base	3.025g
400mM L-arginine-HCl	21g
2mMNaEDTA	[1ml from500mM stock]
5mM red. glutathione	380mg
0.5mM oxid. glutathione	76.5mg

3M GuCl (100ml, ph 4.2)

3M GuCl	28.7g
10mM Na acetate	82mg
10mM Na EDTA	0.372g

Procedure followed for Refolding:

- We mixed 1ml of eluted protein of (510ug/ml) + 1ml of GuCl solution.
- Divided this 2ml solution into three slots equal volume.
- Then 50 ml of refolding buffer was kept at 4^0C with constant stirring.

- 0.4ml PMSF (phenylmethylsulfonyl fluoride) was added in 50 ml of refolding buffer
- Then the first slot of protein was added drop by drop with constant stirring.

  NOTE: reduce the stir speed while incubating. Add protein drop by drop.
- Another slot was added after 10 hrs. Again the third slot was added after 10 hrs.
- The solution was kept on stirring for 24 hrs at 4^0C. And then the solution was concentrated using concentrator of. Finally the protein concentration was checked using UV spectrometer and SDS-PAGE.

  - **Electro-elution of resolved ecdGPR114:**

  In our attempts at purifying ecdGPR114 we, were consistently getting some minor contaminating bands that could hinder in generating specific hyper immune serum against it. Therefore, we tried the purifying it with **electro-elution**. It's a method to extract a protein sample from a poly-acrylamide electrophoresis gel by means of electric field. Because we wanted the pure form of ecdGPR114 of high intensity without any contaminating bands and we were unable to get it from batch purification and from refolding also. So it's a time saving method for eluting multiple bands in pure form [*Paula Sa-Pereira July 2000, vol. 27].*

  Procedure followed for electro-elution:

  Requirements: 1M KCl, gel cutter, Dialysis bag [fill it with electrode buffer], Laemmlis 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].
- The gpr114 protein from stock was treated 1:1 with 5X Laemmli's sample buffer.
- Boiled on thermo-mixer at 95^0C for 10 mins. The sample was cooled and loaded on 15% SDS gel.

- After completion of the run, the gel was put in a solution of 1M KCL for about five minutes.(the polypeptide were visible as white band in a slightly pale white background).
- Then the band corresponding to 25kda was excised with gel cutter and kept at -20^{0}C until electro-elution.
- For electro-elution, the strips were cut into smaller pieces and put in dialysis bag containing appropriate amount of electrode buffer.
- The bag was then submerged in Lammeli's electrode buffer in a horizontal gel electrophoresis apparatus unit].
- The electrophoresis was run at constant 3W overnight at room temperature.
- Then the bags were washed with distilled water and again put in electrophoresis unit containing freshly prepared buffer [10mMTris-HCL, 5M Urea, 1% 2ME] for removal of SDS from the sample.
- The electrophoresis was run at constant 1W for 12-14 hrs at 4^{0}C. Finally the dialysis was done in dialysis buffer [Tris buffer ph 8] for the removal of urea and 2ME.
- For confirmation of eluted band, the 20ul sample was electrophoresed on 15% SDS PAGE. The remaining sample was stored at -80^{0}C
- The gpr114 protein from stock was treated 1:1 with 5X Lammeli's sample buffer.
- Boiled on thermo-mixer at 95^{0}C for 10 mins. The sample was cooled and loaded on 15% SDS gel.

a) Characterization of ecdGPR114:

CD (Circular Dichroism): To check if the protein is present in folded form, we did CD (Circular Dichroism). It is a spectroscopic technique that characterizes the secondary and tertiary structure of a protein. It gives the graph between wavelength and molar elipticity.The negative bend at wavelength 208nm and 222nm shows the helical structure of protein, if it's at 218nm the structure is beta-sheet. The positive bend at 198nm shows the beta-turn structure of protein.

b) Generation of antibodies against ecdGPR114:

- **Immunization:**

The antibodies against GPR114 were not available therefore we venturued to generate a polyclonal anti sera that can be used for different immunoassays. Our main aim of immunization was to generate antibodies against ecdGPR114 so that we could use these antibodies to check the expression of ecdGPR114 protein on naïve, effecter and memory CD8 T cells.

Requirements: Freund's adjuvant, complete/incomplete, naïve mice, purified ecdGPR114.

For first immunization, two naive BALB/c mice were injected subcutaneously with the emulsion containing 20ug in 120ul/mouse of ecdGPR114 protein mixed with CFA (Freund's adjuvant, complete) in equal ratio. The CFA is an adjuvant as it consists of heat killed *Mycobacterium* in a water-in-oil emulsion. It causes an intense inflammatory reaction at the site of injection and as an adjuvant it helps in slow release of antigen.

Then the booster dose was given after 20 days. NOTE: For a booster dose half the concentration of antigen (i.e., 10ug ecdGPR114) and IFA (Freund's adjuvant, incomplete) was used. IFA is also an adjuvant that lacks *Mycobacterium*. The reason of using IFA is because we don't want an intense inflammation. The reason of using half concentration was the expansion of only specific and high affinity BCR expressing cells and a subsequent production of high affinity class switched antibodies. After 15 days of boosting the same mice were given a re-boosting dose as the previous one (i.e. 10ug ecdGPR114 +IFA in 1:1)

In between, on 11^{th} day of first boosting the mice were bled to collect 500ul of peripheral blood. The serum was collected from the clotted blood by centrifuging at 3000 rpm for 30 minutes at 4^0C. The serum was stored at -20^0C.

- **Immuno-assays to check antibody generation and specificity:**

To check whether the HIS contains antibodies against ecdGPR114, we used a number of immunological techniques

Agar gel immune-diffusion (AGID): In order to visualize the interaction of specific antibody and antigen, AGID was used. The procedure for AGID is as follows

- Agarose of 1.2% in Tris-Tricine buffer of pH 8.6 was prepared and poured on a glass slide using a pipette.
- Three wells were made using pipette tip with 3mm-5mm gap between the wells.
- In middle well, we loaded HIS. In one well we added electro-eluted protein andin another one we loaded urea-eluted fraction.
- Incubated overnight at RT temp.

Enzyme linked immune-sorbent assay [ELISA]: It is a sensitive and specific assay that can detect proteins or antibodies at pico-molar to nano-molar range. We performed indirect ELISA in which following reagents were used antigen (ecdGPR114, 5ug/ml), blocking agent (3%BSA in PBST), HIS (contains antibody of interest), PIS, secondary antibody conjugated with enzyme i.e. Alkaline phosphatase-conjugated Affinity pure Donkey Anti-mouse and substrate (4-Nitrophenyl phosphate disodium salt hexahydrate), substrate buffer (glycine 100mM, $ZnCl_2$ 1mM, $MgCl_2$ 1mM) and stop solution (3M NaOH).

HIS: Hyper-immune Serum, PIS: Pre-immune Serum, BSA: Bovine serum albumin.

Experimental setup: Three dilutions of PIS and eight dilutions of HIS, all in triplicates, only antigen and no antigen in another two wells as a control.

Procedure followed:

- The wells in the plate were coated with antigen (electro-eluted gpr114 protein of concentration 5ug/ml, 100ul/well). Incubated overnight at 4 degree C.

- Washing was done with PBST 3 times for 3 minutes with 200ul volume each time.
- Blocking was done with 3% BSA in PBST, 150ul/well. Plate was incubated at room temperature for 2 hrs.
- Again washing was done with PBST 3 times for 3 minutes with 200ul volume each time.
- The required dilutions of PIS and HIS were prepared and added 50 ul/ well. Incubation for 1 hr.
- Again the plates were washed with PBST and then PBS 2 times for 3 minutes.
- Secondary antibody solution was prepared and added 50ul/well. Incubation for 1 hr at RT.
- Washing was done with PBST 3 times for 3 mins. Substrate was prepared and added, 200ul/well. Incubation for 15-20 mins. The plate was observed for a change in color of the substrate thereafter stop solution was added.
- The plate was read using ELISA plate reader at 405nm.

Western Blotting or immune blotting: The western blot is a widely used analytical technique used to detect specific proteins in a sample of tissue homogenate. This was used to ensure that the anti-sera raised was specific to the GPR114.

Procedure followed:

- The samples were electrophoresed on 12% SDS PAGE.
- Incubated the gel in transfer buffer for 5 min.
- The resolved polypeptides were transferred to PVDF membrane using iBlot^{R}.
- The PVDF membrane was incubated overnight with 3% BSA in PBST at 4^{0}C for blocking. Then washing with PBST 3 times was done for 5 min each time.

- Further incubation was done with primary antibody i.e a particular dilution of HIS for 2 hrs at RT. Again washing with PBST.
- Final incubation with secondary antibody i.e. anti-mouse antibody conjugated with enzyme, for 1 hr at RT using (1:20,000) dilution.
- Three washings were done with PBST and then two with PBS.
- The ECL substrate was added in dark and the membrane was kept in the X-ray cassette.Above it, the X-ray film was kept for 3-30 mins.
- Detection of signal was done by X-ray detector and developed on the film.

c) Kinetics of expression of GPR114 in CD4 and CD8 T cells:

After establishing the specificity of HIS, we next checked the expression of gpr in purified naive $CD4^+$ and $CD8^+$ T cells and its expression pattern in stimulated cellsby western blotting.

d) Expression of *gpr114* at transcript level

To confirm the expression of gpr114 in T cells at transcript level, we measured its expression using a pair of primers for which sequence is given below.

Primers for real time PCR:

Reverse primer:	***NEB Tm calcu.***	***oligo-analizer***
5'TGCCAGAAGCTGATGTTCAC 3'		
Length 20		
Tm 41.1 ^{0}CTm 56 deg C	Tm 55.5 deg C	
Ta=51^{0}C		
Hairpin loop at 9.5deg C		
%GC 50		
Forward primer:		
5'CAGCCTGCTGAACAACTACG 3'		
Length 20		
Tm 41 ^{0}C	Tm 57 deg C Tm 56.2 deg C	
%GC 55		
Product size = 90		

As a control, the primers for housekeeping genes were also designed.

Primers for house keeping gene (beta-actin and Carbonic anhydrase III in mouse)

1. B-actin (Mus musculus)

B-actinforward (Musmusculus)	**NEB calcu.**
5' CAA AAG CCA CCC CCA CTC CTA AGA 3'	
Length 24	
Tm = 60.9^{0}C	Tm = 62^{0}C
Ta = 61^{0}CTa = 57^{0}C	
B-actin Reverse (Musmusculus)	
5' GCC CTG GCT GCC TCA ACA CCT C 3'	
Length 22	
Tm = 65^{0}C	Tm = 67^{0}C
Ta = 61^{0}C	
Product = 258	

2. Carbonic anhydrase III (Musmusculus)

CA3 Forward	**NEB calcu.**
5' CAA GGG CAG GGT GGT GAG 3'	
Length 18	
Tm = 58.6^{0}C	Tm = 60^{0}C
Ta = 58 ^{0}C	Ta = 55^{0}C
CA3 Reverse	
5' GGA GCA CAG GAG GAG ACA AGC 3'	
Length 21	
Tm = 60^{0}C	Tm = 61^{0}C
Ta = 58^{0}C	
Product size 105	

Purification of cells by Cell sorting:

The lymph nodes of BL/6 naive mice were isolated. We prepared a single cell suspension using cell strainer. The cells were washed and re-suspended in RPMI and then counted using cell countess (mix the cells with trypan blue dye 1:1, load on slide and read on cell countess). Then the surface staining was done with CD8, $CD44^{lo}$ and $CD62L^{hi}$ to sort out naïve CD8 T cells. A purity of 87% was obtained. Some of the cells were used to isolate the RNA and the remaining cells were used to prepare lysate. The RNA was converted into cDNA to proceed with real time PCR whereas the lysate was used for western blot, to check the expression of gpr114 on the surface of CD8 T cells.

1.3 Results and Discussion:

After Ni-NTA column purification of ecdGPR114, the band of interest (25kDa) was prominently visible (Fig--). In addition other multiple bands were also visible. Despite multiple attempts at purification of ecdGPR114, the desired level of purity could not be achieved. (So we tried to dialyze this elution in buffer with a lower urea concentration at every step. With each change one molar lower concentration was achieved as compared to that of the elution buffer to remove Urea from the sample step by step. But in case of Urea concentration below 2M, the protein got precipitated).

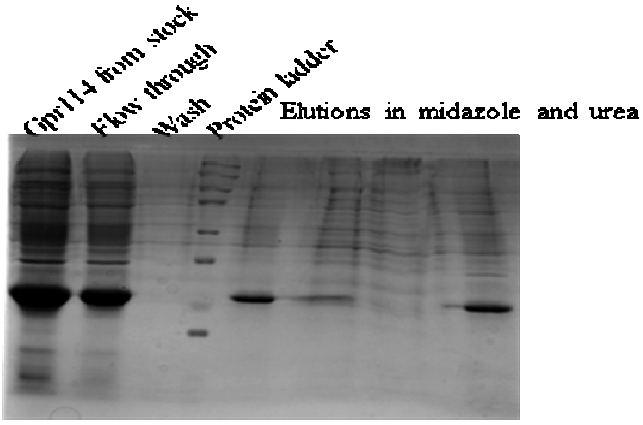


Fig 1.3: Ni-NTA purification of gpr114

Upon batch purification of ecdGPR114, we got an intense band of interest of ~25 kda with many other unwanted bands (Fig---). Then we again attempted to purify the inclusion bodies with an imidazole buffer but in absence of urea. As shown below, the greater level of purity was obtained but still some contaminating bands were visible.

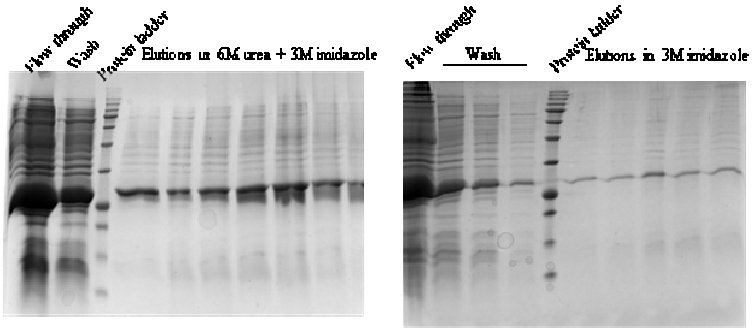


Fig1.4: Batch purification of gpr114

But since the elution contains imidazole, so the beads have more affinity for imidazole instead of ecdGPR114 protein. So before re-purifying it, we did the dialysis, to remove the imidazole from elution samples, in dialysis buffer for 30-40 hrs by changing the buffer three times.

After dialysis, 20-30ul was used to electrophores on 12% SDS-PAGE.

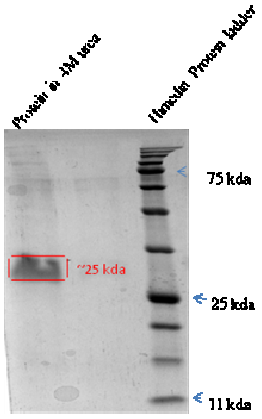


Fig1.5: SDS-PAGE of dialyzed gpr114

After dialysis in 4M Urea, we tried to re-purify half of the sample and electrophoresed on 12% SDS PAGE. And in this we got 90% purified intense band of interest of 25 kda.

We again tried to purify another set of protein in soluble as well as pure form using batch purification. We got intense band of ~25 kda eluted in Urea and 3M imidazole with the same protocol as above mentioned [just the wash buffer used was without urea ph 7.8].

Then we measured the concentration of protein using bio-spectrophotometer, it was 510ug/ml. So we proceed with refolding of our eluted protein as it was in Urea.

Because we wanted the pure form of ecdGPR114 of high intensity without any contaminating bands and we were unable to get it from batch

purification followed by refolding. So we resorted to the next approach called **electro-elution.** Using this approach the specific band could be cut from the gel and eluted using electrophoresis.

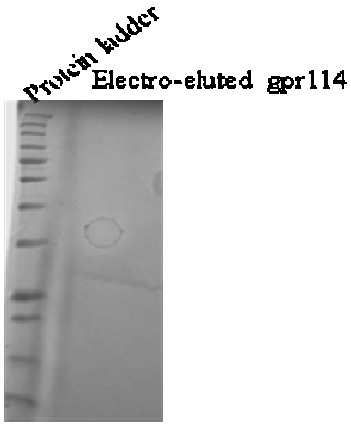


Fig1.6: Purification of gpr114 by electro-elution

The concentration of electro-eluted protein was **168ug/ml** measured using bio-spectrophotometer (refolding buffer as blank). The electroeluted and dialysed protein thus obtained was soluble in the buffer used. Therefore we were interested in estimating the refolding structure.

To check if the protein is present in folded form, we did CD (Circular Dichroism). We noticed that our protein was consists of both α-helical and β-sheets where β-sheet was dominating over alpha-helices. The results from CD spectrum showed that the protein was in a refolded form that could be used for generating antibodies some of which may recognize conformational epitopes as well.

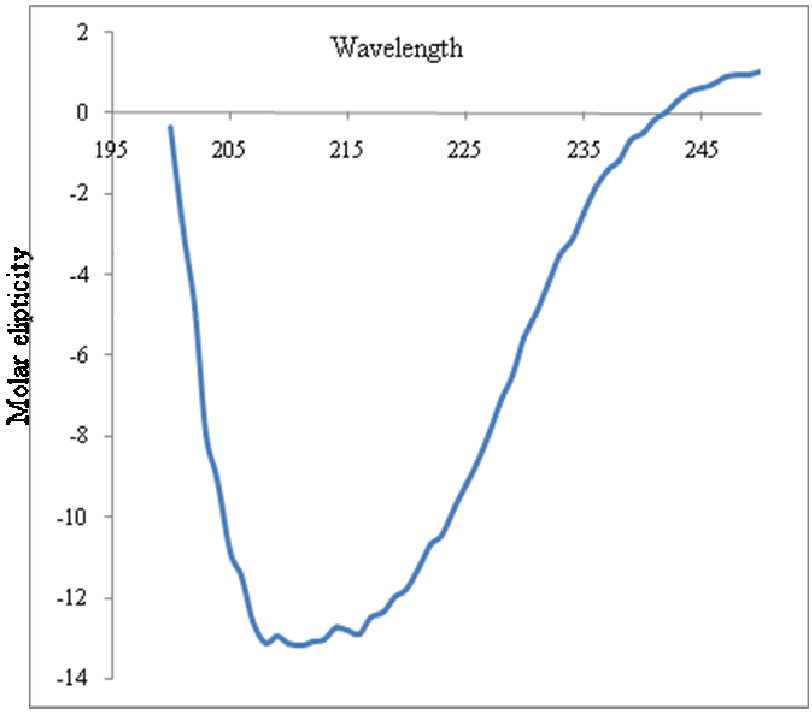


Fig 1.7: Refolded structure of electro-eluted gpr114

So we proceed with immunization and generation of hyper-immune serum against ecdGPR114.

In results of Agar immuno-difussion, we didn't see any precipitation might be because, this assay is not very sensitive and the anti-sera may not be of high titer.

But in results of ELISA, we observed the immune reaction that were visualized by the development of color. The extent of color development was more in those wells in which HIS serum was added as compared to pre-immune serum. The titer of HIS was calculated as per the formula.

Then the western blot results showed that the antibodies generated in the mouse against gpr114 are specific to this protein.

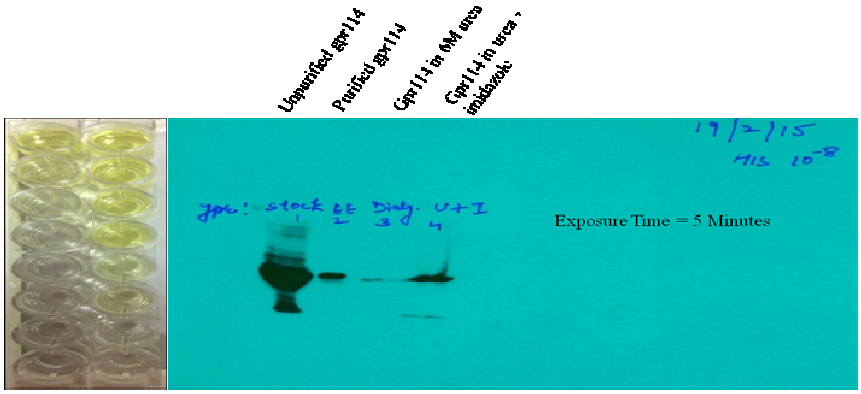


Fig 1.8: ELISA and Western-blott of the antibodies generated against gpr114 in mouse to check the specificity and sensitivity

Then we checked the expression of gpr114 on different immune cells like naïve CD4 and CD8 T cells, stimulated CD8 T cells using anti-gpr114 antibodies. We found the expression of gpr114 on naïve CD8 T cells as expected and it was not there on stimulated CD8 T cells.

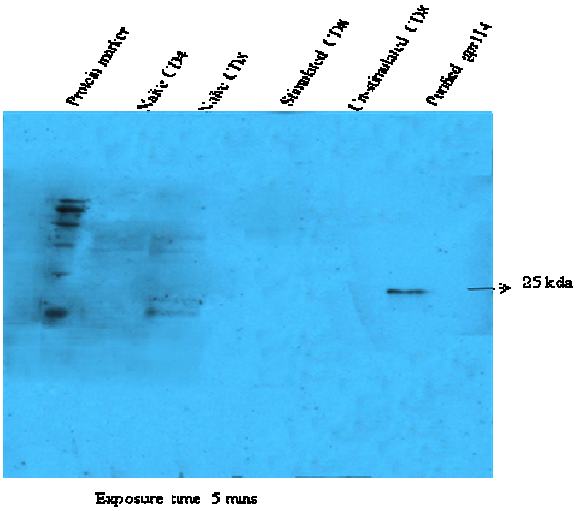


Fig 1.9: Western Blotting to check the expression of gpr114 on different immune cells

1.4 Conclusion:

We attempted at exploring the probability to prove gpr114 as memory CD8 T cell marker. For that we purified the protein in soluble and refolded form. Then the antibodies against this protein were generated in mouse.

The specificity and sensitivity of these antibodies was checked by ELISA and western blotting. To prove it as a marker, its expression was checked on different immune cells like naive CD4 and CD8 T cells, stimulated CD8 T cells using these anti-gpr114 antibodies. We found thatnaïve CD8 T cells express this protein and not stimulated CD8 T as well as not naïve CD4 T cells,as the expression was checked by western blot. Our next step is to check its expression on memory CD8 T cells and also to use an expression control (use of antibody against house-keeping protein) and then to go for RT-PCR.

Chapter 2

Immunomodulation by Russell Viper Venom

2.1 Introduction

Snakebites constitute a major public health problem in tropical and subtropical countries such as India. Large majorities of poor people who reside in rural settings, farm workers, fishermen, construction workers as well as people in uninhabitable locations etc. are among the most affected population. Therefore, the snakebites have an important implication in halting the growth of economy of countries where they occur. According to a recent conservative estimate, around 5 million snakebites with 50% rate of envenoming and quarter of a million deaths are reported annually on a global basis. About 3 million snakebite survivors develop some permanent disabilities like paralysis, necrosis, loss of vision, chronic renal failure as well as immunosuppression and depression. So the number of people affected by snake bite is more than the number of people affected from infectious disease such as HIV/AIDS. Every year people dying from snake bite is around one lakh in India alone.

Russell viper is one of the four poisonous snake species responsible for most human snake bite cases in India. It is often found in agricultural regions. Once bitten, people experience a variety of symptoms including pain, swelling, dizziness, blood coagulopathies and kidney failure. Then depending on the extent of envenoming and management procedures followed either recovery begins or the patient dies. So one of the long terms goal that is still elusive is to develop a cure for snakebite using immunotherapeutics with minimal damage to the host.

Recently researchers have described an association between Russell Viper snake bite and development of Herpes labialis. Herpes labialis is caused by Herpes Simplex virus I and is characterized by development of painful blisters on or around lips. The patients during early stages of labialis is a source of viral spread. These sores typically heal within 2-3 weeks but the

herpes virus remains in dormant state in facial nerves and ganglionic tissues. Depending on the immunological status of host HSV the virus gets reactivated. Some of the factors such as stress, trauma, fever, other systemic concurrent infections etc can trigger the reactivation. Recently researchers have found that Russell's viper envenomation as another triggering factors for HSV activation.

From many eligent studies over many decades it is clear that adaptive immune cells such as CD8+ T cells are responsible for the latency of virus. As HSV gets reactivated upon the snake bites, we reasoned that the components of snake venom could interfere with CD8 T cells and potentially CD4 T cells response which then can precipitate viral reactivation from latency. Therefore we designed this study, to study the effect of Russell viper venom components on immune cells that could potentially lead to the reactivation of virus causing debilitating disease.

2.2 Materials and Methods:

a) Mouse model:

BALB/c mice were used. CD4 and CD8 T cells were isolated from spleen and lymph nodes using 'Dynabeads for untouched mouse CD4/8 T cells' kit.

b) Russell's Viper Venom Fractionation by Gel Filtration:

Gel filtration: It is a technique that separates proteins on the basis of molecular size. This separation is achieved using a porous matrix to which molecules have different degrees of access. Hence, proteins are eluted from gel filtration column in decreasing order of size (*Hagel L, 2001).* Furthermore this approach causes minimal loss of the activity of the purified protein.

Venom of a Russell 's viper snake composed of different proteins. Depending on the zoogeographic origins and the age of Russell's vipers, their venom composition may vary (*Jayanthi and gowda, 1988).* To isolate different proteins from this venom, 10mg/ml venom was dissolved in

PBS(10mM) and passed through the Sephacryl S 200-HR column. The different collected fractions were first concentrated and then electrophoresed on reducing and non-reducing SDS-PAGE.

c) Procedure of isolation of mouse CD4/8 T cells:

Requirements: Heat inactivated Fetal Bovine Serum (FBS), Isolation buffer: PBS supplemented with 2% FBS and 2mM EDTA.

Protocol: The protocol provided in the cell isolation kit (cat no and make) was followed. Briefly, the beads were washed in the isolation buffer (PBS supplemented with 2% FBS and 2mM EDTA). The single cell suspension obtained from LN and spleens of mouse were mixed with antibody mix. and incubated for 20 min at 2^0C to 8^0C. After washing, cells were mixed with depletedDynabeads and incubated for 15 min at RT with gentle tilting and rotation. The desired population was isolated by negative selection using a magnet and cells were re-suspend the cells in RPMI.

d) Experimental setup:

We used 96 well flat bottom plates, coated overnight with anti-CD3 and anti-CD28 antibodies. The cells were stimulated through their co-receptors to find out the relative susceptibility of venom in stimulated and un-stimulated cells. Different dilutions of undiluted Russell's viper venom were used and in one well no venom was added serve as a control. Then cells were added and incubated for one hour at 37 degree. The viability of cells was checked using trypan blue dye exclusion method. Then the cells were stained for Annexin V and PI to check Apoptosis and necrosis by flow cytometry.

In another set of experiments, different dilutions of five peaks (obtained from gel filtration chromatography) and whole venom were used. Then, cells were added and incubated for different time slots (one hour or two hour) at 37 degree. The apoptosis and necrosis was checked using FACS or by the electrophoresis of their genomic DNA. (*see protocol e) and f)).*

e) Apoptosis and Necrosis Assay:

Apoptosis:Annexin V is used to stain the cells undergoing apoptosis. In apoptotic pathway, loss of plasma membrane asymmetry is one of the early events. So the membrane phospholipid phospatidylserine (PS) translocates from inner to the outer leaflet of plasma membrane. Annexin V is a phospholipid binding protein that has a high affinity for PS and binds to cells with exposed PS.

Necrosis: It is a premature death of cells and living tissue caused by factors external to cells or tissue such as toxins or infection. Propidium iodide (PI) is used to stain the cells undergoing necrosis. So in this case, membranes of dead and damaged cells are permeable to PI. It binds to nucleic acids. PI is used to differentiate between early and late apoptotic cells.

Requirements for the assay: 1X Annexin binding buffer, PI (10ug/ml), Annexin V

Protocol:

Incubated cells were harvested and washed with cold PBS. The cells were then resuspended in 1X Annexin binding buffer and 2 µl of Annexin V and 1ul of (10ug/ml) of PI was added. The cells were incubated at RT for 15 min. The cells were then added with 200ul 1X Annexin buffer and kept on ice. After this cells were immediately acquired using BD FACS calibur.

f) DNA Isolation:

This experiment was based on the theory that if cells are undergoing apoptosis then we see bands of smallest nucleosomal fragments. If they are undergoing necrosis then bands of irregular size resulting in smear formation are visible.

Protocol:

To 20ul protease in a 1.5 ml MCT 200ul of previously treated cells were gently added. The lysis of cells was performed using 200ul of AL Buffer by vortex for 15 sec and incubate at 56^0C for 10 min. 200ul ethanol (100%) was added to sample and vortexing was done for 15 sec. A short spin was performed. The samples were transferred to Mini spin column to perform a centrifugation at 8000rpm for 1 min. The filtrate was discarded

and 500ul AW1 buffer was added which was followed by centrifugation at 8000rpm for 1 min. Again the filtrate was discarded and 500ul of AW2 buffer was added followed by centrifugation at 8000rpm for 3-4 min. The filtrate was then discarded and an empty spin was performed for 2 min. Then DNA was eluted using 20-50ul AE buffer followed by centrifugation at at 8000rpm for 2-3 min.Extracted DNA was electrophoresed on agarose gel.

2.3 Results and Discussion:

Gel Filtration of Russell's Viper Venom: 10mg/ml venom dissolved in 10mM PBS passed through Sephacryl S 200-HR gel filtration column. We observed five peaks of different absorbance.

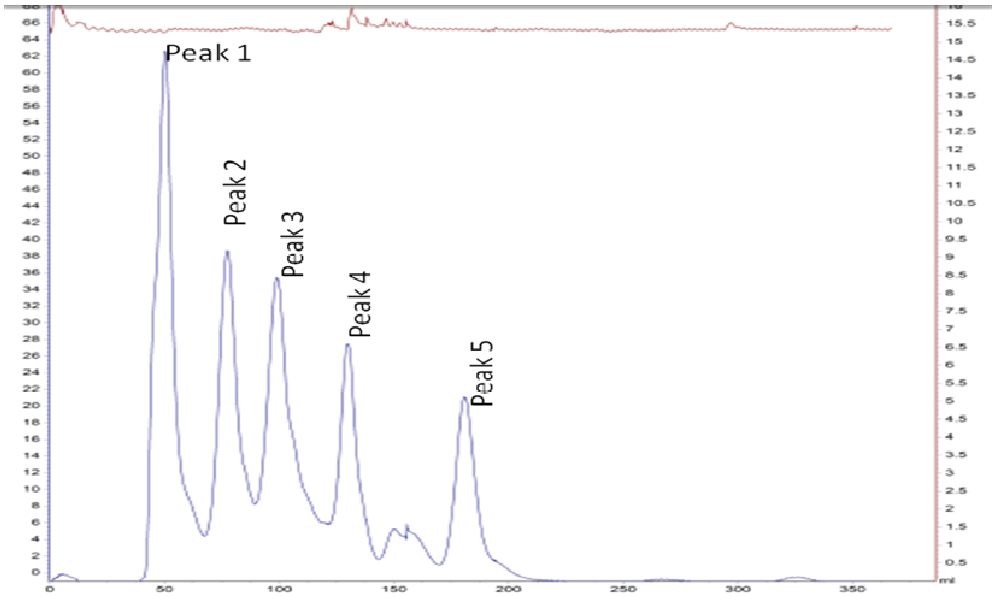


Fig 2.1: Gel filtration pattern of Russell's viper venom

The fractions collected from these different peaks were electrophoresed on a reducing SDS-PAGE. This shows that the venom consists of different proteins of different molecular weight.

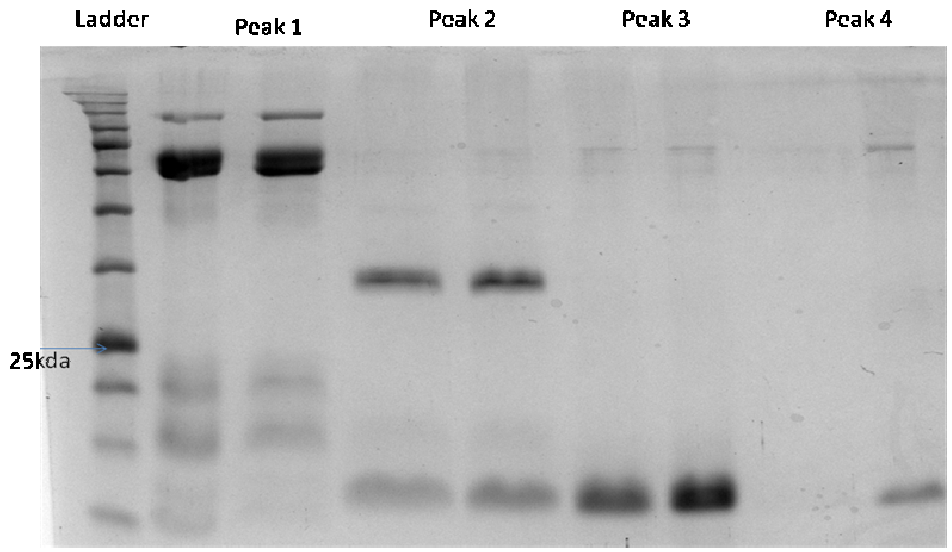


Fig 2.2: Different fractions collected from gel filtration on reducing gel

Apoptosis and Necrosis assay:

Viability of the cells incubated with different dilutions of venom was checked, stained with Trypon blue.

CD4 Stimulated T Cells:

RV (µg)	**Total cells(..x10^5)**	**Live cells(..x10^5)**	**Viability**
10	3.2	1.7	52%
1	4.4	2.4	54%
0.1	5.2	3.5	67%
0.01	3.8	2.6	69%
0	4.4	2.8	64%

CD8 Stimulated T cells:

RV (µg)	Total cells(..x10^5)	Live cells(..x10^5)	Viability
10	4.7	2.7	57%
1	7.7	5.7	74%
0.1	2.9	1.9	65%
0.01	4.1	2.5	62%
0	4.0	2.7	68%

PI and Annexin V staining to check apoptosis and necrosis: The cells were stained with Annexin V and PI and checked for apoptosis and necrosis. We found that protein from peak 1 is causing more apoptosis than when we used whole venom. Protein of peak 2 has similar observation as that of control where no venom was added.

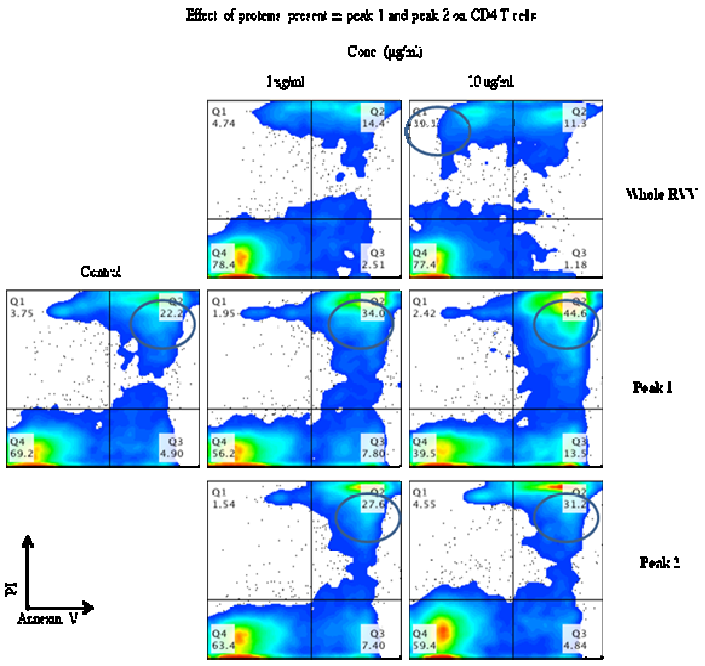


Fig 2.3: FACS of CD4 T cells undergoing apoptosis or necrosis

Agarose gel picture:

DNA from CD4 and CD8 T cells at different time points ranging from 2-15 hrs was isolated and electrophoresed on agarose gel. The results showed that proteins from some of the peaks are causing apoptosis to cells but some proteins are responsible for necrosis also.

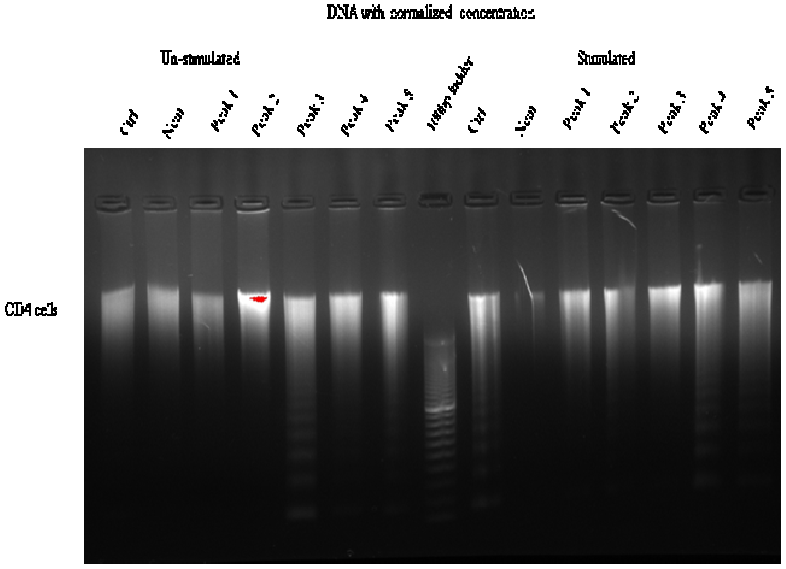


Fig 2.4: DNA isolated from CD4 T cells to check apoptosis or necrosis

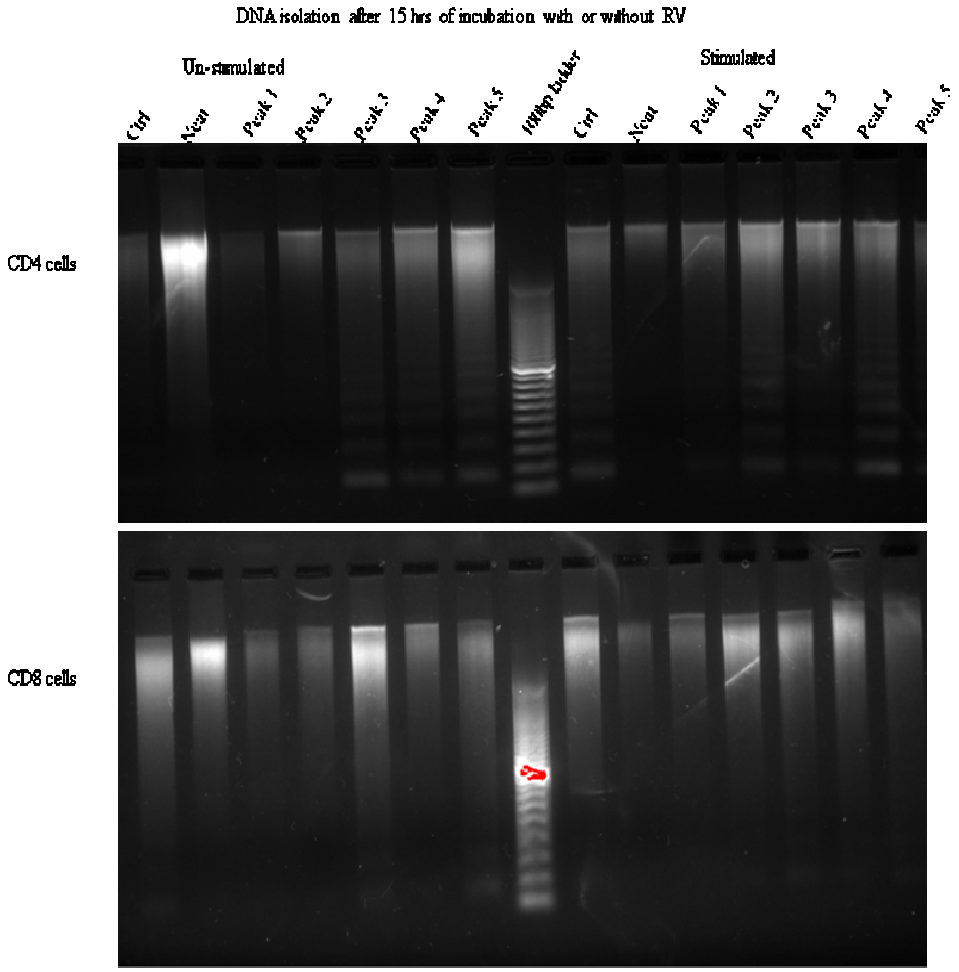


Fig 2.5: DNA isolated from CD4 andCD8 T cells to check apoptosis or necrosis

2.4 Conclusion:

We attempted at studying the effects of Russell Viper venom on immune cells. For that we isolated the different proteins present in venom using gel filtration chromatography and collected five different peaks of proteins. We then tried to check the effect of each protein as well as the whole venom on immune cells. We found that fraction 1 from gel filtration of intact venom is most effective in causing apoptosis as compared to other

fractions. Some of the fractions are found to induce necrosis in immune cells.Intact venom was found to be less efficient in inducing apoptosis in immune cells when compared with fraction 1 probably because of the presence of proteins that have pro and anti-apoptotic properties. This implies (our estimation is) that the whole venom contains some proteins or molecules that are shielding the cells from apoptosis/necrosis causing proteins. Our next step will be to do the mass spec. of these different unknown fractions to find the identity of these protein/s and devise strategies to counteract their effects.

Chapter 3

Additional Work

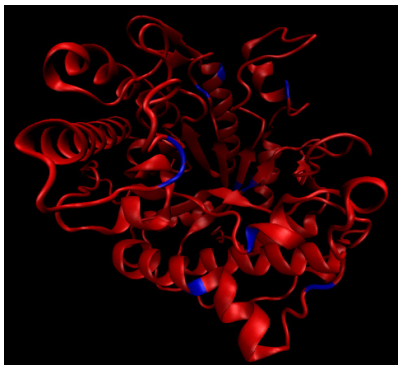
Cloning effort and biophysical characterization of protein

3.1 Introduction

We were working with a bacterial proteins: CelCCA protein from *Clostrodium cellulolyticum* (mesophile)**.**

***Clostridium Cellulolyticum*Cellulase (CelCCA)**

*Clostridium Cellulolyticum*is an anaerobic, motile, gram-positive bacterium. It's a mesophilic bacterium. We are focused on Cellulase gene of CelCCA consists of 1140 base-pairs.



The amino acid sequence of Cel CCA is:

Amino acid seqences of CelCCA –

MRGSHHHHHHGSMRMNIQKHGEDWKGTVVIVHGLGEHSGRYRR
LVREFVSEGVQVVTFDLPGHGKSPGRRGHLRFDDVFKILNEITKDL
ERFVLFGHSLGGLIAIRFTQIFQPENQKGLVVSAPAILLPDTHSPVLE
FMVRFLSFFVPFLTMSNGINPSDLSRNREAVEAYIRDPLVHDRISFK
LASDMLSHMKKVLKDAERIKVPVLIFHGTDDRVVSFEGSKKFFEAL
STEKKLVSFPGGYHELFEDPEHQKEFFKTIVEWSLEKLGGK#

3.2 Materials and Methods:

a) Protein purification of CelCCA :

A process by which information from a gene is used in the synthesis of a functional gene product is called gene expression. These products are often proteins. The genes were expressed in expression vector and preceded with protein formation.

Method:

Primary culture of 5 ml was inoculated by using CelCCA containing plasmid in expression host of *E.coli* glycerol stock. Tetracycline and ampicillin antibiotic of final concentration of 0.0125mg/ml and 0.1mg/ml respectively. and it was followed incubation at 37^0C with shaking overnight.

The secondary culture (500ml) was inoculated from primary culture, at 37^0C with shaking for 6hrs. The culture was centrifuged at 8000 rpm for 10 min to pellet down.

CelCCA was purified under native condition by using IMAC (immobilized metal affinity chromatography)..The pellet was dissolved in native lysis buffer and then sonicated for 1 hr at 20 amplitude by pulse of 10 second on and 20 second off. The sonicated solution was centrifuged at 12000 rpm for 1hr. The supernatant which contains protein was loaded on pre equilibrated by native lysis buffer Ni- NTA resin column. washing was done with 20mM imidazole and finally protein elution was done in 250mM imidazole.

After elution of protein, the imidazole was removed by dialysis against 20mM Phosphate buffer , pH8 by using 10kD cutoff dialysis membrane (Thermo scientific).

Finally the samples were electrophoresed on SDS PAGE to check the purity of protein.

The protein concentration was checked using UV spectrometer at 280nm.

b) Biophysical characterization of CelCCA:

- **Circular dichroism (CD) measurement:**

  Circular dichroism (CD) is an excellent tool for rapid determination of the secondary structure and folding properties of proteins that have been obtained using recombinant techniques. The most widely used applications of protein CD are to determine whether an expressed, purified protein is folded, or if a mutation affects its conformation or stability (*Greenfield 2009*). It gives the graph between wavelength and molar elipticity. The negative bend at 208nm and 222nm shows the alpha-helical structure and the same bend at 218nm shows the beta-sheet structure.

- **Size exclusion chromatography**

  Gel filtration (GF) chromatography separates proteins solely on the basis of molecular size. Separation is achieved using a porous matrix to which the molecules, for steric reasons, have different degrees of access--i.e., smaller molecules have greater access and larger molecules are excluded from the matrix. Hence, proteins are eluted from the GF column in decreasing order of size. (*Hagel L, 2001).*

  The superdex G-200 10/300GL of bead volume 23.8 ml with void volume 8-9ml obtained from GE healthcare was used on a GE Akta purifier 10 chromatography workstation. The column had optimum separation range from 10 KDa to 600 KDa. The column was pre equilibrated with those respective buffers in which protein was kept. Approx equal volume 400 µl of protein concentration of 1 mg/ml were loaded.

- **Protein fluorescence measurement**

  Protein fluorescence is measured by variancary eclipse steady state fluorimeter using 3mm of path length cuvette. Excitation is on 290nm and emission recorded between 300 to 500nm and slit width kept is 5nm.

- **Dynamic Light Scattering (DLS):**

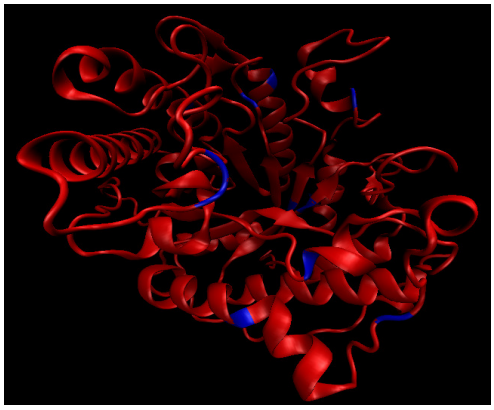
  Dynamic light scattering (DLS), sometimes referred to as Quasi-Elastic Light Scattering (QELS), is a non-invasive, well-established technique for measuring the size and size distribution of molecules and particles or proteins.

A DAWN 8 from Wyatt technology was used to determine the hydrodynamic volume and poly-dispersity profile of protein, using protein samples of 2.0 mg/ml concentration, for dynamic light scattering experiment.

3.3 Results:

Purification and characterization of Cel CCA from *Clostridium cellulolyticum*

Cel CCA is 44.36 kda protein constituting eleven Tryptophan, in which some are surface exposed.



The protein was purified using IMAC (immobilized metal affinity chromatography), elution in immidazole. The elution was electrophoresed on SDS PAGE. Band of interest at ~45 kda confirms the presence of purified protein.

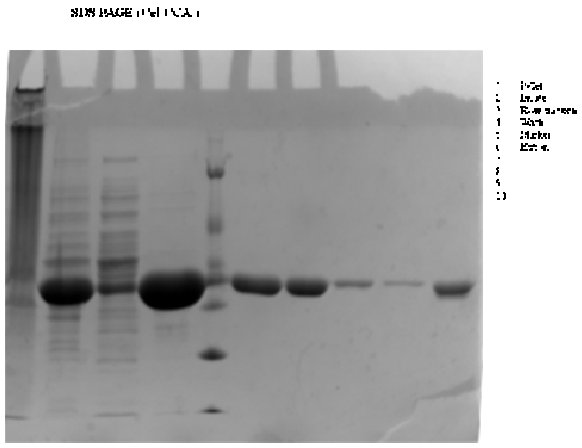


Fig 3.1: Ni-NTA purification of Cel CCA protein

The protein concentration was measured at 280nm using UV spectrometer and found 1.67mg/ml. The protein was further characterized for secondary structure, fluorescence and chemical stability using different techniques:

Structure analysis:

200ul of protein was analyzed in CD in cuvette of 1mm pathlength. The structure was consisting of 41% helix and 26% beta-sheet band at 208nm and 220nm.

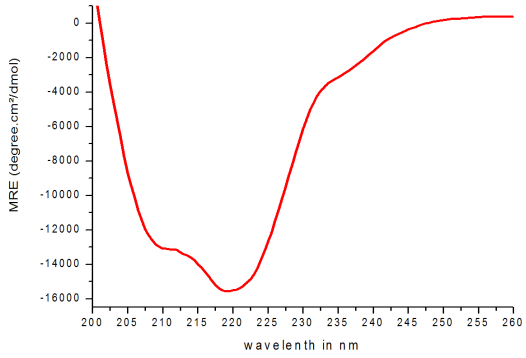


Fig 3.2: Refolded structure of Cel CCA

Fluorescence spectra obtained at 280nm (excitation) emission at ~350nm with intense fluorescence intensity which can be attributed to tryptophan molecules present on the surface.

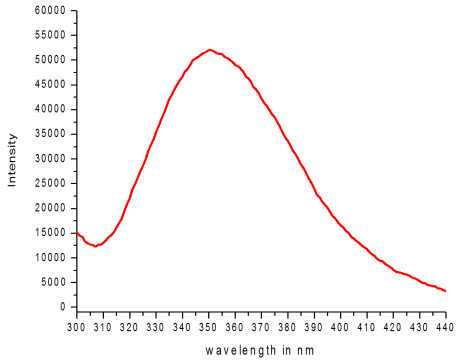


Fig 3.3: Fluorescence spectra of Cel CCA (excitation at 290nm)

For chemical denaturation study the protein was incubated with different concentrations of guanidium chloride and the scanned on cary eclipse

steady state fluorimeter using 3mm of path length cuvette. An inverse relation was found in the concentration of GuCl and fluorescence intensity of protein Cel CCA. The fluorescence intensity was nearly stable upto 2M of GuCl which then showed a sharp decrease in the range of 2M to 2.8M of GuCl and it was stable upto 4M with decreased intensity.

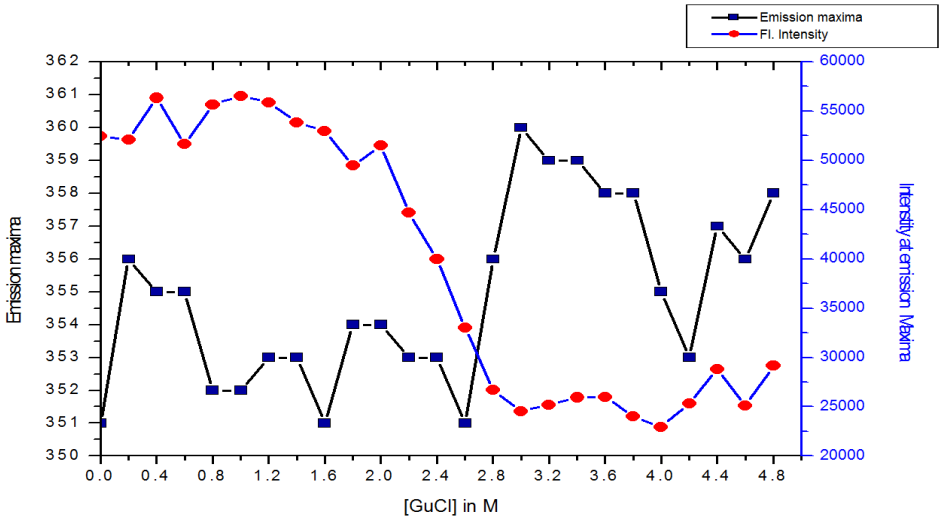


Fig 3.4: DLS data showed that the protein is in monomeric form of ~3nm.

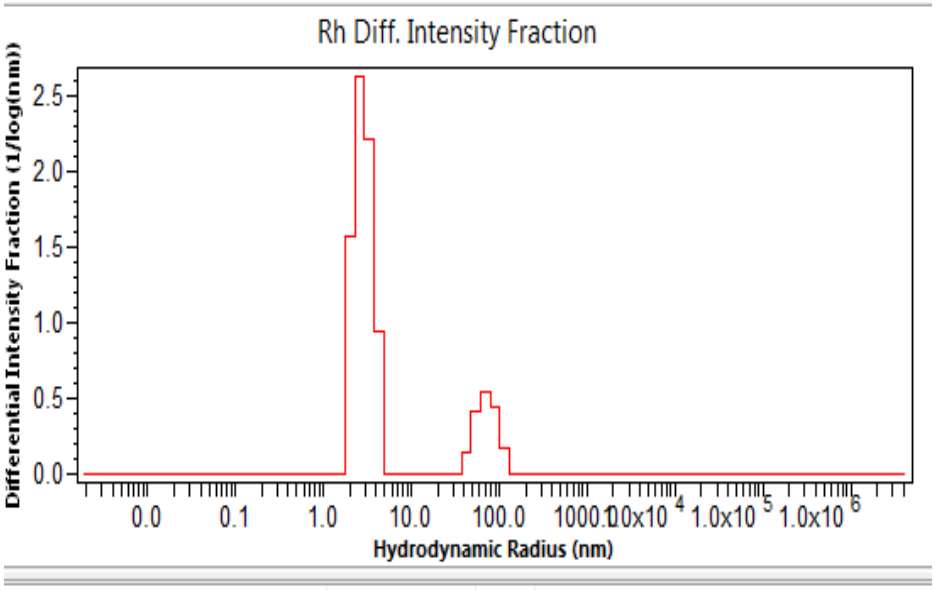


Fig 3.5: DLS of Cel CCA

3.4 Conclusion and Discussion:

Cel CCA, the purified protein has helical and beta-sheet structure and is of monomeric form of size 3nm as observed from DLS data. The protein has inverse relation with the concentration of GuCl and fluorescence intensity of protein Cel CCA.

Conclusion:

We tried exploring the probability of GPR114 serving as a memory CD8 T cell marker. For that we purified the protein in soluble and refolded form. Then the antibodies against this protein were generated in mouse. The specificity and sensitivity of these antibodies was checked by ELISA and western blotting. To prove it as a marker, its expression was checked on different immune cells like naive CD4 and CD8 T cells, stimulated CD8 T cells using these anti-gpr114 antibodies. We found that naïve CD8 T cells express this protein and not stimulated CD8 T as well as not naïve CD4 T cells, as the expression was checked by western blot. Our next step is to check its expression on memory CD8 T cells using various immunological and molecular biology approaches followed by *in vivo* studies.

We attempted at studying the effects of Russell Viper venom on immune cells. For that we isolated the different proteins present in venom using gel filtration chromatography and collected five different peaks of proteins. We then tried to check the effect of each protein as well as the whole venom on immune cells. We found that fraction one from gel filtration of intact venom is most effective in causing apoptosis as compared to other fractions. Some of the fractions are found to induce necrosis in immune cells. Intact venom was found to be less efficient in inducing apoptosis in immune cells when compared with fraction one probably because of the presence of proteins that have pro and anti-apoptotic properties. This implies (our estimation is) that the whole venom contains some proteins or molecules that are shielding the cells from apoptosis/necrosis causing proteins. Future studies will focus on characterizing and identifying the venom components using Mass Spectrometry. The overall aim is

to devise strategies to counteract their effects using immunotherapeutics.

Additional work focused on cloning and characterization of some of the biologicals from bacterial system but we were not able to clone the polymerase of *P. furiosis* and lipase of *T. maritima*, might be due to various problem. For characterization of Cel CCA, the purified protein was found to have helical and beta-sheet structure and is in monomeric form of size 3nm as observed by DLS analyses. The protein has inverse relation with the concentration of GuCl and fluorescence intensity of protein Cel CCA.

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