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*RESEARCH ARTICLE*

# A XENOGENIC IMMUNE RESPONSE TOWARDS STZ-RINM5F CELLS REVEALS CYTOKERATIN18 AS A NOVEL IMMUNOGENIC ANTIGEN THAT MAY PREDISPOSE TOWARDS TYPE-1 DIABETES

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## *Abstract*

Studies suggest certain stimuli like apoptosis behind the aberrant expression of circulating specific auto-antibodies in Type-1 diabetic patients. In the present study, we investigated whether Streptozotocin (a diabetogenic compound) induced apoptosis in Rat RINm5f Insulinoma cells can expose immunogenic cryptic antigens. A time course treatment of RINm5f cells with 4 mM Streptozotocin revealed 6hrs as minimal time period to induce apoptosis without much effect on viability.Subsequent immunization of mice with the untreated and Streptozotocin treated RINm5f cells revealed highly reactive sera from 6hr STZ treated cells. Hybridoma technique showed a highly reactive clone named sup160which secretedIgG1 type monoclonal antibody.Parallely, we show that 6hr STZ-RINm5f challenged mice displayed both humoral and cellular immune response, as shown by increased presence of IgG1 / IgG2a subclass of antibodies and overexpression of IFNγ, IL-4 and TNFα in splenocytes. FACS and confocal imaging further established the reactivity of sup160 antibody with RINm5f cell surface antigen. The antigen was identified to be cytokeratin18 protein as detected by 2D gel electrophoresis and mass spectrometry. In conclusion, we have successfully established a novel xenogenic mice model system for identification of new auto-antigen in Type-1 diabetes, although further studies are warranted in human subjects.



## Introduction:-

Type-1 diabetes (T1D) is an autoimmune disease characterized by humoral (which involves B cell secreted antibodies) and cell mediated immune response (which involves CD8+ cells, CD4+ cells, antigen presenting cells and innate cells) towards beta cells of pancreas which ultimately predispose people to persistent or recurrent hyperglycemia (Maahs et al., 2010; Richard and Diana, 2010). The humoral system in T1D produces auto-antibodies against some cellular antigens such as insulin, Glutamic Acid Decarboxylase (GAD), Glucose-6-phosphate, zinc-transporter 2 and Islet cell antigens (Baekkeskov et al., 1990; Wenzlau and Hutton, 2013). The exact etiology of these auto-antibodies is unknown; however, it is believed that some may be pathogenic in nature. Interestingly, more than 95 % of Type-1 diabetic patients have one or more types of islet auto-antibodies that

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precede the clinical manifestation of type-1 diabetes (Parikka et al., 2012). According to The Diabetes Prevention Trial-Type 1 study the risk of developing T1D increases 50% after 5 years if the patient has three or more auto-antibodies (Krischer et al., 2003).

Various reports suggest that despite negative selection or anergy of auto-reactive B cells, there exist certain low affinity self-reactive pool of peripheral B cells which are constantly patrolling for antigenic epitopes present on cellular proteins (Nemazee, 2017). Consequently, a high concentration of IgM antibody is found in autoimmune disorders like T1D which may lead to activation of polyclonal natural antibodies (Decraene et al., 1992; Casali and Schettino, 1996). In neonates there exist classes of B-cell repertoire known as ($CD5^+$) B1 cells and marginal-zone splenic B cells that produce pathogenic natural auto-antibodies (IgM, IgA and IgG3) with high binding avidity, independently of T cell help (Duanand Morel, 2006; Sidman et al., 1986). The exact mechanism of generation of these auto-antibodies is unknown but several studies suggested somatic hypermutation &class-switch DNA recombination, beta cell turnover, genetic abnormalities or alterations, abnormal survival due to alteration in molecules and defective removal of apoptotic cells (which can shed antigens that can elicit autoimmune response) as major culprits (Schettino et al., 1997; Ichiyoshi et al., 1995; Straus et al., 1999; Sakaguchi et al., 2003; Peng et al., 2007)

A number of strategies have been planned to prevent T1D; however major focus has been directed in the early diagnosis. Most of the Type-1 diabetic patients are screened on the basis of the presence of auto-antibodies to several antigens. Due to some limitations like detection methods, antibody titer, threshold limits, model systems and genetic heterogenicity; many antigens either remain unexplored or are not detected (Lampasona and Liberati, 2016). Studies in Non-Obese Diabetic (NOD) mice have shown that there occurs increased binding of T splenocytes to xenogenic Rat Insulinoma (RIN) cells and subsequent co-incubation with RIN cell membrane extracts inhibits the binding (Martignat et al., 1993). The structure formed by RIN adherent splenocytes is termed as “diabetic rosette”. Further studies showed that the “diabetic rosette” was higher in female NOD mice than male NOD mice which also signify the severity of the insulitis (Martignat et al., 1993). Similarly, incubation of rat pancreatic beta-cell extracts and various subcellular fractions with NOD splenic T cells demonstrated large array of proteins that can act as potential beta cell auto-antigens (Bieg et al., 1993).In yet another study, it was observed that lymphocytes isolated from Type-1 diabetic patients showed increased binding to RIN cells compared to control subjects (Gouin et al., 1996).

All these studies indicate that auto-antigens may not be newly synthesized but are already present in cryptic form that is inaccessible to auto-reactive immune cells. In fact, studies have demonstrated that tagging of 1-fluoro 2, 4-dinitrobenzene to normal human leukocyte surfaces, results in exposure of antigenic sites specific to human leukemic cells and disappearance of receptor / antigenic sites normally seen on untagged leukocyte surface (Joshi et al., 1981). Likewise, cross-linking of surface-bound monoclonal antibody by horse alpha-mouse IgG in resting peripheral T cells and thymocytes causes cell membrane perturbation which stimulates expression of certain antigens that are not normally expressed (Mittler et al., 1983).

In diabetes, and other autoimmune diseases apoptosis has been suggested as a mechanism to present antigens that can induce cell mediated or humoral response (Eguchi, 2001). Thus it will be worth to uncover new agents in T1D that can alter the dynamics of cell without altering much to its functional state. Moreover, the factors responsible for triggering T1D in an individual are at dispute. Streptozotocin (STZ) is a commonly used diabetogenic compound which has a long history related to beta cell death associated events in diabetes. Its specificity to beta cells enhances its application to study diabetes in animal models. STZ is taken specifically through Glut-2 receptor and the mice that received multiple low doses of STZ displayed internalization of Glut-2 receptor (Wang and Gleichmann, 1995). So it is quite possible that STZ may be affecting the topography of cell which in turn may lead to exposure of new antigen(s) or antigenic sites. RINm5f cell line is a very good in-vitro model for the study of biology of pancreatic islet cells and the pathogenesis of T1D. In the present study we have exploited hybridoma technique to identify autoantibody or auto-antigen by heterlogous immunization of mice with STZ treated RIN cells. The heterologous immunization is the best choice to evoke a good immunological response against an antigen. The ability to detect specific anti-beta auto-antibodies provides the anabolic window of opportunity to develop diagnostic and prognostic therapies.

## Materials And Methods:-

### Animals and cells:

The Balb/c mice were obtained from experimental animal facility of National Centre for Cell Science, Pune, India. During the period of experiments, the animals were housed at $21^0$C with 12 hours' light and 12 hours' darkness cycle. Food and water were available to animal's ad libitum. Rat Insulinoma (RINm5f) cells line was obtained from American Type Culture Collection, Manussas, VA, USA and maintained in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal calf serum, penicillin (100 IU / ml) and streptomycin (100 µg / ml). All incubations were performed at $37^0$C in 5% $CO_2$, in humidified air. The mouse myeloma SP20.Ag14 obtained from ATCC, Manussas, VA, USA was used as fusion partner. The cells were grown in the DMEM supplemented with 10% fetal calf serum, penicillin, streptomycin, sodium pyruvate (100 mM) and 2-mercaptoethanol (0.1mM).

### STZ preparation:

500 mM Stock solution of Streptozotocin (Sigma Aldrich) was reconstituted in 0.01 M citrate buffer (pH 4.5) just before treatment. 100 ml of 0.1 M sodium citrate buffer was prepared by mixing 55.5ml of 0.1M trisodium citrate (Sigma Aldrich, USA) and 44.5ml of 0.1M citric acid monohydrate (Sigma Aldrich, USA). pH was adjusted with 1 N NaOH and measured by pH meter (Thermo Fischer Scientific, USA). The buffer was filter sterilized prior to treatment.

### Annexin V/ propidium iodide (PI) double-staining assay:

Apoptosis was measured by flow cytometry using Annexin V/PI (Sigma Aldrich, USA) double staining method (Vermes et al., 1995). Briefly, 4 mM STZ treated RINm5f cells were collected, washed with ice-cold PBS twice, and resuspended in 500 µl 1X binding buffer containing Annexin V (1:50) and 40 ng / sample propidium iodide for 15 min at 37°C in the dark. Then, the number of viable, apoptotic and necrotic cells was quantified by flow cytometer (Becton Dickinson, USA) and analyzed by the CELLQuest software. Cells were excited at 488 nm and the emissions of Annexin V at 525 nm and PI were collected through 610-nm band-pass filters. At least 10,000 cells were analyzed for each sample. Apoptosis rate (%) = (number of apoptotic cells) / (number of total cells observed) × 100.

### DNA ladder assay:

For DNA ladder assay, $2 \times 10^6$ RINm5f cells were plated into Nunc cell culture dishes (Thermo Fischer Scientific, USA) 24hrs prior to STZ treatment. Following treatment with STZ for 6 hr, 12 hr and 24 hrs, the cells were washed with phosphate-buffered saline (PBS) and harvested. Cells were then resuspended in 0.5 ml of lysis buffer for 10 min at room temperature. After centrifugation at 12,000 x g at room temperature for 5 min, the supernatant was transferred to a new eppendorf tube and 700 µL chloroform-isoamyl alcohol (Sigma Aldrich, USA) was added, before centrifuging at 2,000 x g at room temperature for 30 sec. DNA was acquired by centrifugation of the samples, washed, dried and dissolved in loading buffer, and separated by electrophoresis during 90 min at 60 V on 1.5% agarose gels, containing ethidium bromide (Sigma Aldrich, USA). Bands were visualized under ultraviolet light in Gel documentation system (Biorad, USA) (Rahbar. Et al., 2015)

### Cell viability assays:

RINm5f cells were cultured in 96-well plate at a density of $1.5\times10^4$cells per well in 150 µl of complete medium and treated with 4 mM STZ for 6, 12 and 24 hrs. 15 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide from Sigma Aldrich] reagent at a concentration of 5 mg/ml in PBS was added to each well and plate was incubated at 37°C for 3 hours. Now 150 µl DMSO (Sigma Aldrich) was added in each well to fully dissolve the MTT formazan crystals. Absorbance was recorded at a wavelength of 590 nm in spectrophotometer (Biorad, USA).

### Immunization schedule:

$1 \times 10^6$ RINm5f cells were seeded in eight 60 mm petri plate's containing complete RPMI medium (Thermo Fischer Scientific, USA). Four plates were treated with only citrate buffer and rest were treated with 4 mM STZ for 6, 12 and 24 hrs. The cells were detached from the petri plate using scrubber. $1 \times 10^6$ STZ treated RINm5f cells were used as dose of antigen per animal (mice) per week. The immunization schedule performed to immunize the mice is shown below:

1. First week (withFreund's Complete Adjuvant; subcutaneously)
2. Second week (withIncomplete Freund's Adjuvant; subcutaneously)
3. Third week(withoutAdjuvant; intra-peritoneally)

All the animals were bled after 48 hrs of last injection. ELISA was performed with RINm5f cell extract to check the titer of antibody. A booster dose of 1 x $10^6$ RIN / STZ-RIN cells per animal was given intra-peritoneally to increase the antibody titer. Again the titer of the antibodies was checked by ELISA.

**Indirect ELISA for detecting reactivity of sera against RINm5f cell extract:**
The maxisorp 96 well ELISA plate (Thermo Fischer Scientific, USA) was coated with 100 µl / well of 5 µg / ml RIN soluble cell extract overnight at $4^0$C. The plate was washed with PBS-Tween-20 (Sigma Aldrich) and blocked with 100 µl / well of 1% BSA (Sigma Aldrich, USA) at $37^0$C for 1 hr. The plate was washed with PBS-Tween-20 twice. The respective sera i.e., un-immunized, RIN immunized and STZ-RIN immunized sera were diluted (1: 100) and 100 µl / well of each serum was put in respective wells in triplicates. The plate was incubated at $37^0$C for 3 hrs and washed thrice with PBS-Tween-20. The plate was incubated with 100 µl / well of goat anti-rat HRP secondary antibody (Santa Cruz Biotechnology, USA) at $37^0$C for 1 hr and washed with PBS-Tween-20 thrice. The reaction was developed with ABTS substrate (Sigma Aldrich, USA) and read at 410 nm in spectrophotometer.

**Fusion protocol:**
The animals showing highest titer were selected for fusion with SP20.AG14 cells (mouse myeloma cells) and antibody secretory clone was screened as per established protocol (Galfre and Milstein, 1981). 24 hrs prior to the fusion, the myeloma cells were fed with fresh complete DMEM medium (Sigma Aldrich, USA). The animals showing the highest titer of antibodies were given booster dose of 6hr STZ-RIN cells (1 x $10^6$ cells), 24hrs prior to fusion. For plating out the fused cells peritoneal macrophage feeder layer was prepared in four plates of 24 well tissue culture plates. The procedure for preparing peritoneal macrophages is as follows------

Four 6 – 8 weeks old Balb/c mice of either sex were sacrificed by cervical dislocation and were cut open with sterile scissors and forceps. 5 ml ice cold, sterile, plain DMEM was injected in the peritoneal cavity of animal and peritoneal lavage was taken out with the help of 5 ml disposable syringe (Dispovan, India). The peritoneal lavage of all the animals was pooled and centrifuged at 2000 x g for 3 minutes. The pelleted cells were again washed with plain DMEM. The pellet was resuspended in complete medium containing DMEM + 15% fetal calf serum + 1X HAT media supplement (Sigma Aldrich, USA) and about 5 x $10^4$ cells were plated in each well of four 24 well plates in the same medium.

Animals showing high antibody titer were dissected and spleen was removed. The mouse SP2 / 0 cells (ATCC, Manussas, VA, USA) were collected from the three 75 $cm^2$ tissue culture flasks and washed twice with plain DMEM. Both the cells (splenocytes and SP2 / 0) were washed separately twice with plain DMEM medium and total count and viability was recorded. Both the cell population were then mixed in 1: 1 proportion and again washed with plain DMEM medium. The mixed pellet now ready for the fusion, was held in 50 ml centrifuge tube and diluted with the fusion mixture (50% PEG in DMEM / DMSO mixture) over a period of 1 minute in beaker containing water at $37^0$C. The fused pellet was mixed well and fast diluted with 50 ml plain DMEM over a period of 3 minutes. During this the fused cell suspension was handled very gently and was spun at 1000 x g (rpm) for 5 minutes. The cell pellet was diluted gently with 40 ml of complete medium (i.e., DMEM + 15% fetal calf serum + 1X HAT). The homogenous cell suspension was then spread gently drop wise over the plates containing peritoneal macrophages feeder layer and the plates were incubated at $37^0$C at 5% $CO_2$ in water jacketed $CO_2$ incubator. The culture plates were monitored every day for chance contamination. The plates were fed with fresh complete medium every alternate day. This routine was followed every alternate day till colonies of hybrids reached 70% confluence. These wells were marked and supernatants were collected and ELISA was performed as described earlier using RIN cell extract as an antigen.

**Screening of hybrids:**
The hybrids giving the high readings (three times the reading of plain DMEM) to RIN cell extract were selected for further cloning. The secretory hybrids were further cloned with limiting dilution technique on peritoneal macrophages feeder layer in 96 well tissue culture plates. After achieving 50% confluence, the single clones were expanded in 24 well tissue culture plates. The supernatants were again tested by ELISA.

**Cryopreservation:**
Rest of the clones were then cryopreserved in cryomed at cooling temperature of $1^0$C per minute with freezing mixture [fetal calf serum (90): DMSO (10)] and stored in liquid nitrogen. The procedure used for freezing the clones was as follows:

The hybridoma cells were pelleted by centrifuging at 1200 x g rpm for 5 minutes. The pellet was held on ice and the pre-cooled freezing mixture was added dropwise, mixed properly to avoid cell aggregation.

**Ascites preparation:**
The clones showing highest reactivity on ELISA were expanded in 75 $cm^2$ tissue culture flask. Parts of cells (1 x $10^6$ cells / ml / animal) were injected intra-peritoneally in adult pristine SCID (severe combined immunodeficiency) mice. After about 8 – 10 days nearly 2 – 3 ml of ascites was collected from tapping peritoneal cavity of mice. The ascites was centrifuged at high speed (5000 x g, 15 minutes at $4^0$C) and the supernatant was stored at – $70^0$C.

**Purification of ascites:**
The ascites was thawed and diluted with PBS in equal proportion. The flask containing the diluted ascites was kept on ice on the magnetic stirrer and saturated ammonium sulfate was added dropwise to make the final concentration of 45%. The flask was held on ice for 60 minutes with constant stirring. The precipitated mixture was then centrifuged at 5000 x g for 60 minutes at 4 – $10^0$C. Next day the contents were collected and precipitate, if any, was discarded. 10 µl of it was used for determining the protein concentration by BCA kit method (Pierce) and rest was aliquoted and stored at – $20^0$C till further use.

**Isotyping of antibody secreted by sup 160:**
The isotyping analysis of the antibody secreted by sup 160 was analyzed by using following procedure. Briefly, maxisorp immunoplate was coated with 100 µl of soluble extract of RINm5f cells overnight at $4^0$C. The plate was blocked with 1% BSA for 1 hr at $37^0$C and washed with PBS-Tween20. Sup160 supernatant was diluted 1: 100 in PBS-Tween20. 100 µl of diluted sera were added in triplicate on the plate. The plate was then incubated for 3 hrs at $37^0$C. After incubation the plate was thoroughly washed thrice with PBS-Tween. 100 µl of the anti-mice IgG1, IgG2a, IgG2b, IgG2c, IgM, IgE, IgD and IgA antibodies conjugated to HRP (1: 1000 dilution) were then put on the plate and incubated for 1 hr at $37^0$C. The plate was extensively washed three times with PBS-Tween and incubated with O-phenylenediaminedihydrochloride (Sigma, USA). Finally, the reaction was stopped with 20% $H_2SO_4$ and the Optical Density (OD) measured by an ELISA reader at 492 nm. Similarly, with 6 hr SRS sera, the isotyping for IgG1 and IgG2a was performed

The antibody titer of secretory clone (sup160) was estimated by serial dilution method and the signal was read at O.D 450 nm. The inverse of highest dilution in which antibody still gives positive result indicates antibody titer.

**Splenocyte isolation:**
The spleen was washed twice with sterile plain DMEM medium and was minced with the help of sterile frosted slides. After this the cell suspension was centrifuged at 400 x g for 10 minutes at room temperature. Aspirate supernatant and suspend pellet in ACK lysis buffer (Sigma Aldrich, USA) for 1 minute. Centrifuge again at 400 x g for 5 minutes and discard supernatant. Resuspend cells in PBS for a final wash and centrifuge at 400 x g for 5 minutes. Discard supernatant and suspend pellet in known volume of DMEM medium. Mix cell suspension with equal volume of 0.2% trypan blue and count live cells in hemacytometer under microscope.

**RT-PCR:**
Total RNA was isolated from spleen and pancreas using commercially available Trizol reagent (Thermo Fischer Scientific, USA) and cDNA was prepared according to the protocol mentioned in SuperScript First-Strand Synthesis System (ThermoFischer Scientific, USA). The PCR was carried out with 5µl of cDNA, Taq DNA polymerase (New England Biolabs, UK), dNTP's (Sigma Aldrich, USA), a magnesium ion containing Taq buffer (New England Biolabs, UK) using following set of primers:

**IL-1 β** [Forward primer: 5'- GCAGCTATGGCAACTGTTCCT -3'; Reverse primer: 5'- GGTGGGTGTGCGGTCT -3'; annealing temperature: $55^0$C]
**TNF α** [Forward primer: 5'- GGATCATCTTCTCAAAACTCG -3'; Reverse primer: 5'- TCACAGGAGCAATGACTCCAAA -3'; annealing temperature: $52^0$C]
**IFN γ** [Forward primer: 5'- GCTCTGAGACAATGAACGCT -3'; Reverse primer: 5'- AAAGAGATAATCTGGCTCTGC -3'; annealing temperature: $53^0$C]
**IL-10** [Forward primer: 5'- CTGGCTCAGCACTGCTAT -3'; Reverse primer: 5'- ATTCATGGCCTTGTAGACAC -3'; annealing temperature: $53^0$C]

**IL-4** [Forward primer: 5′- TCGGCATTTTGAACGAGGTC -3′; Reverse primer: 5′- GAAAAGCCCGAAAGAGTCTC -3'; annealing temperature: $53^0$C]
**IL-5** [Forward primer: 5'- TCACCGAGCTCTGTTGACAA -3'; Reverse primer: 3'- CCACACTTCTCTTTTTGGCG -5'; annealing temperature: $53^0$C]
**TNFα** [Forward primer: 5'- GCCTCTTCTCATTCCTGCTTG -3'; Reverse primer: 3'- CTGATGAGAGGGAGGCCATT -5'; annealing temperature: $61^0$C]
**IL-6** [Forward primer: 5'- ACGGCCTTCCCTACTTCACA -3'; Reverse primer: 3'- CATTTCCACGATTTCCCAGA -5'; annealing temperature: $65^0$C]
**β Actin** [Forward primer: 5′- TGGAATCCTGTGGCATCCATGAAAC -3′; Reverse primer: 5′- AAAACGCAGCTCAGTAACAGTCCG -3'; annealing temperature: $59^0$C]
The PCR for each gene was subjected to 35 cycles of amplification.

**FACS analysis:**
The RIN / 6hr STZ-RINm5f cells were harvested in ice cold PBS (containing 10% FCS, 1% sodium azide) and cell number was adjusted to 1-5 x $10^6$ cells/mL in polystyrene round bottom 12 x 75 $mm^2$ Falcon tubes. Thereafter, cells were pelleted by centrifugation at 300 x g for 5 minutes and resuspended in 100 µl of PBS (containing 2% FBS and 0.02% azide). The cell suspension was mixed with 500 µl of fixation buffer (2% PFA in PBS), left for 10 – 15 minutes on an orbital shaker (100 rpm) and permeabilized with 500 µl of 0.5% NP-40 for 15 minutes followed by PBS washing. Un-immunized sera, 6 hr STZ-RINm5f immunized sera and sup 160 sera were added at appropriate dilution followed by incubation at 4°C for 30 minutes. Cells were washed and incubated with 100 µL fluorescein isothiocyanate (FITC)-labeled goat antirat IgG (diluted 1:100) for 30 min at 4°C. Subsequently, the cells were analysed on FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using CELLQuest software (Hogg et al., 1981).

**RIN cell immunofluorescence staining:**
Poly L-lysine coated slides were prepared by dipping them in poly L-lysine solution (prepared in 10 mM Tris pH8) for at least 10 minutes. The slides were allowed to dry and RIN cells were grown on them. PBS washing was performed and cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature. Thereafter, 0.25% Nonidet P-40 induced permeabilization was performed for 10 minutes and cells were blocked overnight at 4°C with 5% fetal goat serum, 1% bovine serum albumin, and 0.1% Triton X-100. Respective sera and sup 160 was added, incubation was performed for 1 hour and cells were washed before incubation with fluorescent labeled secondary antibody (anti-mice FITC secondary antibody) for 1 hour (Paddock and Eliceiri, 2014).

**2D gel electrophoresis:**
RINm5f cells were solubilized in SD buffer [urea (7 M), thiourea (1 M), beta-mercaptoethanol, CHAPS (4%), ampholytes (1%) and protease inhibitor cocktail (Roche, Switzerland)] with the help of pipette and centrifuged at 14,000 rpm for 15 minutes at $4^0$C. IPG (immobilized pH gradient from Sigma) strips of pH range 3 – 10 were rehydrated under silicon oil and kept in Bio-Rad equipment for IEF (Iso –electric focussing) run. Around 70 µg of total protein subjected to IEF run at 50mA at $20^0$C. The strip was equilibrated in two steps (10 – 15 minutes). In first step, the strip was incubated with equilibration buffer (SDS, Glycerol, Tris, Urea, bromophenol blue, pH 6.8) + DTT to inhibit reformation of disulfide bridges. In second step, the strip is incubated with equilibration buffer + iodoacetamide which alkylates the proteins and reacts with any unreduced DTT. Now the strip was placed 12.5% SDS-PAGE gel and sealed with agarose. The run was performed at 70 V. The gel was incubated with 6 hr STZ-RINm5f immunized sera and sup 160 and respective proteins were detected with anti-mouse secondary antibody conjugated to HRP by ECL reagent (Pierce Biotechnology, USA) (WenzlauandHutton, 2013).

**Protein sequencing:**
For Gel pieces having protein band showing reactivity to sup 160 monoclonal antibody were cut. The proteins were identified at RGCB, India. Briefly, the gel pieces were transferred to siliconized tube and destained in 300 µl of 50% methanol overnight. The gel pieces were dehydrated in acetonitrile, rehydrated in 30 µl of 10mM dithiothretol in 0.1 M ammonium bicarbonate and reduced at room temperature for 0.5 hrs. The DTT solution was removed and the sample was alkylated in 30 µl of 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 0.5 hrs. The reagent was removed and the gel pieces rehydrated in 100 µl of 0.1 M ammonium bicarbonate. The pieces were again dehydrated in 100 µl acetonitrile, the acetonitrile was removed and the gel pieces were rehydrated in 200 µg / µl trypsinin 50 µM ammonium bicarbonate on ice for 10 minutes. Any excess trypsin solution was removed and 20 µl of 50 mM ammonium bicarbonate added. The sample was digested overnight at $37^0$C and the

peptides formed were extracted from the polyacrylamide in two 30 µl aliquots of 50% acetonitrile / 5% formic acid. These extracts were combined and evaporated to 25 µl for MS analysis.

The LS-MS system consisted of a Finnigan LCQ ion trap mass spectrophotometer system with a protonspray ion source interfaced to a self-packed 8 cm x 75 µm id Phenomenex Jupiter 10 cm C18 reversed phase capillary column. 0.5 – 5.0 µl volumes of the extract were injected and the peptides eluted from the column by an acetonitrile / 0.1 M acetic acid gradient at a flow rate of 0.25 µl / minute. The nanospray ion source was operated at 2.8 KV. The digest was analyzed using the double play capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans. The data were analyzed by database searching using the sequest search algorithm. Peptides that were not matched by this algorithm were interpreted manually and searched vs the EST databases using search algorithm (Karpievitch et al., 2010).

**Statistical analysis**

For each experiment three independent readings were performed. Results were expressed as the mean ± standard deviation. Statistical analysis was done by the Student's t-test and calculations were performed by SPSS 16.0 software. $P < 0.05$ was considered to indicate a statistically significant difference.

## Results:-

**Time course effect of 4 mM STZ on RINm5f cells as measured by apoptosis and cell viability assay**

In our previous paper we demonstrated that 4 mM STZ induced G0 / G1 cell cycle arrest in RINm5f cells as early as 12hrs (Singh et al., 2012)In this study, we used same concentration of STZ to study apoptosis in RINm5f cellstill 24 hrsby two approaches i.e., annexin V / PI staining and DNA ladder assay. Annexin V-FITC staining showed6hrs as minimal time period to expose phosphatidylserine on RINm5f cell surface **(Figure 1A)**. The percentage of apoptotic cells in each scatter plot procured from UR and LR panels are as follows: control (5.82 ± 0.5%), 6hr STZ (16.9 ± 0.7%), 12hr STZ (35.03 ± 1.2%) and 24hr STZ (47.4 ± 1.9%) **(Figure 1B)**. In DNA ladder assay, 4 mM STZ induced DNA fragmentation as early as 12hrsand no DNA laddering was observed in non-STZ and 6hr STZ treated cells**(Figure 1C)**. Parallely, we also studied the effect of STZ on RINm5fcell viability and observed a major decline in viability after 12hrs **(Table 1)**.

**High reactivity of 6hr STZ-RINm5f sera with RIN cell extract and identification of secretory clone sup 160 of IgG1 type**

Our next aim was to find the immunological response against RINm5f cells and 4 mM STZ treated RINm5f cells. Untreated RINm5f cells (Control) and RINm5f cells were treated with 4 mM STZ for 6hr, 12hr and 24hr were injected into Balb/c mice and immunization schedule was performed over a period of 3 weeks (as described in materials and methods). The sera from respective animals were subjected to indirect ELISA to detect any reactivity against RINm5f cell extract. A significant reactivity was observed with 6hr SRS (≃ 5.5 fold) and 12hr SRS (≃1.8 fold) (**Figure 2A)**. No significant change in reactivity towards RINm5f cell antigens was observed among the sera from US, RS and 24 hrs SRS.

We went ahead to identify auto-reactive antibody / s in 6 hr SRS which show specific reactivity towards the RINm5f cell antigens. A hybridoma approach was used to raise monoclonal antibodies against the reactive antigen / s (as described in material and methods). ELISA was performedand 20 positive clones were obtained. Among these clones the highest reading was observed with a secretory clone named sup160 and the reactivity of this clone was almost > 1.6 fold higher than 6hr SRS**(Figure 2B)**. So, we decided to carry our further studies with this clone and other clones were cryopreserved for future use. After performing a serial dilution method, the titer value of sup160 came out to be 1600 U / ml **(Figure 2C)**. ELISA based isotyping of this monoclonal antibody revealed IgG1 type **(Table 2)**.

**6hr STZ-RINm5f immunized Balb/c mice evoke both humoral and cell mediated immunological response**

In this context, we also examined the kind of immunological response towards the antigen / s exposed in 6hr STZ-RINm5f cells. RIN cells in mice represent a xenogenic model to study immune related phenomenon. For humoral response, IgG1 / IgG2a antibody levels were measured in sera obtained from mice immunized withRINm5fcells and 6 hr STZ-RINm5f cells and compared with unimmunized serum.As shown in **Table 3**, no significant difference was observed between the levels of these antibodies between unimmunized and RINm5f immunized animals. For cell mediated immune response we measured Th1 / Th2 cytokines (IFNγ, IL-4, IL-10, IL-5) and TNFα (a

proinflammatory cytokine)(Maahs et al., 2010)in splenocytes ofun-immunized and 6 hr STZ-RINm5f cell immunized mice by RT-PCR. As shown in **Figure 3A & 3B**, IL-4 (≃ 6.4 fold), TNF α (≃ 7.2 fold)and IFN γ (≃ 9.0 fold) were significantly increased in splenocytes of 6hr STZ-RINm5f immunized animals compared to splenocytes of un-immunized mice. Gene expression of IL-5 and IL-10 cytokines showed no significant change.

**sup160 monoclonal antibody shows strong reactivity on RINm5f cellsurface**

To gain insight into the localization of antigen, the RINm5f cells were incubated with unimmunized serum (control serum), 6hr STZ-RINm5f immunized serum and sup160 monoclonal antibody and analyzed by FACS and confocal imaging. In FACS, we found that RINm5f cells showed higher reactivity with sup160 monoclonal antibody and 6hr STZ-RINm5f immunized serum as compared to un-immunized sera. The histogram in **Figure 4A**, shows that sup160 monoclonal antibody showed higher peak shift towards right followed by 6hr STZ-RINm5f immunized serum.

In the next step, we performed confocal imaging to visualize the antigen localization. As shown in **Figure 4B**, sup160 monoclonal antibody and 6hr SRS showed fluorescence which was diffused in cytoplasm and concentrated more towards cell surface of RIN cells. No visible staining was observed in controls. The nuclei were stained with DAPI. The comparative fluorescence intensity is shown in bar chart **(Figure 4C)** wherein sup160 monoclonal antibody showed nearly 2-fold higher fluorescence intensity than 6hr SRS.

**2D Gel Electrophoresis revealed Cytokeratin-18 as the target antigen of Sup 160**

Now we were interested to decipher the nature of protein identified by sup160 monoclonal antibody. Total RINm5f cell extract was subjected to 2D gel electrophoresis (as described in material and methods) and incubated with sup160 monoclonal antibody. Simultaneously, another piece of 2D gel was stained with coomassie blue stain. As shown in **Figure 5A & 5B,** sup160 monoclonal antibody spotted a single protein which had molecular weight near to 48 kD (pH range 4.0 – 6.0). Further analysis of this spotted protein by mass spectrometry revealed cytokeratin18 as a target protein.

## Discussion:-

Most of clinical studies suggest that the presence and number of auto-antibodies correlate with the progression and severity of type-1 diabetes (WenzlauandHutton, 2013; Parikka et al., 2012). However, the exact etiology of these auto-antibodies in T1D has remained ambiguous. Among various mechanisms apoptosis has been suggested a vital event that can expose or leach antigens to evoke an immune response (Peng et al., 2007). However, the experimental methods that prove such presumptions have not been explored much. Moreover, there are some challenges in screening the autoantibodies in individuals. The most commonly encountered challenge is antibody titer that limits the detection of auto-antigens. Thus there is a need to design some strategies that can be used to harness the antibody response against some unknown antigens.

In this work, we have attempted to generate a STZ-RINm5f xenogenic cell model system to identify a monoclonal antibody that might act as biomarker for T1D. In the previous work we had shown that 4 mM STZ leads to cell cycle arrest in RIN cells (Singh et al., 2012) In this context, we have used same concentration of STZ to study early and late apoptotic stages in RINm5f cells. We show by annexin V / PI staining that 6hr represents the early apoptotic phase wherein the STZ exposes phosphatidylserine on RINm5f cell surface. On the other hand, the later phase of apoptosis started from 12hrs onwards as shown by DNA fragmentation assay. Simultaneously, cell viability assay revealed that STZ preserved functional state of cell till 6hrs. All this data suggests that 6hr time period might represent a critical window for STZ to induce bio-physical changes in RINm5f cells of immunological significance. This lead us to investigate whether STZ treated RINm5f cells evoke any humoral response against some antigens.

We believe that functional state of cell plays an important role to evoke a proper humoral response against self-antigen(s). Indeed, posttranslational modifications, altered tissue specific expression patterns or levels, coding DNA mutations have been suggested to be mechanisms that can expose tumor associated antigens of immunological relevance (Houghton., 1994)Pre-apoptotic stage represents an early state wherein such events can happen at physiological level. In order to establish the fact, we studied immunological response in mice immunized with RINm5f cells alone and STZ treated RINm5f cells. The idea behind using mouse as an in-vivo immunogenic model system was based on the fact that heterologous transfer of cells generally evokes an efficient immune response. By indirect ELISA, we found that sera from 6hr STZ-RINm5f immunized animals displayed a significant reactivity with RINm5f cell extract. This clearly implies that STZ leads to exposure of new antigenic sites (epitopes) during

early phase of apoptosis. It should be noted that beta cell in itself can act as antigen presenting cell which are evident from the fact that beta cells express MHC and ICAM-1 expression and IL-6. In multiple low dose STZ induced type-1 diabetes it was found that STZ led to upregulation of MHC Class I molecules on beta cells which are normally expressed at lower levels in normal pancreas (Campbell and Harrison, 1990). These MHC class I molecules present antigen epitopes through proteosomal pathway. There exists a bi-directional talk between proteasome pathway and apoptosis. There are situations wherein late stages of apoptosis (i.e., caspase activation) inhibit the activation of proteasomes in order to deactivate inducer of apoptosis (IAP) (Li et al., 2000). For example, Caspase 6 cleaves UFD2 (polyubiquitination factor) and caspase 3 / 7 cleaves the proteasome activator PA28γ (Yang et al., 2000; Mahoney et al., 2002). These studies were interesting in our case as this justifies why STZ treated RINm5f cells did not evoke any humoral immune response in late apoptosis. We did not go much deep to understand the kind of modifications because our goal was limited.

After establishing the fact that 6hr STZ-RINm5f immunized sera (SRS) has the potential antibodies which recognize the RIN cell antigens, we were interested to identify a suitable highly immunogenic clone. By hybridoma approach we identified sup 160 as a highly secretory clone (reactivity was mostly 3 times than other clones as measured by ELISA technique) from the splenocytes of 6hr STZ-RINm5f immunized rats. Many researchers have used this approach to raise monoclonal antibodies against expressed protein(s) or whole cells (Araya et al., 2002; Rezaei and Ghaderi, 2017; Dreyer et al., 2010). This clone was studied further and all other clones were cryopreserved for future screening. The sup160 clone was expanded, purified and the isotyping method revealed IgG1 subtype. Interestingly, IgG1 subclass is a predominant auto-antibody that shows reactivity towards type-1 diabetic autoantigens (Rose et al., 2010; Soto-Aguilar et al., 1991). This indirectly indicates that we have raised an auto-antibody against an antigen that might behave as auto-antigen. Alongside, we also show that the antibody titer value of this clone is 1600 which is highly significant from serological point of view.

Having established the effective immunological response against the 6hr STZ-RINm5f cells, we sought to examine the kind of immunological response in these mice. Though immune response in type-1 diabetes is controversial but studies suggest that both humoral and cell mediated immune response occur (WenzlauandHutton, 2013). Following markers were assessed to study humoral and cell mediated response: IgG1 / IgG2a levels for humoral response and, IFN γ, IL-4, IL-10, TNF α and IL-5 for cell mediated immune response. These markers were chosen because of their well-established role in type-1 diabetes and STZ induced diabetes (Bonifacio et al., 1999; Lukic et al., 1998; Shehata et al., 2011). A significant increase in IgG1 and IgG2a antibody levels was found in serum of 6hr STZ-RINm5f immunized and STZ animals. This clearly implies that we have developed an in-vivo system that mimics the humoral response in T1D. No significant change of IgG1 and IgG2a antibody levels in sera of un-immunized and RINm5f immunized animals reinforces the fact that apoptosis like stimuli play a pivotal role to mediate the antigen – antibody crosstalk. In the previous result, we noticed that RINm5f cell extract shows some significant reactivity towards RIN immunized sera. So, theoretically IgG1 and IgG2a antibodies should have displayed reactivity with RIN immunized sera. We speculate that the reactivity we observed in our previous result might be due to the presence of IgM antibodies. During a humoral immune response, IgM is the first antibody that is secreted by activated B cells in response to an antigen(s) (Campbell and Harrison, 1990). Due to their polyclonal nature, they can detect multiple antigens based on affinity. In fact, reports also show that IgM antibodies are present at clinical onset of Type-1 diabetes (Gronwall and Silverman, 2014). This is followed by IgG antibody response that generally peaks after 3 weeks of immunization against a specific antigen. However, we have not measured IgM response which we believe will be quite obviously there. Furthermore, our aim was to identify an IgG secreting clone because most of the auto-antibodies in T1D are of this nature.

Coming to markers for cell mediated immune response, we observed significant increase in mRNA levels of TNFα, IFNγ and IL-4 cytokines in splenocytes from 6hr STZ-RINm5f immunized mice. The expression of these cytokines clearly indicates that an efficient Th1 or CD8+ T Cell response has been generated against STZ treated RINm5f cells. On the other hand, no significant change in IL-5 and IL-10 cytokines signifies that heterologous RINm5f cells don't evoke Th2 response in mice. The expression of these cytokines clearly signifies T cell immunological response which is in accordance with previous reports (Decraene et al., 1992). The over-expression of IFNγ and IL-4 was important to note because both these cytokines are required to mediate antibody isotype switching (IFNγ induces IgG2a isotype switching and IL-4 induces IgG1 isotyping switching) (Schoenbornand Wilson, 2007). TNFα on the other hand is an inflammatory cytokine that plays an important role in pathogenesis of autoimmune diseases like T1D (Takatasu, 1997). In our case over-expression of TNF α also indicates efficient immune response against heterologous RINm5f cells. Taking into account the functions of IL-5 and IL-10 cytokine, (which include anti-

inflammatory, downregulation of MHC class II antigens, Th1 cytokines and co-stimulatory molecules); no change in its expression further supports the above data (Lee et al., 2005; Kiyoshi, 2011). Also no significant change in IL-5 and IL-10 expression signifies that it might have some other housekeeping roles. In fact, IL-10 promotes activation of B cells alone or in combination with IL-4 (Couper et al., 2008; Itohand Hirohata, 1995; Rousset et al., 1992)The expression of these markers is in accordance with the previous reports which suggest that inflammation and adaptive immunity go hand-in-hand during beta cell destruction in type-1 diabetes.

So far we had observed the reactivity of 6hr SRS and sup160 monoclonal antibody with RINm5f cell extract which presents the antigen in denatured state. Also, xenogenic transfer of RIN cells might expose certain antigenic sites which may not mimic the actual antigenic property of protein of interest or in other words we can say cross-reactivity. So, we analyzed the reactivity of sup160 monoclonal antibody and 6hr SRS with whole RINm5f cells by flowcytometry. Interestingly, in FACS histogram sup160 monoclonal antibody displayed a greater peak shift than 6hr SRS with RINm5f cells which is quite obvious because of purity of antibody. The flowcytometry result with RINm5f cells was further analyzed by confocal imaging which revealed that reactive antigen(s) were localized diffusely in cytoplasm and more intensely near plasma membrane. Moreover, comparative analysis of the relative level of fluorescence intensity revealed higher reactivity of sup160 monoclonal antibody. The higher reactivity of sup160 monoclonal antibody demonstrates the specificity of this monoclonal antibody towards the target antigen. 6hr SRS represents a pool of polyclonal antibodies which in itself explains its lower reactivity than sup160 monoclonal antibody. The distribution of antigen(s) as revealed by confocal imaging signifies that antigen(s) are somewhat hydrophilic / hydrophobic. This is in support of the fact that our antibody recognizes both denatured and native state of antigen. At this point of time we can't comment whether the antigen(s) are over-expressed or post-translationally modified as this is beyond the scope of this work. However, we have raised a monoclonal antibody which is quite effective in recognizing an antigen in its native and denatured form. Raising such antibodies has a great potential in serological testing.

Our next important goal was to identify the target antigen recognized by sup160 monoclonal antibody. This we achieved through 2D gel electrophoresis which separates proteins on the basis of pH and molecular weight. 2D gel electrophoresis has been used to study islet proteins, enzymes and autoantigens since 1930 (Chatterjee et al., 1985; Vischer et al., 1987; Escurat et al., 1991). With sup160 monoclonal antibody a single intense spot corresponding to molecular weight of 48 kD was recognized and its pI was somewhat between pH 4.0 – 6.0. Mass spectrometry of this spot revealed a protein similar to cytokeratin18 (type I keratin). On surveying the literature, we came across cytokeratin8 and cytokeratin18 which are expressed at higher levels in pancreatic islets as compared to other keratins (Bouwens, 1998). Their role in pancreas is still unknown but it is believed that they are mainly involved to maintain integrity of cell architecture besides other cellular and tissue-specific functions. A study showed that $K8^{-/-}$ mice are protected from STZ induced stress as depicted by increased glucose tolerance, lower fasting glucose levels and insulin sensitivity (Alam, 2013). Similarly, another study showed that transgenic mice over-expressing human K18 mutations sensitize them to Streptozotocin (STZ)-induced death (Ku et al., 2010). Comparative proteome analysis of pancreas from STZ treated Sprague-Dawley rats and genetically obese mice also revealed keratin like proteins (Park et al., 2012). Also, pancreatic exocrine autoantibodies against cytokeratin's of molecular weight 40kD have been found in Type-1 and Type-2 diabetic patients (Kobayashi et al., 1990).

We further went ahead to find the similarity between rat, mice and human cytokeratin18 using BLASTP 2.8.0+ program for sequence alignment(data not shown here). To our surprise rat / mice cytokeratin18 displayed very high similarities (almost 96%) while as rat / human cytokeratin18 showed only 84% identity. Such a high degree of identity between mice and rat cytokeratin18 was important to note as this can be helpful in developing serological assays to study role of sup160 monoclonal antibody in both animal models. How good this monoclonal antibody may work in human serological assays remains a question? But 84% identity human cytokeratin18 with rat species gives us the opportunity to believe that it might work with human serological assays also.

## Conclusion:-

In conclusion our finding reinforces the fact that STZ-RINm5f xenogenic model system can be used as a new tool to identify any unknown pathogenic antibodies in T1D. Unfortunately, we did not have access to sera of type-1 diabetic patients to validate our model system. Also immune tolerance studies would be worth to decipher the role of these antigens or antibodies in autoimmunity. In conclusion, we have been successful to identify an auto-reactive or pathogenic sup160 monoclonal antibody against cytokeratin18 (a beta cell antigen) that may have potential of

biomarker in T1D. We also provide a technical aspect which can be harnessed to identify new antigens during autoimmunity.

**Table 1:-** Time course percent survival of RINm5f cells treated with 4 mM STZ.
The cells were treated with STZ for 6 hr, 12 hr and 24 hrs. The cell viability was measured by MTT assay at 590 nm. Values represent mean ± S.D of three independent experiments. *p < 0.05 was significantly different compared to control.

| | **% SURVIVAL ALONWITH WITH S.D** |
|---|---|
| **CONTROL** | 100 ± 14.5 |
| **6hr STZ** | 90 ± 8.2 |
| **12hr STZ** | 58 ± 10.9 |
| **24hr STZ** | 30 ± 11.05 |

**Table 2:-** Isotyping of sup 160 monoclonal antibody
An indirect ELISA method was used to detect the isotype of sup 160. The HRP conjugated secondary antibodies were used to identify the isotype by measuring absorbance at 492 nm.

| | **IgG1** | **IgG2a** | **IgG2b** | **IgG2c** | **IgM** | **IgE** | **IgD** | **IgA** |
|---|---|---|---|---|---|---|---|---|
| **Sup 160** | 0.9 | 0.2 | 0.2 | 0.17 | 0.18 | 0.2 | 0.1 | 0.14 |
| **NEGATIVE CONTROL** | 0.14 | 0.16 | 0.15 | 0.19 | 0.14 | 0.18 | 0.1 | 0.1 |

**Table 3:-** Comparative analysis of IgG1 and IgG2a levels in serum of Un-immunized, RINm5f immunized, 6 hr STZ-RIN immunized and MLD-STZ group of animals.

An indirect ELISA method was used to estimate IgG1 and IgG2a isotypes in serum of un-immunized, RIN immunized and 6 hr STZ-RINm5f immunized mice. The reactivity was detected by HRP-conjugated secondary mouse antibody and absorbance was read at 492 nm. The mean ± S.D represents data from 5 animals in each group. *p < 0.05 significantly different from un-immunized group and $^{\#}$p < 0.025 significantly different from RINm5f immunized group.

| | IgG1 | IgG2a |
|---|---|---|
| UNIMMUNIZED | 0.092 ±0.020 | 0.085 ±0.007 |
| RINm5f immunized | 0.18 ± 0.022$^{*}$ | 0.12 ± 0.019$^{*}$ |
| 6hr STZ IMMUNIZED | 2.35 ± 0.179$^{*,\#}$ | 1.809 ± 0.190$^{*,\#}$ |

**Figure Legend's**
**Figure 1:-** Time course assay of apoptosis and cell death in RINm5f cells treated with 4 mM STZ.
The cells were treated with 4mM STZ for 6hr, 12hr and 24hrs and in each assay the data represents means ± SD of three independent experiments. **A)** Two dimensional dot plot showing percentage of Annexin V-FITC and PI (propidium iodide) labeled cells in FACS analysis. On x-axis, FL1-H represents the annexin V-FITC labeled cells and on y-axis, FL2-H represents PI labeled cells. The lower left quadrant displays cells negative for PI and annexin V-FITC; the lower right quadrant displays cells positive for annexin V-FITC (early apoptotic); the upper left quadrant displays cells positive for PI (necrotic); and the upper right quadrant displays cells positive for annexin V-FITC and PI (late apoptosis cells). Compared to control (without STZ), there occurred gradual increase in apoptosis in time dependent manner. **B)** The corresponding apoptosis rate (5.82 ± 0.5% for control, 16.9 ± 0.7% for 6hr STZ, 35.03 ± 1.2% for 12hr STZ and 47.4 ± 1.9% for 24hr STZ) is represented in bar graph. *P < 0.05 represents significantly different from control **C)** DNA ladder assay performed on 2% agarose gel containing ethidium bromide. The bands were visualized under UV light in gel documentation system and 100 bp DNA ladder was used as marker. No DNA fragmentation is observed in control and 6hr STZ treated cells. A clear DNA laddering pattering is observed after 12hrs of STZ treatment.

**Figure 2:-** Enzyme immunoassay of RINm5f and STZ-RINm5f sera with RINm5f cell extract and identification of highly reactive clone (sup 160) by hybridoma technique.
**A)** Error bar graph showing fold change in reactivity between **US (**un-immunized sera),**RS (**RINm5f immunized sera) and **SRS (**STZ-RINm5f immunized sera) with RINm5f cell extract after performing ELISA. The data correspond to the standard deviation of triplicate measurements of the optical density of the wells at 410 nm. The

dotted line represents the reference fold difference. *P < 0.05 represents significantly different from US. Around 6 animals were used in each group for experimentation. **B)** Bar graph showing fold change in reactivity of supernatant in different clones after enzyme immunoassay. The clones were obtained through hybridoma technique and the cells used for fusion were splenocytes from 6 hr STZ-RINm5f immunized rats and SP20 myeloma cells. Among the clones sup 160 showed the highest reactivity. **C)** Line graph showing antibody titer of sup 160. X-axis represents the antibody serial dilution and y-axis represents the O.D at 450 nm. The antibody titer from graph is calculated as inverse of highest dilution at which reading is almost zero. From line graph the antibody titer value of sup 160 is 1600 U / ml.

**Figure 3:-** Immune response in 6hr STZ-RINm5f immunized animals.

**A)** RT-PCR (reverse transcriptase polymerase chain reaction) of genes i.e., Tumor Necrosis Factor alpha (TNFα), Interferon Gamma (IFNγ), Interleukin 4 (IL-4), Interleukin 10 (IL-10) and Interleukin 5 (IL-5) from splenocytes of unimmunized mice group (lane 1) and 6hr SRS group (lane 2). β-actin was used as loading control. The mRNA was converted to cDNA by reverse transcriptase and PCR for the corresponding genes was carried out by Taq DNA polymerase in PCR machine. **B)** Error bar graph shows the fold change in the expression of corresponding genes normalized to β-actin. The data was analyzed in Gel Documentation system by using image analysis software.The data is expressed as mean ± SD of six independent experiments. Around six mice were used in each group. P < 0.05.

**Figure 4:-** Comparative reactivity of 6hr SRS and sup160 monoclonal antibody with 6hr STZ-RINm5f and RINm5f cells and identification of cytokeratin18 antigen.

**A)** Histogram showing reactivity of 6hr SRS and sup 160 monoclonal antibody with RINm5f cells after performing FACS with FITC conjugated secondary antibody. An un-immunized (US) serum has been used as negative control. The reactivity is presented as shift towards right in fluorescence relative to control (US). Peak 1 represents negative control (un-immunized serum), Peak 2 represents 6hr SRS reactivity and Peak 3 represents sup160 monoclonal antibody reactivity. Peak 3 shows greater shift than peak 2 relative to control **B)** Confocal image showing localization of antigen recognized by sup160 monoclonal antibody and 6hr SRS in RINm5f cells as visualized by FITC anti mouse secondary antibody. US (un-immunized sera) has been used as negative control. No immunofluorescence is observed with US while as both sup160 monoclonal antibody and 6hr SRS show bright immunofluorescence. **C)** Error bar graph showing relative level of FITC fluorescence intensity between 6hr SRS and sup 160. The fluorescence intensity was analyzed by ImageJ software. The intensity of sup160 monoclonal antibody is almost 2.5 times than 6hr SRS. The data represents means ± SD of three independent experiments. P < 0.05.

**Figure 5:-** Identification of cytokeratin18 antigen.

**5A)** The gel on the left side shows coomassie staining of the total RINm5f cell extract after 2D gel electrophoresis. **5B)**The gel on right side shows immunoblot with sup160 monoclonal antibody that recognizes a 48 kD antigen in pH range 4.0 – 6.0.

**Figure's**

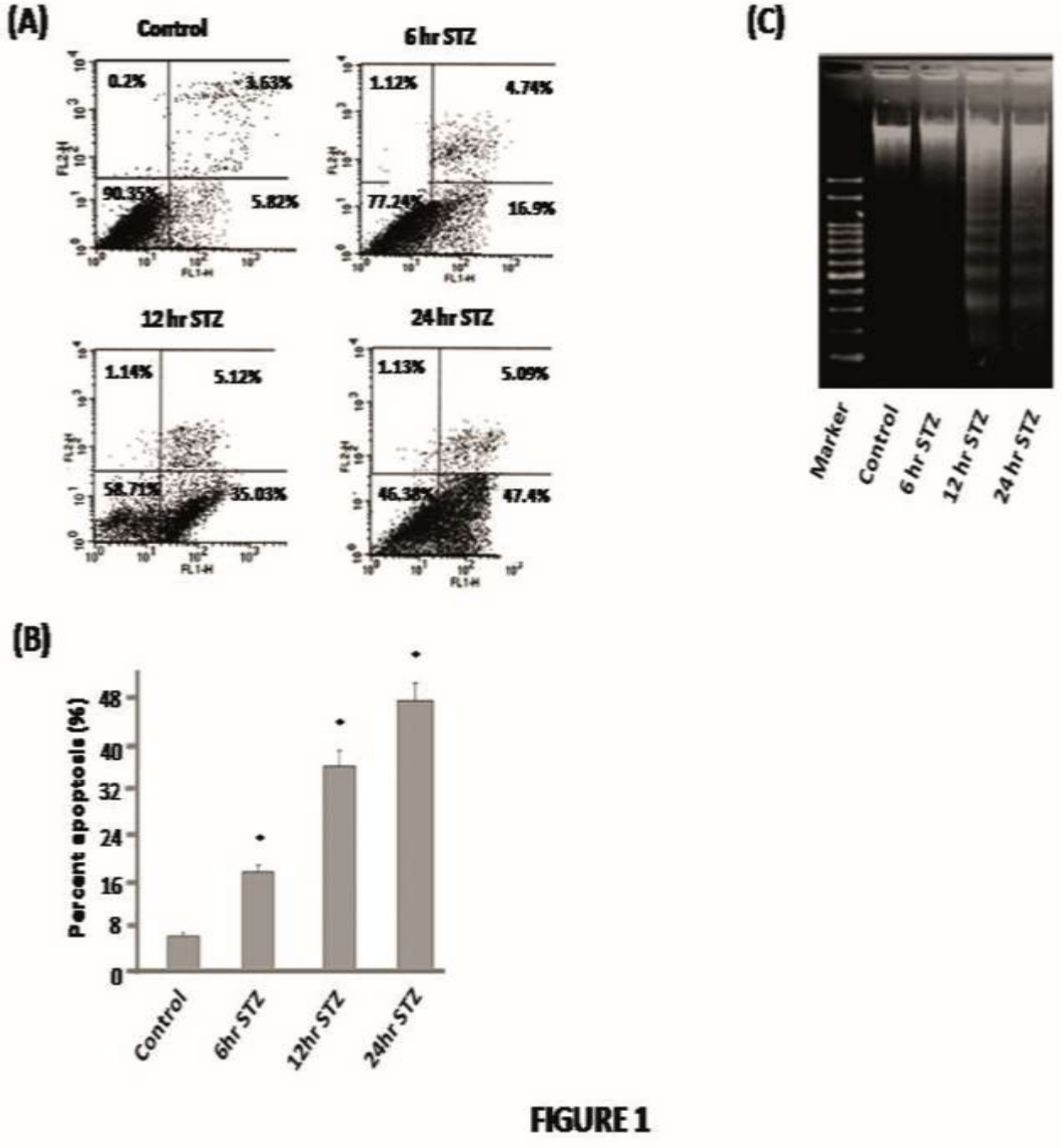


**FIGURE 1**

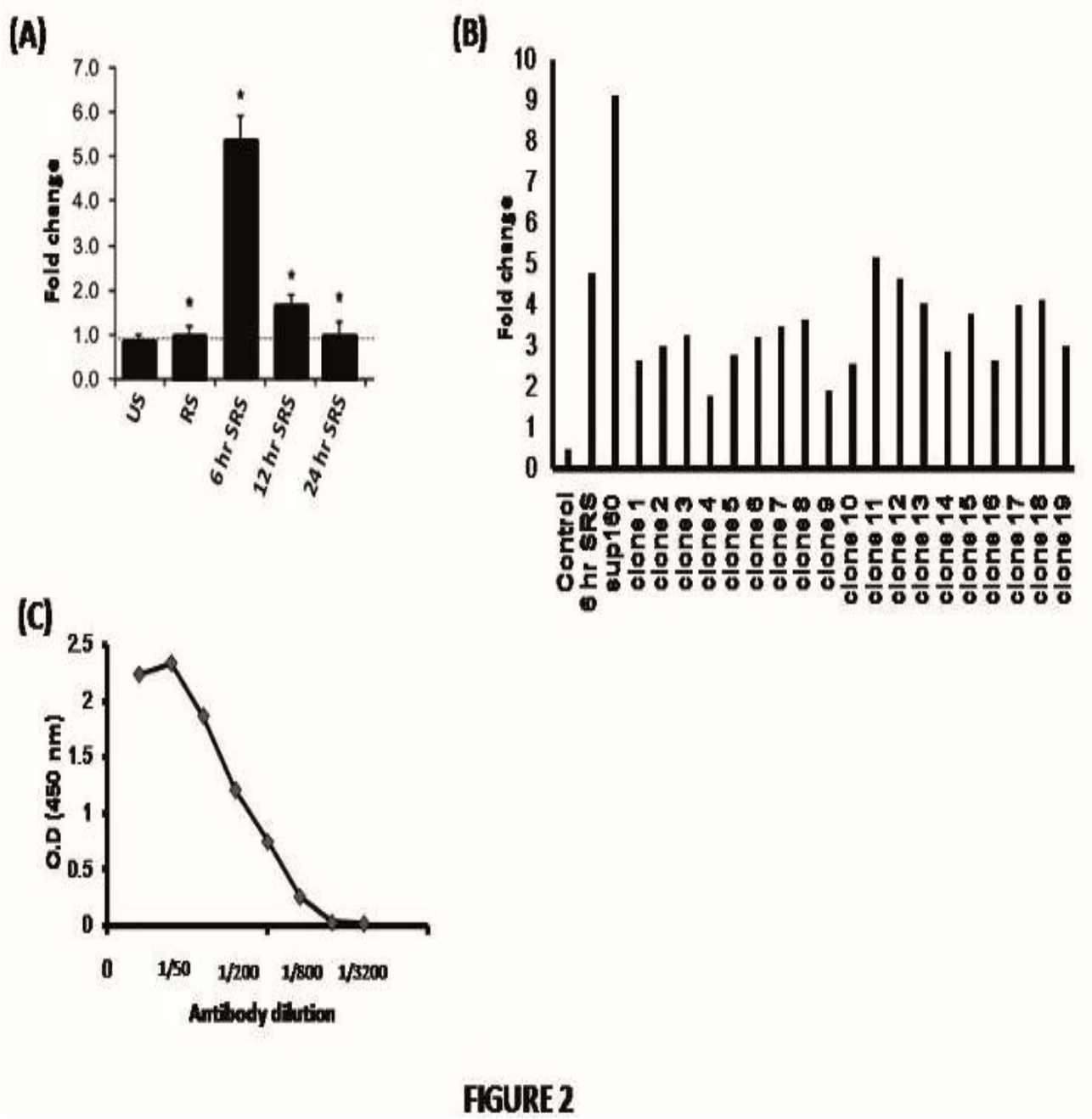
(A)
Fold change
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
US
RS
6 hr SRS
12 hr SRS
24 hr SRS
(B)
Fold change
10
9
8
7
6
5
4
3
2
1
0
Control
6 hr SRS
sup160
clone 1
clone 2
clone 3
clone 4
clone 5
clone 6
clone 7
clone 8
clone 9
clone 10
clone 11
clone 12
clone 13
clone 14
clone 15
clone 16
clone 17
clone 18
clone 19
(C)
O.D (450 nm)
2.5
2
1.5
1
0.5
0
0
1/50
1/200
1/800
1/3200
Antibody dilution

FIGURE 2

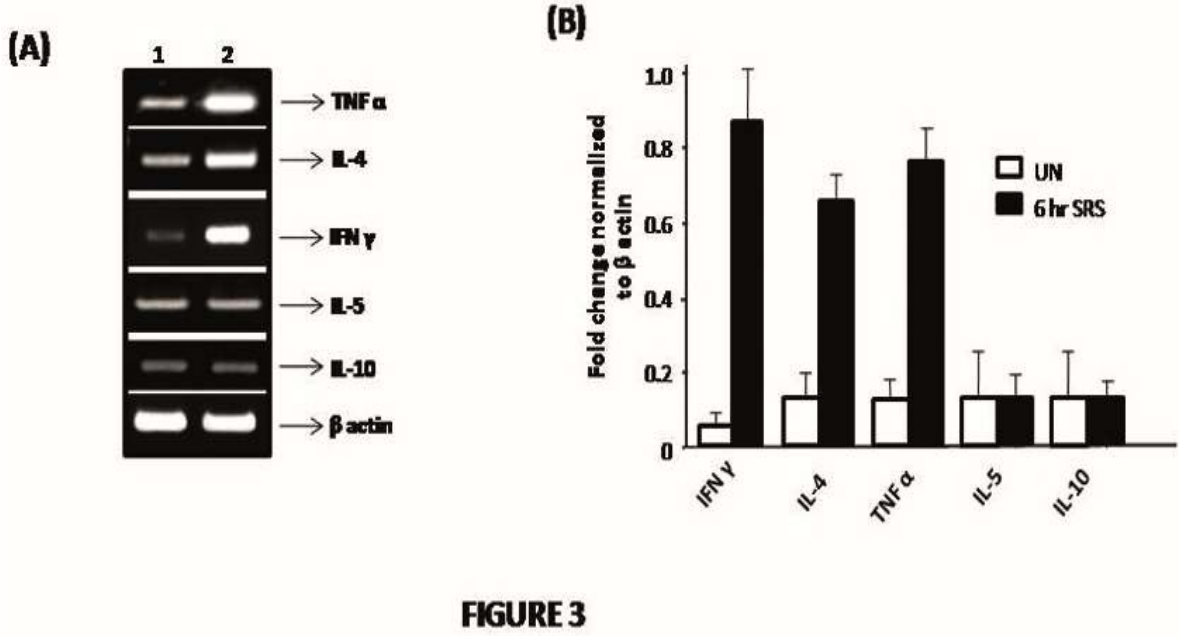


FIGURE 3

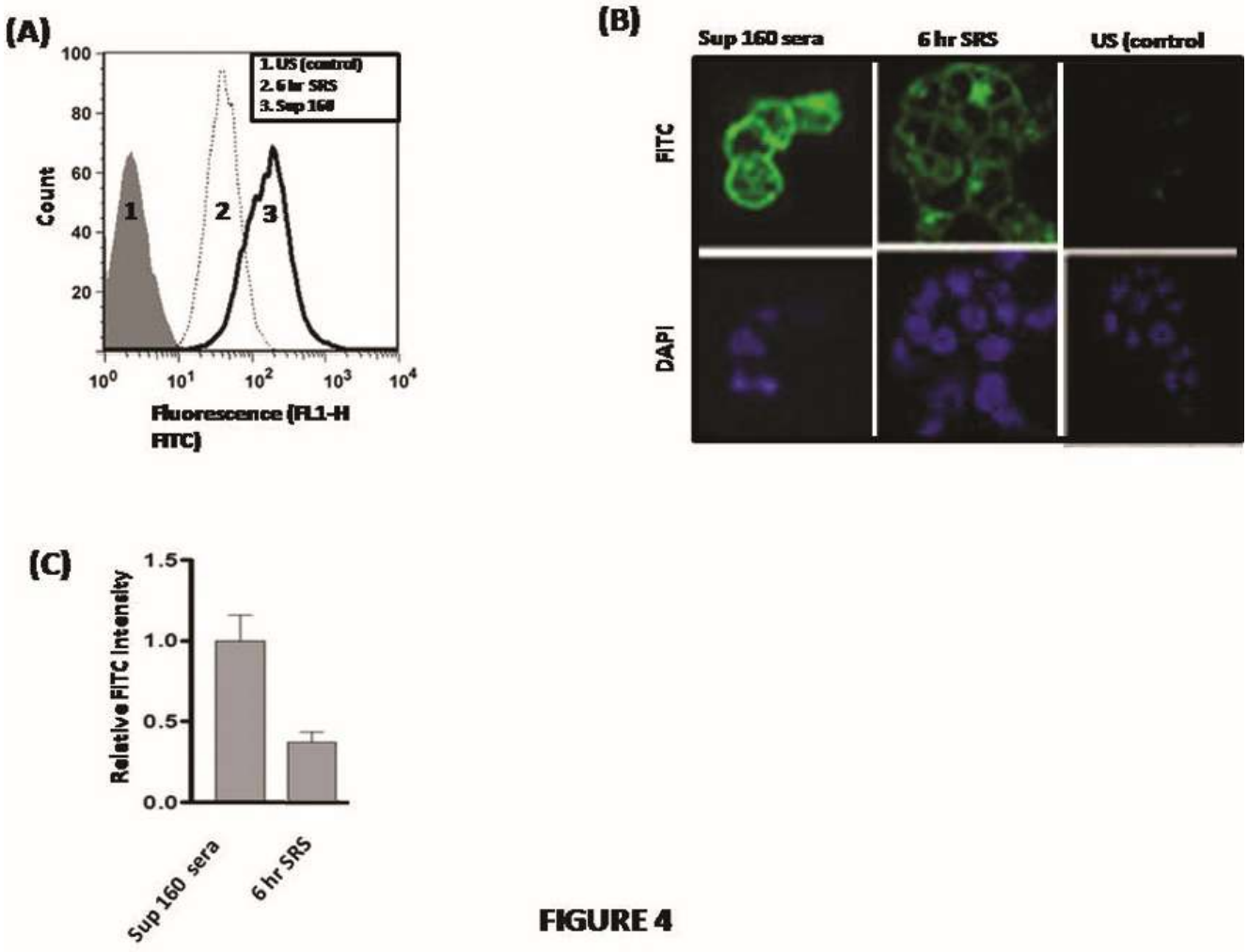


FIGURE 4

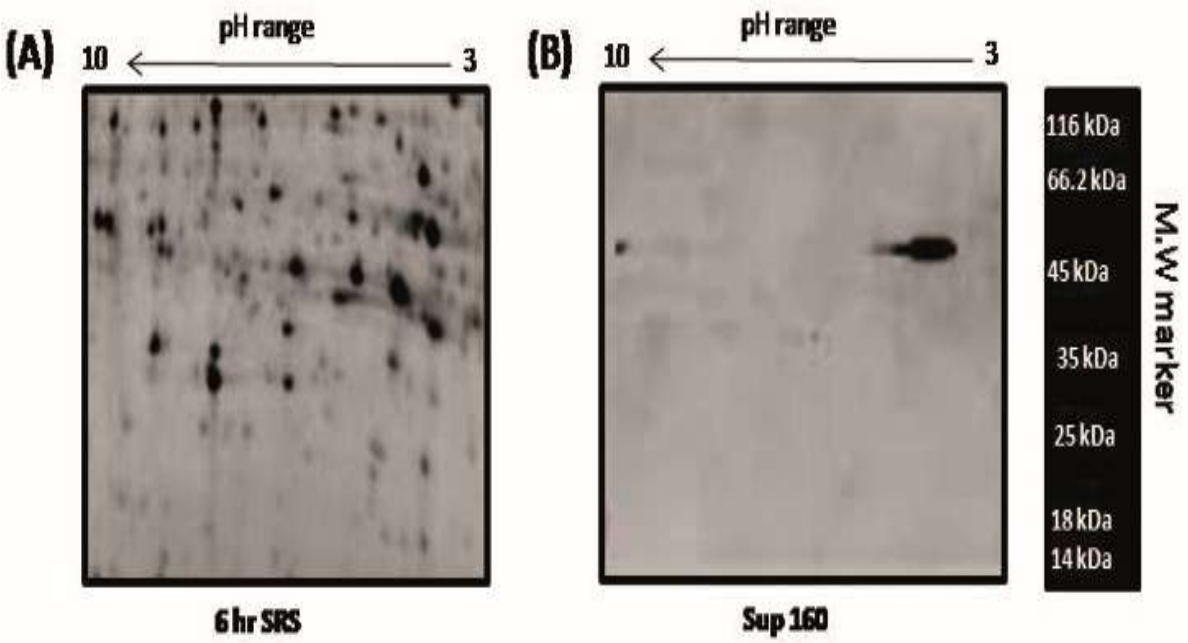


FIGURE 5

**Funding:**
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**Competing interests**
The authors declare that they have no competing interests.

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