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In vitro Immunogenicity Risk-assessment of Therapeutic Proteins in Preclinical Setting

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Abstract

Immunogenicity against therapeutic proteins is a clinical problem in the successful treatment of many diseases and as such, immunogenicity risk assessment in preclinical setting would be useful to improve safety and efficacy of protein based therapeutics in the product development stages. Here, we attempted a mechanism based *in vitro* studies as screening tool to capture clinically observed antibody based immune response against two representative therapeutic proteins; recombinant human Erythropoietin-alpha (rHuEPO) and recombinant Factor VIII (rFVIII). Flow cytometry was used to determine the maturation level of dendritic cells (DCs), a primary initiator of T-cell responses. Studies to capture T-lymphocyte proliferation upon challenge with free rFVIII were performed and secretion of immunomodulatory cytokines was analyzed by ELISA assay. These *in vitro* techniques could be used as risk assessment tool to determine the immunogenic potential of formulations of recombinant proteins in preclinical setting.

Keywords

Cytokine; dendritic cell; ELISA; Erythropoietin; Factor VIII; flow-cytometry; immunogenicity; recombinant protein; T-cell proliferation

1. Introduction

With the advent of recombinant DNA technology, tremendous breakthrough is achieved in developing therapeutic proteins for the treatment of many severe diseases. Unfortunately, many untoward effects were observed in patients including the development of clinical immune responses against the therapeutic proteins. It is recognized that the immune response towards recombinant proteins is a complex interplay (Fig. 1) between antigen presenting cells (APC), T-lymphocytes and B-lymphocytes. The proposed steps that are involved in the immunological cascade were illustrated by Chirino et al (1) wherein, (a) the exogenously administered protein is taken up, is processed and the immunogenic epitopes are presented by APC in the context of major histocompatibility complex (MHC), (b) APC-T-cell interaction, (c) interaction between T-cell and B-cell and (d) differentiation of B-cells into antibody secreting plasma cells. Thus, *ex vivo / in vitro* antigen presentation and cell interaction studies could be useful to gauge the immunogenicity risk against a therapeutic protein. Here, we have used two recombinant proteins viz. Erythropoietin-alpha and Factor VIII (rFVIII) as representative therapeutic proteins and determined their influence on APCs and T-cells respectively.

Flow-cytometry is a well-established technique that was used to determine differences in the expression level of APC-surface molecules *in vitro*. The activation and proliferation of T-

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cells in the presence of antigen was studied by performing *in vitro* by using ^{3}H-Thymidine-based T-cell proliferation studies. It has been demonstrated that cytokines play a critical role in directing the immune response either towards immunity or regulation. TGF-β (2) and IL-10 (3) are two important cytokines that are known to induce the generation of regulatory T-lymphocytes whereas; cytokines such as IFN-γ, IL-6, IL-17, and IL-23 are known to contribute to the immunity arm of the immune response. Pertinently, the level of immunomodulatory cytokines can be characterized by an ELISA assay. The use of such techniques as well as development of other *in vitro*-based techniques, for e.g., B-cell based assays will enhance the early assessment of immunogenicity of the next generation of therapeutic proteins.

2. Materials

Factor-VIII knock-out mice which bear a targeted deletion in exon 16 of the FVIII gene, was established using breeding pairs provided by Drs. Kazazian and Sarkar from the University of Pennsylvania, PA. Normal mice were used for Erythropoietin studies. All animal studies were pre-approved by the Institutional Animal Care and Use Committee of the University at Buffalo.

2.1 Cell culture

1. RPMI-1640 medium with L-glutamine and without calcium or magnesium (GIBCO, Carlsbad, CA). Supplemented with Penicillin (100U/ml). Streptomycin (100 μg/mL)
2. 2-mercaptoethanol (*see* Note 1).
3. Heat-inactivated and sterile filtered premium fetal bovine serum (Lonza Inc., Walkersville, MD) (*see* Note 2).
4. Recombinant murine Granulocyte Macrophage Colony Stimulating Factor (rmGMCSF, 200 U/ml) (Peprotech Inc., Rocky Hill, NJ) (*see* Note 3).
5. 0.4% Trypan blue dye
6. Neubauer Chamber (Hemocytometer)
7. Sterile tissue culture dishes (100 mm × 20 mm).
8. Sterile Phosphate Buffered Saline: (10x PBS) and DNase-RNasa-free distil water. The sterile 10x PBS is diluted 10x with the distil water and pH adjusted to 7.2 and sterile filtered via 0.22 μm filter. The solution is stored at 4°C.
9. 25 gauge sterile hypodermic needle (25 gauge): 10 mL sterile syringe and sterile cell-strainer with 100 μm pore-size.
10. Sterilized 15 and 50 mL centrifuge tubes, sterilized 0.22 μm bottle-top filter.
11. 10 mL serological pipettes

[1]Penicillin-Streptomycin is aliquoted and stored at −20°C. 2-mercaptoethanol is stored at 4°C and away from light.
[2]The frozen bottle is thawed by initially keeping it at 4°C for 24 hr and later thawing at room temperature. It is advisable not to thaw FBS by directly keeping the bottle at 37°C atmosphere. Upon, complete thawing, the bottle is opened in aseptic condition in a sterilized cell-culture hood and aliquoted into sterilized 50 ml conical tubes. The tubes are then stored at −20°C.
[3]The lyophilized powder is reconstituted with the manufacturer's recommended reconstitution buffer or with sterile water for injection. The reconstituted protein is aliquoted into microcentrifuge tubes as a single-use volume and stored at −20°C. Do not use any stocks which are more than 6 months old, as it has been observed that the activity of reconstituted rmGMCSF decreases significantly over time.

12. Aerrane™ isoflurane (Henry Schein Inc., Melville, NY): used as a mixture with oxygen to anesthetize the animals.
13. Centrifuge: which can hold 15 and 50 mL centrifuge tubes and run at 4°C for 10 min and centrifugal speed of up to 1000g is required.
14. Cell culture incubator.

2.2 DC maturation study

1. Albumin-free recombinant human erythropoietin-alpha (rHuEPO; Prospec-Tany TechnoGene Ltd.) (Reconstitute and store at −80°C).
2. Fc-Block®, FITC-anti-MHC-II, FITC-anti-CD11c, PE-anti-CD40, PE-anti-CD80, PE-anti-CD86 and corresponding isotype controls (BD Biosciences and eBioscience Inc., San Diego, CA).
3. 10% ultrapure Paraformaldehyde (diluted with sterile and ice-cold 1x PBS as required).
4. Calibrite® calibration kit, FACS cleaning solution and Sheath fluid
5. FACS Calibur Flow-cytometer
6. CellQuest softwares (BD Biosciences).

2.3 T-cell proliferation and cytokine analysis

1. Full-length, purified, excipients-free recombinant Factor VIII (rFVIII; Advate) (store at −80°C).
2. 0.5 mL sterile syringe with 29 gauge hypodermic needle (used to administer formulation to the animals).
3. Sterilized, flat-bottom glass pestle
4. Dynal® $CD8^+$ (Lyt 2) T-cell depletion kit and Dynal® magnet (Invitrogen). The kit is stored at 4°C.
5. 30% Bovine serum albumin (BSA)
6. 96-well flat-bottom plates with lids.
7. Concanavalin A (Con A; Sigma-Aldrich).
8. ^{3}H-Thymidine (6.7 Ci/mmol, Perkin Elmer, Waltham, MA) is stored at −20°C in a freezer which is distinctly labeled and used for storing radioactive materials.
9. Unifilter-G 96-well plate, Scintillation ‘O’ fluid and plate seals
10. Cell-dyn®-1700 cell counter (Abbott labs, Abbott Park, IL)
11. Plate harvester, plate sealer and Top-count™ scintillation counter (Perkin Elmer)

2.4 Cytokine analysis

1. Duoset® ELISA development kit for Transforming growth factor-beta (TGF-β), Interleukin (IL)-2, 6, 10, 17, 23 and Interferon-gamma (R&D systems, Minnesota, MN). Store at 4°C (*see* Note 4).

[4]The ELISA standards and antibodies are aliquoted as per the manufacturer’s instructions and stored in microcentrifuge tubes at −80°C.

2. NUNC maxisorp 96-well flat-bottom plates with lids.
3. Wash buffer: 0.1% Tween-20 in PBS, pH 7.0 and 0.22 μm filtered. Prepare fresh and store at room temperature.
4. Stop solution: 2 N Sulfuric acid.
5. Plate washer (Tecan US, Inc., Durham, NC).

3. Methods

3.1 Dendritic cell culture

Murine bone marrow isolation and dendritic cell (DC) culturing method was followed using the procedure of Lutz et al (4).

1. Use naïve normal mice aged between 8 to 12 weeks old for the bone marrow isolation.
2. Transfer the animal to a chamber that has an inlet and outlet for a constant flow of oxygen and isoflurane. Anesthetize the animal and verify complete anesthesia by the lack of righting-reflex of the animal as well as by monitoring the slowness of breathing.
3. Take the animal out of the chamber and place on a surgery board covered with cotton-lined underpad. Keep the animal under anesthesia using a nose-cone which is supplied with constant supply of isoflurane and oxygen. Keep monitoring the breathing of the animal. If the animal starts to gasp then, reduce the flow of isoflurane accordingly.
4. To verify that the animal is completely anesthetized, press the paws of the hind limbs as a form of stimuli. Mice paws are very sensitive to stimuli and hence, a lack of ‘twitching’ reflex action will indicate that the animal has been completely subdued.
5. Lay the animal on its dorsal side with its forelimbs and hind limbs stretched and fastened using a fastening tape to keep the limbs from interfering with the surgery.
6. Spray 70% alcohol using a squirt bottle onto the chest, abdomen and hind limbs of the animal to disinfect. Alcohol also wets the skin hairs and makes it easier to do incisions without loose hair contaminating the procedure.
7. With the help of a pair of sterilized forceps and scissors, make an incision in the skin near the abdomen area and slowly cut the skin up to the sternum. Then, carefully cut the diaphragm and sacrifice the animal by cardiac puncture.
8. Upon sacrifice, turn off the flow of isoflurane and oxygen.
9. Make two skin incisions on the two hind limbs starting from the pelvic region till the ankle.
10. Then, carefully clear the muscle surrounding the proximal region of the femur near the pelvic joint by scraping using the pair of scissors. One can also make use of wet alcohol wipes or 2 in × 2 in cotton gauze to remove the muscle tissue by hand.
11. Cut the pelvic bone so as not to damage the head of the femur. Keep the ball-&-socket joint intact.
12. Once the two hind limbs are isolated, clear the muscle attaching to the bones by carefully scraping it using a pair of scissors. Take care not to break or cut the

bones. Discard the fibula, part of the pelvic bone and the feet. Collect only the femurs and tibiae.

13. Put the femurs and tibiae in a 15 ml centrifuge tube containing 70% alcohol for 2–3 min to disinfect.
14. Turn on the centrifuge and let the temperature come down to 4°C.
15. All of the following steps involving cells must be carried out in a cell-culture hood with laminar airflow and HEPA filters. The hood should be sterilized by turning on the UV light for 30 min. All the material that is moved into the hood must be wiped with 70% alcohol to maintain sterile conditions inside the hood.
16. Also keep a pair of sterilized forceps and scissors, two sterilized 50 mL tubes, a cell-strainer, 10 mL syringe with a 25 gauge needle, a serological 10ml pipette and sterile, ice-cold PBS into the hood.
17. Transfer the bones along with the alcohol into a sterilized Petri dish.
18. Then immediately take the bones out of the alcohol with forceps and place them in another Petri dish. This step is required to make sure the alcohol does not come in contact with the bone marrow otherwise; the bone marrow cells will die in presence of alcohol.
19. Transfer approximately 40 mL of PBS to one of the 50 mL tube and aspirate 10 mL of it using the 10 mL syringe with an attached needle.
20. With a sterile pair of scissors, cut both the ends of the bones, thus making a open-ended cylinder with the bone marrow inside it.
21. Flush the bone marrow using the PBS filled syringe into an empty Petri dish. All the bones are flushed in a similar manner till all the bone marrow is collected in the Petri dish.
22. Place the cell-strainer atop the second empty 50 mL tube.
23. Using a serological pipette, disperse any cell clumps present in the flushed cell suspension and transfer the cell suspension to the tube via the cell-strainer.
24. Centrifuge the tube at 300 g for 10 min at 4°C.
25. After the centrifugation, discard the supernatant and resuspend the cell pellet in 2 mL of sterile RPMI-1640 media and mix thoroughly.
26. Take 50 μL of the cell suspension into a 1.5 mL microcentrifuge tube containing an equivalent volume of 0.4 % Trypan blue dye (1:1 dilution, i.e. dilution factor of 2) and mix thoroughly.
27. Load approximately 15 μL (or an appropriate volume to cover the entire counting area of the slide) of the mixture onto a Hemocytometer (Neubauer chamber) and place under a light microscope for cell counting.
28. There will usually be two types of cells visible under the microscope, transparent and blue colored cells. Transparent cells are the ones which have not taken up the dye and are viable cells whereas the cells that have taken up the dye are dead cells. Count only the transparent cells.
29. The viable cell count is done manually by counting the cells present in the five squares on the hemocytometer grid as described in Fig. 2.
30. Calculate the concentration of viable cells/mL present in the reconstituted cell suspension as per the following formula:

Viable cells/ml of suspension = (Cell count in five squares/5) × dil. factor × 10^4

where;

dil.factor = 2 (50 µL of cell + 50 µL of Trypan blue)

10^4 = Dilution factor of the hemocytometer as per the manufacturer.

31. Accordingly, add 10 mL of DC media (RPMI-1640 containing Penicillin/streptomycin and 2-mercaptoethanol and 0.22 µm filtered) containing 2×10^6 viable cells, 1 mL (10%) heat-inactivated FBS and 200U/mL of rmGMCSF to sterile 100 mm × 20 mm Petri dishes. This day is considered as 'Day 0'. Incubate the plates in a humidified cell culture incubator at 37°C and 5 % CO_2.
32. Add 10 mL of fresh DC media (containing 10% FBS and rmGMCSF) slowly, to each of the plates on Day 3 without disturbing the basal cell layer.
33. On day 6 & 8, aspirate half of the supernatant from each plate (approx 10 mL) and add to 15 mL centrifuge tubes. Centrifuge the tubes at 300g for 10 min at 4°C. Discard the supernatant and resuspend the cell pellet in 10ml of fresh DC media (containing 10 % FBS and rmGMCSF).
34. Carefully add the resuspended cell suspension back to the plates without disturbing the basal cell layer.
35. On day 9, harvest the loosely attached cells from the plates without scraping the plates and collect in 50ml centrifuge tubes. Centrifuge the tubes at 300g for 10 min at 4°C and discard the supernatant.
36. Resuspend the cell pellet in 5 mL of sterile and ice-cold 1x PBS.
37. Determine the concentration of viable cells as described in steps 25–29.
38. Characterize the dendritic cells by determining the cell-surface expression level of key molecules such as Major Histocompatibility Complex II (MHC-II), CD11c (a marker of DC), CD86, CD80 and CD40 (for detailed flow-cytometry sample preparation look at section B).
39. Based on our numerous flow-cytometric analyses, it was found that the cell suspension contains approximately 80% of DCs.

3.2 DC maturation study

1. On day 9 of DC culture, harvest the cells and count viable cells as described previously.
2. Take a 24-well sterile tissue-culture plate with lid and plate 2×10^6 DCs/well in DC media.
3. Thereafter, add 2 µg/mL of free rHuEPO to each of the wells and mix well. Keep four wells untreated to serve as negative control. Incubate the plate at 37°C and 5 % CO_2 for 24 hr.
4. After 24 hr of incubation, harvest the DCs from each well and transfer to appropriately labeled 15ml centrifuge tubes. Centrifuge the tubes at 300g for 10 min at 4°C.
5. Discard the supernatant and resuspend the pellet in another 10 mL of RPMI-1640 media. Centrifuge the cells for a second time and discard the supernatant.

6. Resuspend the resulting cell pellet in 2 mL of sterile, ice-cold 1x PBS and count viable cells by Trypan-blue exclusion method as described earlier.
7. Accordingly, transfer 1×10^6 viable cells to appropriately labeled microcentrifuge tubes such that each treatment group has two tubes. One tube will be used for analyzing the cell-surface expression whereas the other tube will serve as isotype control. If the cell volume is greater than 500 μL then use 5ml flow-cytometry tubes instead of microcentrifuge tubes.
8. Add PBS to fill the tubes and centrifuge the tubes at 300g for 10 min at 4°C.
9. Discard the supernatant carefully and resuspend the cell pellet in residual volume (100 μL) and keep on ice.
10. Add 3 μL of Fc-Block® antibody to each of the tubes, mix well and let tubes incubate for 15 min on ice (*see* Note 5).
11. Add 5 μL of either FITC-anti-MHC-II or PE-anti-CD86 or PE-anti-CD40 antibody to one of the two tubes from each group. Add 5ul of the corresponding isotype control to the other tube from each group (*see* Note 5). Mix the tubes well and incubate the tubes on ice for 30 min in dark.
12. Add PBS to fill the tubes and centrifuge the tubes at 1000g for 5min at 4°C and discard the supernatant. This step will remove any unbound antibodies.
13. Resuspend the cell pellet in 0.5 mL of ice-cold and ultrapure 2% Paraformaldehyde to fix the cells and keep the tubes on ice. The cells are now ready to be analyzed using flow-cytometry.
14. Flush the flow-cytometer with the manufacturer provided cleaning solution or 10% filtered bleach for 10 min. Then run distil water for 10–15 min.
15. Connect the flow-cytometer to the computer and start the FACScomp software. Calibrate the machine using the Calibrite® calibration kit and follow the manufacturer's protocol.
16. Upon calibration, start the CellQuest program for analyzing the samples.
17. Create a template with four plots; (A) SSC vs FSC, (B) Cell count vs FL1 (FITC), (C) Cell count vs FL2 (PE) and (D) FL1 vs FL2.
18. Run the auto-fluorescence tube for optimizing the settings such that all of the cells show up on the plot A, observe peak in plot B & C and the peak is towards the left of the plot, and most of the cells are present in the lower left quadrant of plot D. This can be optimized using 'Detection & Threshold' window which can be selected from the 'Cytometer' tab.
19. Once the optimization is complete, run the samples in order to capture 50,000 events.
20. After completion of analysis, follow step 14 before turning the flow-cytometer off. The results of the study are shown in Fig. 3 (A, B and C).

3.3 T-cell proliferation study

1. Divide naïve FVIII-knockout mice into two groups. Immunize one group of animals with 2 μg of free rFVIII in 100 μL of total injection volume using 29 gauge

[5]Prior titration of the antibodies with the cells is required in order to add optimum concentration of the antibodies to bind to all the respective binding sites.

sterile needle and 0.5ml syringe via the sub-cutaneous route of administration once a week for two consecutive weeks. It has been shown that this immunization protocol is able to prime the immune system in these animals towards rFVIII (5,6). The other group will serve as control.

2. On the third day after 2nd weekly immunization, sacrifice all mice by cardiac puncture and collect the individual spleens in 10ml of sterile and ice-cold RPMI-1640 media in 15 mL centrifuge tubes.
3. Transfer the spleen into a Petri dish inside the sterilized cell-culture hood.
4. Homogenize the spleen using a sterile flat-bottom glass pestle.
5. Disperse any cell clumps using a 10 mL serological pipette.
6. Pass the cell suspension through sterile 100 μm cell strainer three times to remove debris.
7. Transfer the filtered cell suspension into a sterile 15ml centrifuge tube and centrifuge the tube at 200g for 10 min at 4°C.
8. Discard the supernatant and resuspend the cell pellet in 3 mL of sterile and ice-cold 1x PBS.
9. After thorough mixing, add 80 μL of the cell suspension to a microcentrifuge tube and determine lymphocyte count using Cell-Dyn®-1700 instrument.
10. Upon determination of total lymphocyte concentration in the sample, take 1×10^7 total lymphocytes for use with Dynal $CD8^+$ (Lyt 2) T-ymphocyte depletion kit and follow the manufacturer's protocol to enrich $CD4^+$ T-lymphocytes.
11. At the end of the $CD4^+$ T-cell enrichment, count the cells as described in step 9.
12. Accordingly, add 2×10^5/ well of $CD4^+$ T-lymphocytes in quadruplicate wells to a sterile tissue-culture 96-well plate with lid. Make up the volume in each cell to 200ul by adding T-cell media (RPMI-1640, Penicillin-Streptomycin, Sodium pyruvate, Polymyxin-B and 10% FBS, 0.22 μm filtered).
13. Challenge the T-cells from immunized animals with 1000ng / ml of free rFVIII.
14. Use T-cells from untreated animals as the negative control.
15. Add 125ng /ml of Concanavalin A (Con A) to another four wells of untreated T-cells to serve as the positive control.
16. Incubate the plates in a humidified cell-culture incubator at 37°C for 72 hr.
17. Add 1 uCi /10 μL of ^{3}H-Thymidine (6.7 Ci / mmol) to each well and incubate the plate for an additional 16 hr in a 'radioactive materials only' labeled humidified cell-culture incubator at 37°C and 5% CO_2.
18. After the incubation, mount the plate on a plate harvester and harvest the cells onto a Uni-filter membrane-backed 96-well plate (*see* Note 6).
19. Add 30 μL of scintillation 'O' fluid to each of the wells of the Uni-filter plate and the plate is sealed using a plate sealer.

[6]Make sure to scrape the bottom of the plate with the harvesting probes in order to deposit as many cells as possible onto the membrane of Unifilter plate. Rinse the plate with water sufficiently to flush any radioactive material. Take appropriate steps to ensure safe disposal of radioactive material.

20. Analyze the plate for radioactivity (counts per minute, cpm) in each well by using a Top-count scintillation counter.
21. Calculate the average count of the quadruplicate wells for each group and accordingly, determine the stimulation index (SI) for each individual group as per the following formula:

    SI = Average count of sample / average count of negative control
22. The results are shown in Fig. 4.

3.4 Cytokine Analysis by ELISA

1. Follow steps (section 3.3: 1–14)
2. After 72 hr, centrifuge the plate at 300g for 10 min at 4°C.
3. Carefully aspirate the supernatant from each of the quadruplicate wells and pool them in a 1.5 mL microcentrifuge tube and keep on ice.
4. Store the tubes at −80°C until further analysis.
5. Perform cytokine analysis for TFG-β, IL-2, IL-6, IL-10, IL-17, IL-23 and IFN-γ using the Duoset ELISA kit (R&D systems, MN).

Acknowledgments

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References

1. Chirino AJ, Ary ML, Marshall SA. Minimizing the immunogenicity of protein therapeutics. Drug Discov Today. 2004;9:82–90. [PubMed: 15012932]
2. Zheng SG. The Critical Role of TGF-beta1 in the Development of Induced Foxp3+ Regulatory T Cells. International journal of clinical and experimental medicine. 2008;1:192–202. [PubMed: 19079658]
3. Hara M, Kingsley CI, Niimi M, Read S, Turvey SE, Bushell AR, Morris PJ, Powrie F, Wood KJ. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. Journal of immunology. 2001;166:3789–3796.
4. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods. 1999;223:77–92. [PubMed: 10037236]
5. Ramani K, Miclea RD, Purohit VS, Mager DE, Straubinger RM, Balu-Iyer SV. Phosphatidylserine containing liposomes reduce immunogenicity of recombinant human factor VIII (rFVIII) in a murine model of hemophilia A. J Pharm Sci. 2008;97:1386–1398. [PubMed: 17705286]
6. Purohit VS, Ramani K, Sarkar R, Kazazian HH Jr, Balasubramanian SV. Lower inhibitor development in hemophilia A mice following administration of recombinant factor VIII-O-phospho-L-serine complex. J Biol Chem. 2005;280:17593–17600. [PubMed: 15728582]

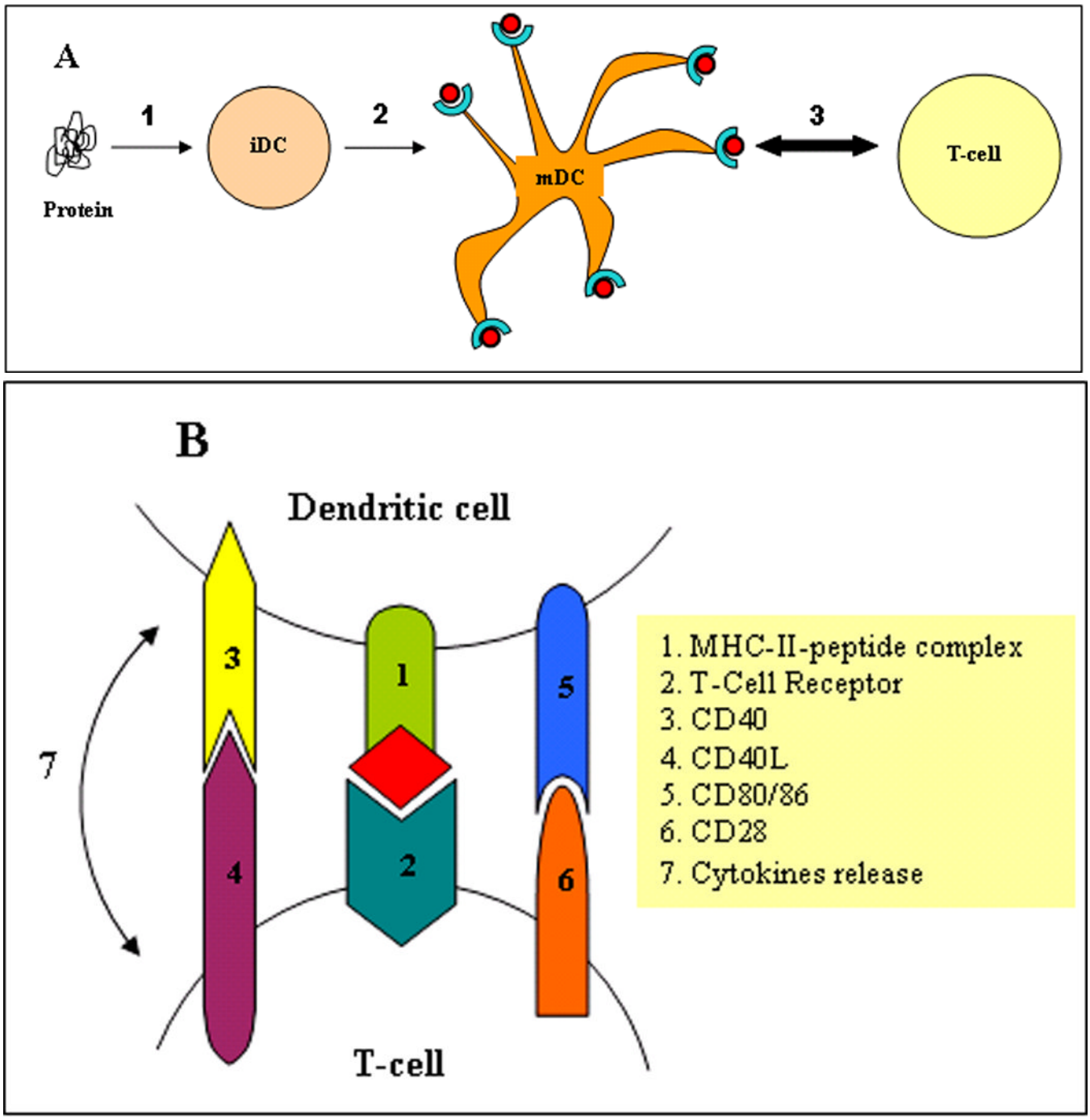


Fig. 1. Protein immunogenicity and cell interaction

(A) Initial steps involved in protein immunogenicity: Step 1: Protein uptake and processing by immature dendritic cell (iDC), Step 2: Maturation of dendritic cell (mDC) and Step 3: Interaction between mDC and T-cell. **(B)** Signaling between mDC and T-cell: *1–2:* peptide-MHC-II and TCR signal, *3–6:* co-stimulation, *7:* release of immunomodulatory cytokines by mDC and activated T-cell.

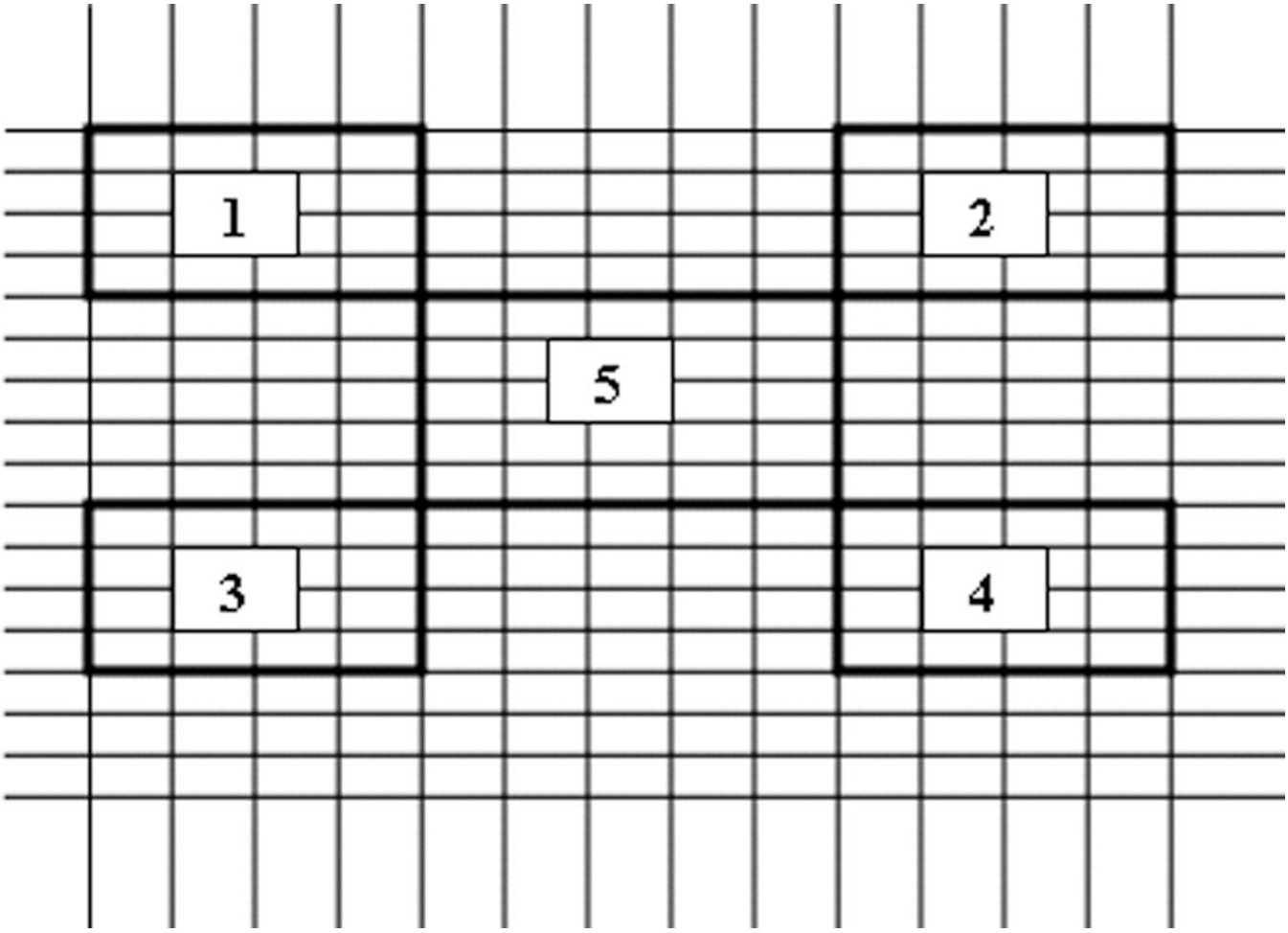
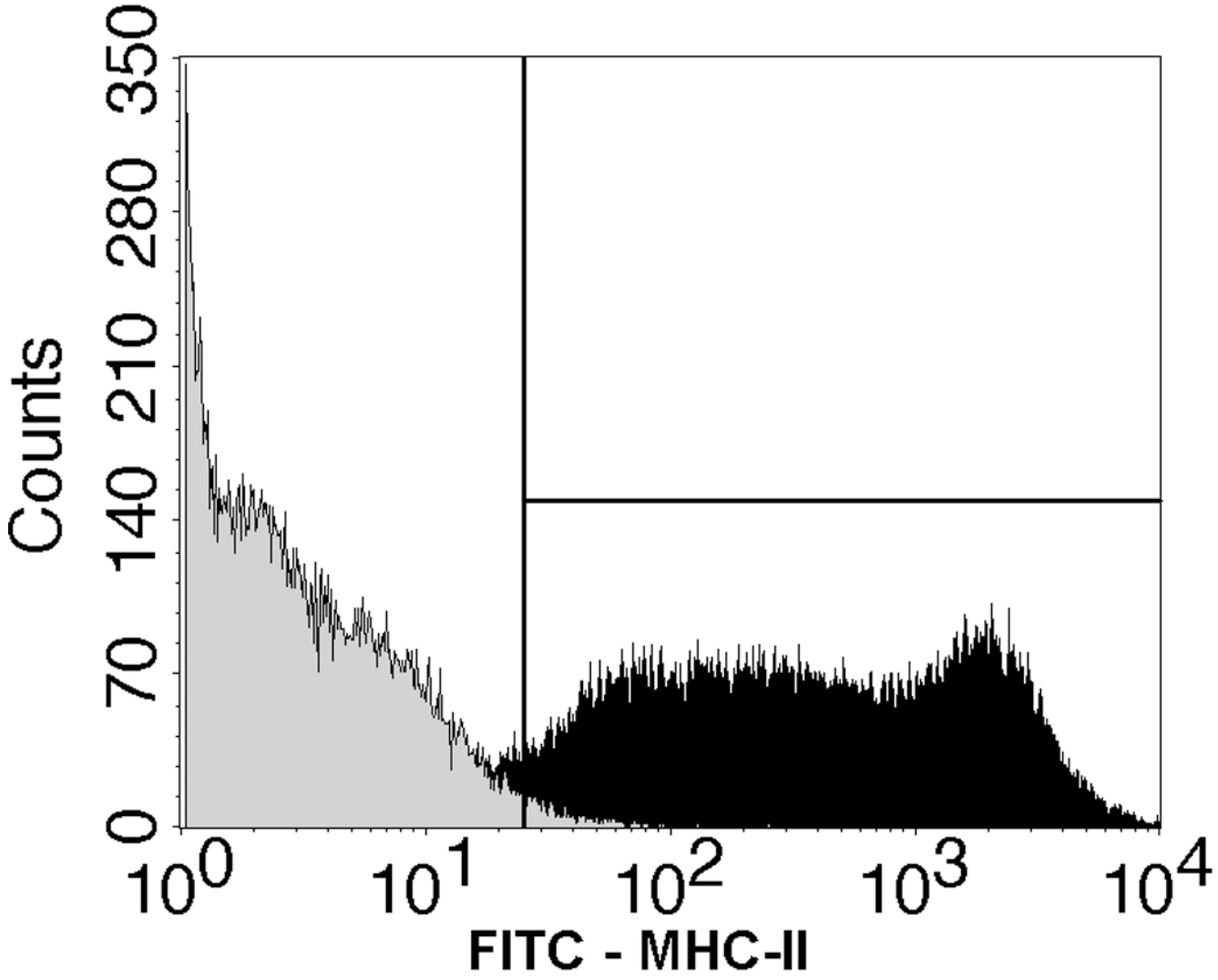
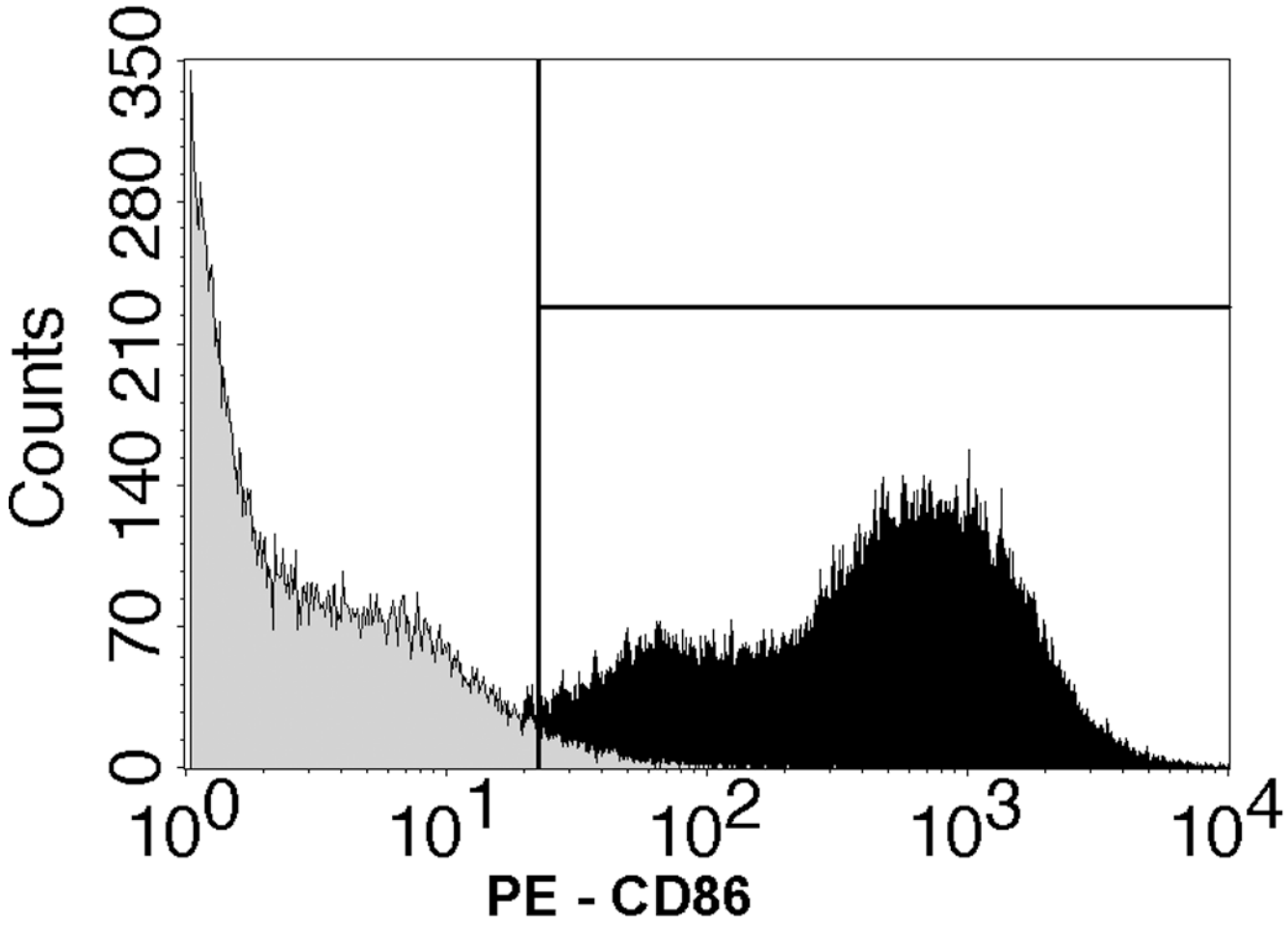


Fig. 2. Diagram of a hemocytometer

All the five large squares (as denoted by numbers 1–5, consisting of 16 smaller squares or 25 smaller squares for the center square) are counted and the average count is determined.





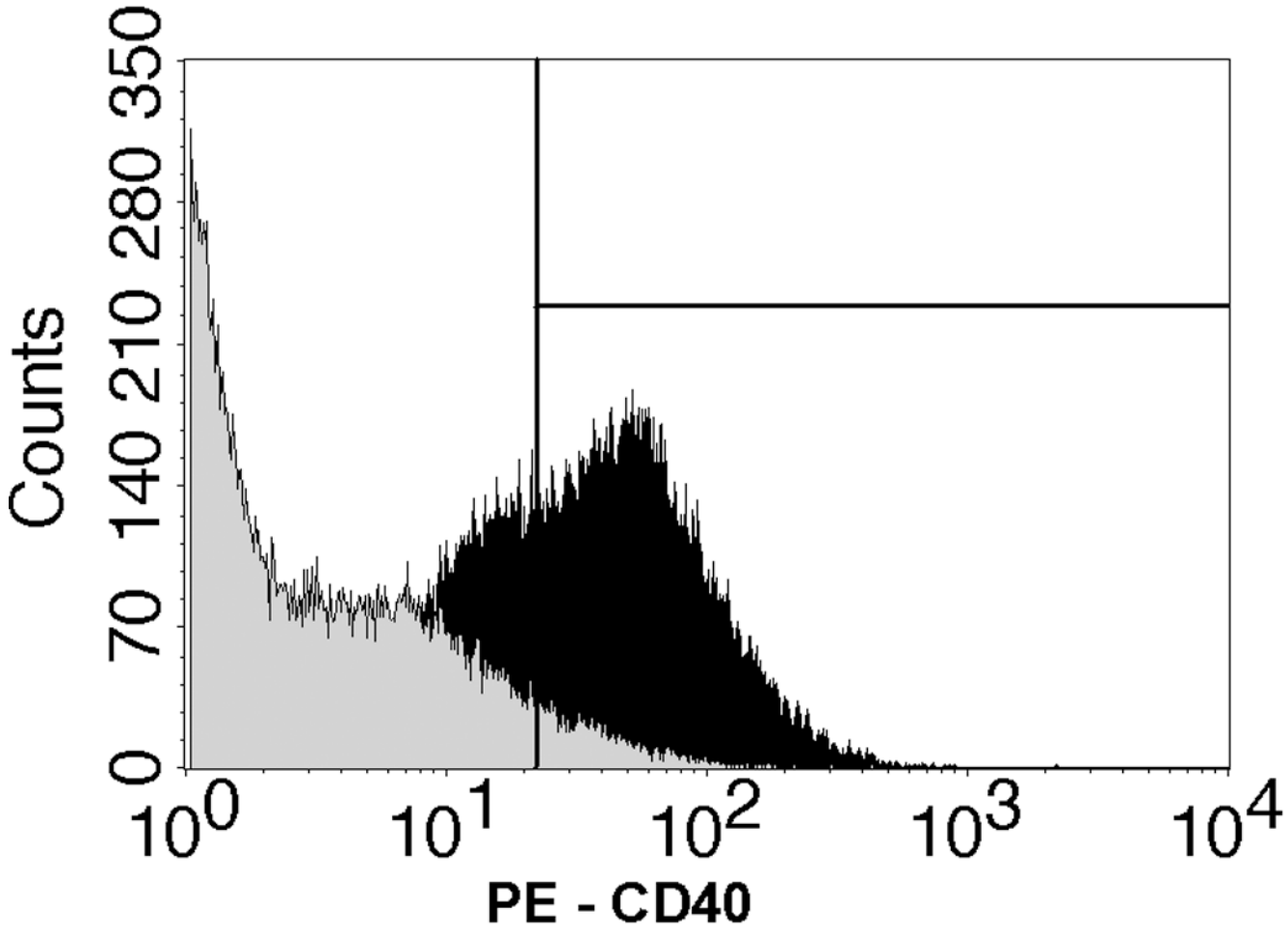


Fig. 3. DC maturation study

Dendritic cell-surface expression level (A) MHC-II, (B) CD86, (C) CD40 as determined by using flow-cytometric analysis upon cell exposure to free rHuEPO.

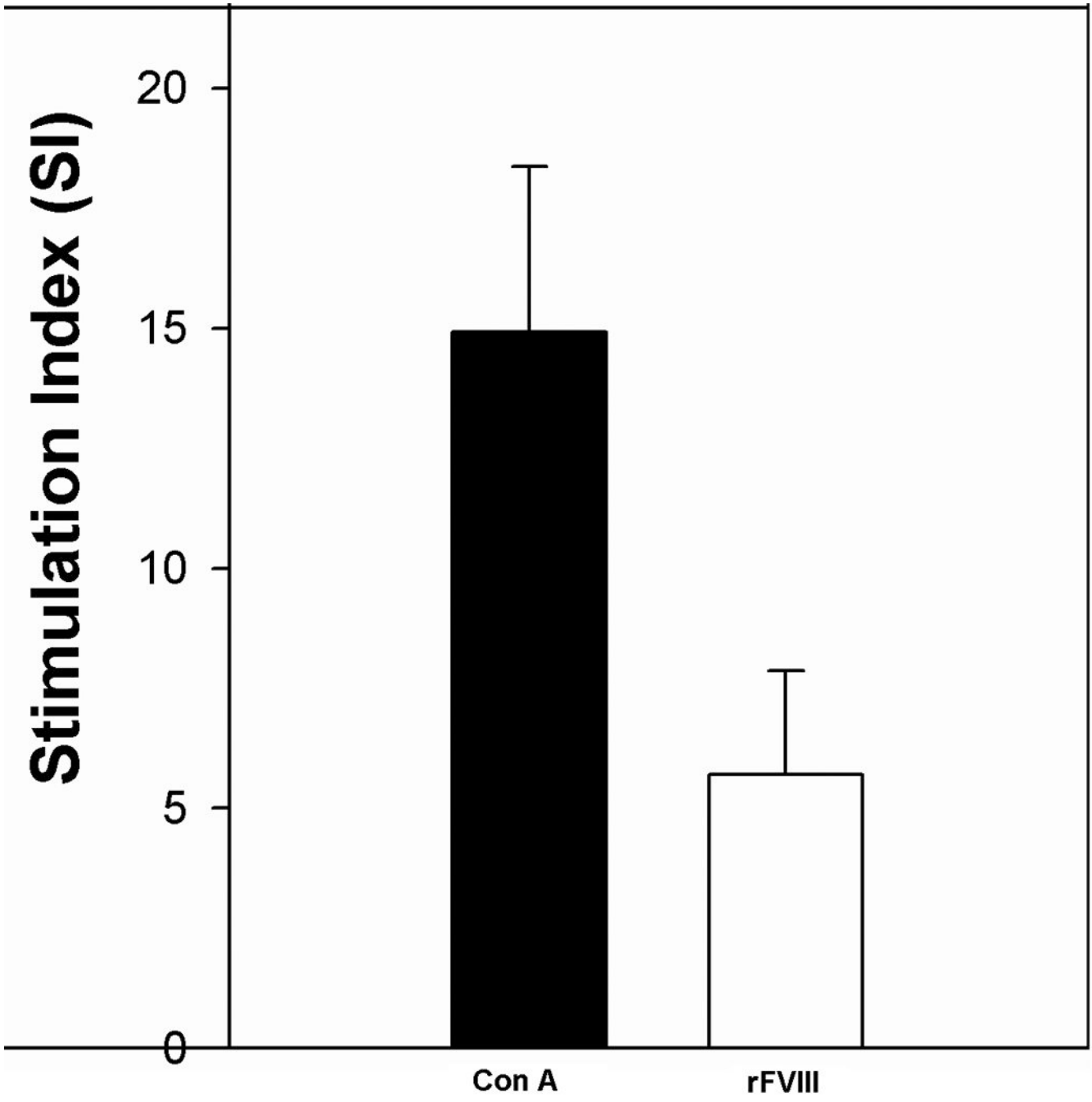


Fig. 4. T-cell proliferation study

Performed by the incorporation of ^{3}H-Thymidine into the cells and measuring the indirect cell-count by radioactivity counts.