

REVIEW ARTICLE

Recent Advancement on isolation, Activation and Cryopreservation of Lymph Node Cells in Mice

Seema Gupta^{1*}, Madhuri Grover², Vasundhara Saxena³

ABSTRACT

Lymph nodes are found within the body have B, T, and other immune cells and help to filter and trap foreign particles. Like any other primary culture, lymph node culture would retain many of the differentiated characteristics of cells *in vivo*; thus, they have the potential for acting as an alternative method to the mammalian model.

For setting up primary lymph node culture in mice, different types of lymph nodes were collected from mice, followed by isolation, activation and cryopreservation of cells from a lymph node. The present review emphasizes various procedures used for isolation, activation and cryopreservation of lymph node cells. Isolation of cells was performed by collagenase digestion, teasing apart of lymph node using dissecting needle or lymph nodes were disrupted between two frosted slides. Concanavalin A has been widely used to stimulate mice lymph node cells. A low dose of Con A has a stimulatory effect on T cells but high dose have inhibitory action and caused suppression of proliferation of T cell. Balb/c mice and C57Bl/6 mice were used for a different dose of Con A. The addition of cryoprotective agents, e.g. dimethylsulphoxide and careful control of cooling rates, affords protection from cell damage during freezing.

Keywords: Balb/c mice, Con A, C57Bl/6 mice, DMSO, Lymph node.

How to cite this article: Gupta S, Grover M, Saxena V. Recent Advancement on isolation, Activation and Cryopreservation of Lymph Node Cells in Mice. *Int. J. Pharm. Edu. Res.* 2021;3(1): 5-9.

Source of support: Nil

Conflict of interest: None

INTRODUCTION

Lymph nodes are important secondary lymphoid organs present at lymphatic intersection in the body, allowing efficient interaction between antigen presenting cells and naive T and B-cell and enhancing the effective initiation of an immune response.¹ Lymph nodes have

three compartments, the cortex, paracortex and medulla. B and T cells are located in different areas within these compartments. They interact with antigen-presenting cells, and undergo clonal expansion.² Lymph node culture is a primary culture in which there is maintenance of growth of cells separated from the parental tissue (such as kidney, liver) using the mechanical or enzymatic methods, in culture medium using suitable glass or plastic containers place under controlled conditions.³

There are many substances have the ability to stimulate RNA, protein and DNA synthesis in lymphocytes. Many of these substances are competent to trigger a lymphocyte, such as phytohaemagglutinin (PHA), Concanavalin A (Con A), bacterial toxins, antilymphocytic serum (ALS) and streptolysin, responded with DNA synthesis at optimal doses of the mitogens but not able to stimulate the cells at lower and higher dose.⁴ Con A is a jack bean lectin,⁵ obtained from *Canavalis ensiformis*, is widely used as T-cell specific mitogen.⁶ Con A bind to glycoprotein, glycolipids⁷ which stimulates the Ca²⁺-dependent step involved in T cell receptor stimulation, leading to interleukin-2 (IL-2) production. Antigen binding of T cell receptor stimulates the secretion of IL-2 and lead to the expression of IL-2 receptor (IL-2R). The IL-2/IL-2R interaction stimulates the growth, differentiation, and survival of antigen (Ag) selected cytotoxic T cells via the activation of specific gene expression.⁸

Cryopreservation of cell is a method which is used for storing a living organism for a long time at extremely low temperature. The process includes the preservation of cells or whole tissues on 77°K or -196°C with cryoprotectant (DMSO).⁹ The present review clearly explained the role of Con A for T cell stimulation. Various techniques have been proposed for isolation, activation and cryopreservation of lymph node cells obtained from different species of mice.

ISOLATION OF LYMPH NODE CELLS

In steady-state conditions, the number and distribution of lymphocyte populations are under homeostatic control. There is continuous generation of new lymphocytes in primary and secondary lymphoid organs and then achieve immune-competence within different tissues. These new lymphocytes must challenge with resident cells for survival. The first step in studying lymphoid

¹Assistant Professor, Rameesh Institute of Vocational and Technical Education

²Associate Professor, B.S. Anangpuria Institute of Pharmacy

³Assistant Professor, Rameesh Institute of Vocational and Technical Education

*Corresponding Author: Seema Gupta, Institute of Pharmacy, Ram-Eesh Institute of Vocational and Technical Education, Greater Noida, Uttar Pradesh, Pin Code: 201310, India., E-mail: seemaguptaggg@gmail.com

tissue cells is their isolation in intact and viable form, appropriate for the establishment of *in vitro* culture systems.

TECHNIQUES INVOLVED FOR ISOLATION OF LYMPH NODE CELLS

The peripheral lymph nodes were isolated from the surrounding tissue and were placed in ice-cold PBS (Phosphate buffer saline) and agarose gel was poured. Embedded nodes were attached to the specimen disk of the vibratome with non-toxic tissue glue. Agar-embedded tissue was sectioned at 320 μm thickness. Incubation of culture was done at 37°C in a 5% CO₂ humidified incubator. It was a very effective and quick technique for generating lymph node slices. These were then used to investigate the behavior of introduced T cells.¹⁰

Various other techniques have been proposed for the isolation of lymph node cells. Animals were anesthetized and the complete gastrointestinal tract was removed by subdiaphragmatic transection of the esophagus and the colon. The tract was then placed in chilled KHB (Krebs-Henseleit buffer). Mesenteric lymph nodes were isolated and collected tissues were centrifuged at 500 g. Single-cell suspension from mesenteric lymph node was prepared, passed through a 100 nm nylon mesh into a 15 ml tube filled with 20% FCS. The collected cells were washed and stored in CMF/20% FCS (Combined Magnetic Field) on ice.¹¹

Other technique include enzymatic digestion. Enzymatic digestion of lymph node was done to produce single cell suspension. The popliteal lymph node was isolated from F1 hybrid rat. Centrifugation of isolated cells were done at 250g for 5 minutes and then cell were plated in RPMI 1640. Collagenase digestion was used to obtain primary lymph node cultures. After 1–2 days the isolated cells proliferated, and it was observed that lymph node cultures contained high endothelial cells, and they show expression of a surface determinant for lymphocytes *in vitro*.¹²

Inguinal, axillary, and cervical lymph nodes from BALB/c mice and C3H mice were isolated. They were rinsed three times in medium and teased apart with dissecting needles. The resulting cell suspension was filtered through gauze to remove large debris. The cells were washed three times in medium supplemented with 10% FCS and total and viable counts (trypan blue) were performed.¹³ Mesenteric lymph node played a pivotal role in mediating food allergy. Mice were killed and lymph node were aseptically removed and disrupted between two frosted glass slides into 3mL of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and passed through a wire-mesh filter. This technique was used to obtain a single-cell suspension.¹⁴

ESTABLISHMENT AND ACTIVATION OF LYMPH NODE CULTURE BY CONCANVALIN A

The protein Con A is a lectin found in the jack bean. Various biological effects of Con A include mitogenesis, cytotoxicity, hepatotoxicity, and teratogenicity in cell cultures. Stimulation of DNA synthesis in animal cells was stimulated by Con A. It has potency as an immunostimulant and effective model agent for hepatic failure. Con A has the ability to induced mitogenesis (an induced mitotic cellular division) in T cells which have been associated with cytokine expression and secretion.¹⁵

The plant mitogen Con A, PHA and PWM expressed their mitogenic response at various doses on cultivating samples of mice in 1 ml of RPMI 1640 media. After 48 hours, when [SH] thymidine was added in culture and kept for 24 hours, the result showed that mitogen have the ability to react with T cells *in vitro* was greater in mitogen-treated nude mice than normal mice.¹⁶ Con A activates peripheral lymph node T cells by activating T cell activating factor (TAF) and proliferate the IL-2 response. In the presence of Con A, TAF stimulated IL-2 mRNA in T cells at steady-state level. TAF has an important role in primary T cell immune responses as it secretes active IL-2 into the culture medium and causes proliferation of the T cells.¹⁷

During mitogenic stimulation, dose range of Con A plays an important role. Low doses of Con A have stimulatory effect on T cells, but high dose have inhibitory action and caused suppression of proliferation of T cell and enhanced T cell death in C57BL/6 mice.¹⁸ Another study was done on lymph node cell cultures stimulated with Con A, which induced either suppression (at high concentrations) or further stimulation (at lower concentrations) of the proliferative activity in C57BL/6J female mice. Gel filtration of the extracts revealed that suppressive activity was due to factors of molecular weight in the 50,000-96,000 range, while stimulation was due to factors in the 13,000-35,000 molecular weight range.¹⁹

Mechanism of lymphocyte stimulation by Con A was studied. Monovalent form of Con A (m-Con A) binds to lymphocyte surface receptor but does not stimulate blast formation or cytotoxicity. It was suggested that Con A bridge binding sites on the lymphocyte surface to induced lymphocyte activation. The mechanism by which Con A exert its biological effect on lymphocyte depends upon various factors like the organization of its subunit, the number and type of binding sites and valence.

In addition to the stimulation of blast transformation, the binding of Con A to lymphocyte surface receptors has other effects on cells, which may be related in part to the lectin's mitogenic properties. These include increased

phospholipid incorporation into the membrane, increased flux of ionic potassium, calcium and increased uptake of nucleotides, sugars, and amino acids.²⁰ In another study, Con A covalently bound to acrylic polymer particles and stimulated rat lymphocytes into DNA synthesis. The average maximum number of Con A that bound was about 5×10^6 per cell. The result indicated that Con A's free sugar-binding site was essential for binding to the cell. Con A was required for an initial interaction of the mitogen with the surface of the cells.²¹

Con A act as a polyclonal T-cell activator and mimics antigen in generating cytotoxic T lymphocytes from normal lymphocytes. As Con A and antigenic activation process are mediated by similar pathway and by same receptors it is possible to use concanavalin interchangeably in the activation of Cytotoxic T lymphocytes. Con A and antigenic activation processes are mediated by similar pathways and by the same receptors and the result reported that the lymphocytes primed to an allograft was activated specifically into secondary cytotoxic T lymphocytes (2°CTL) by Con A.²²

Con A stimulated lymphocytes in cell culture medium and also stimulated the growth of an IL-2 level.²³ However, in another study it was concluded that activating lymphocyte Con A also causes apoptosis of lymph node T cell of Balb/c mice, *in vivo*. Con A induced T cell activation has been associated with apoptosis and death of apoptotic cell.²⁴ Con A's *in vivo* effect on lymphocyte activation was also studied by using ^3H labeled Con A, which s.c injected. route at different concentration (0.1-250 $\mu\text{g}/\text{foot}$) in female Balb/c mice. Popliteal lymph node were isolated and cell suspension was prepared and cultured in 2×10^5 cells/well and viability was counted by using haemocytometer. The result showed that there is a spontaneous proliferation of IL-2 by the activation of IL-2 receptor.²⁵ Balb/c mice infected with the intestinal intracellular parasite *Eimeria papillata* in order to characterize lymphocyte response and cytokine profile throughout primary and secondary infections. Lymphocytes were isolated from the mesenteric lymph node. They showed that during primary infection with *Eimeria papillata*, cells do not proliferate as compared to uninfected controls on day 0, but after Con A stimulation *in vitro* expressed Th2-type cytokines (IL-4 and IL-10) on day 3.²⁶

IMMUNE RESPONSE IN BALB/C MICE AND C57BL/6 MICE

Balb/c and C57BL/6 are widely used mice strains for the study of immune related diseases because of Th1/Th2 shift response pattern. Th1 (T helper 1) response is an essential component of immune related diseases and

produces high amount of IL-2, IFN- γ and IL-12 and low amount of IL-4 and IL-5.²⁷ Various groups have studied the difference between the Balb/c and C57BL/6 mice. In Balb/c mice Thelper cell type response were preferentially developed. They are more susceptible to intracellular parasite infection and have a higher tumor incidence as compared with C57BL/6 mice. Balb/c mice are more resistant to the induction of autoimmune diseases than C57BL/6 mice. The thymus and peripheral lymphoid organs of BALB/c mice has more CD4⁺CD25⁺ T cells than in C57BL/B6 mice.²⁸ C57BL/6 mice induce T-helper type 2 response (IL-4, IL-5, and IL-13) while Balb/c induce Thelper 1 (IL-2, INF-gamma) type of response.²⁹

CRYOPRESERVATION OF LYMPH NODE CELLS

Cryopreservation is a technique that provides optimal conditions for the preservation of mouse lymph node cells in liquid nitrogen using cooling rate techniques and the survival of mouse lymphocytes throughout a procedure for storage at (-196°C) for the improvement of recovery and the possible extension to the mouse system of cell selection by freezing. After thawing, the survival of cells cooled at different rates in dimethyl sulphoxide (DMSO, 5 or 10%, v/v) was assessed from the [^3H] thymidine incorporation in response to PHA and Con A. Before freezing, the protection against freezing damage increased with time (up to 20 min) in DMSO (5%, v/v) at 0°C. During freezing and thawing, the cooling rate giving optimal survival was 8 to 15°C/min for DMSO cells (5%) and 1 to 3°C/min for DMSO (10%). Rapid thawing (>2.5°C/min) was superior to slow thawing. After thawing dilution at 25 or 37°C greatly improved cell survival compared with 0°C; at 25°C, survival was optimal (75%) at a moderate dilution rate of 2.5 min for a 10-fold dilution in FCS (10%, v/v) followed by gentle centrifugation (50g).³⁰

Dimethyl sulfoxide (DMSO) was used for cryopreservation of spleen and lymph node lymphocytes. They were frozen at 1°C/min with DMSO and stored at -196°C for 10 minutes and the functional recovery of the cell populations was monitored by the mitogenic response in culture after thawing. When DMSO was 5 to 10% in the freezing medium, response to mitogen was 40% retained, but the concentrations of DMSO required was ten fold higher to induced this effect than that present in the culture medium after freezing and thawing³¹ and similarly Grant C K also reported that the addition of protective agents, e.g. dimethylsulphoxide and careful control of cooling rates affords protection from cell damage during freezing. Cell samples in glass vials were insulated in polystyrene containers and stored directly in deep freeze at -65°C. Both rat thoracic duct cells and

human blood lymphocytes had been cryopreserved using this method. Frozen cells remained viable for periods of up to 1 year.³²

Maximum survival was obtained by freezing of lymphocytes at 1°C/min by a control procedure. Cooling obtained 91% survival of viable peritoneal exudated cells at 1°C/min by a control, optimized procedure. There was no recovery of viable cells after cooling at 75°C/min.³³

Activation of T and B lymphocytes also depends upon seasonal changes in the mouse. From March through July, recoveries of viable cells were high and it was minimum levels in January and February. In the spring and summer, activation of both T and B lymphocytes by mitogens was maximal and then declined in October to only 40% of unfrozen control levels.³⁴

CONCLUSION

The present review justifies Con A's role, which activates lymphocyte in the same way as antigen-induced lymphocyte stimulation. Thus, Con A has been used to understand the mechanism by which specific antigens stimulate lymphocytes. It was suggested that Con A bridge binding sites on the lymphocyte surface to induce lymphocyte activation and free sugar-binding site of Con A is essential for the binding to the cell. Lymph node cell cultures stimulated with Con A, which induced either suppression (at high concentrations) or further stimulation (at lower concentrations) of the proliferative C57BL/6J female mice activity. C57BL/6 mice induce T helper type 2 response (IL-4, IL-5 and IL-13) while Balb/c induce T helper 1 (IL-2, INF-gamma) type of response. For cryopreservation of cells freezing of lymphocytes at 1°C/min by a control procedure yield maximum survival. Dilution damage during both thawing and post-thaw dilution may be due to osmotic swelling as DMSO and normally excluded solutes leave the cell.

Future aspects of developing a primary culture of lymph node following all the mentioned aspects could be promising strategy not only to identify potential immunotoxicants but also to detect viruses and bacteria, validate disease model, perform mechanistic studies, understand shifts in Th1/Th2 response, to study cytokine activity and immunological tolerance.

REFERENCES

1. Fletcher AL, Malhotra D, Acton SE, Pelletier AB, Curry M, Armant M, *et al.* Reproducible isolation of lymph node stromal cells reveals site-dependent differences in fibroblastic reticular cells. *Front Immunol.* 2011; 2:35.
2. Cynthia LW. Normal Structure, Function, and Histology of Lymph Nodes. *Toxicol Pathol* 2006; 34:409-24.
3. Trowell OA. Experiment on lymph node cultured *in vitro*. *Ann NY Acad Sci.* 1955; 59: 1066-69.
4. Moller G. Induction of DNA synthesis in human lymphocytes: Interaction between non-specific mitogens and antigens. *Immunology.* 1970; 19: 583-98.
5. Philip SS, Jeffrey NK, Dianna JB. Purification and characterization of N-glycanase, a concanavalin A binding protein from jackbean (*Canavalia ensiformis*). *Biochem J.* 1998; 330: 13-20.
6. Sharon. Lectin receptor as lymphocyte surface markers. *Advanced Immunol.* 1983; 34: 213.
7. Goldstein IJ, Poretz RD. Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins. *The Lectins Properties, Functions and Applications in Biology and Medicine. San Diego: Academic.* 1986: 233- 47.
8. Stern J, Smith KA. Interleukin-2 induction of T-cell G1 progression and c-myc expression. *Science.* 1986; 233 (4760): 203-206.
9. Harris CL, Toner M, Cravalho EG. Cryopreservation of isolated hepatocytes: intracellular ice formation under various chemical and physical conditions. *Cryobiology.* 1991; 28:436-44.
10. Salmon H, Caicedo AR, Boursin FA, Lebugle C, Donnadieu E. *Ex vivo* imaging of T cells in murine lymph node slice with widefield and confocal microscope. *J vis Exp.* 2002; 53: 3054.
11. Koscielny A, Wehner S, Engel ER, Kurts C, Kalff J. Isolation of T cell and dendritic cell from peripheral intestinal tissue, peyers patches and mesenteric lymph node after intestinal manipulation. *Protocol Exchange.* 2011; 10:201.
12. Ager A. Isolation and culture of high endothelial cells from rat lymph nodes. *J cell sci.* 1987; 87: 133- 44.
13. Knop J. Influence of various macrophage populations on Con-A induced T-cell proliferation. *Immunology.* 1980; 41: 379- 85.
14. Beong OL, Christopher AJ, Kaliquz Z, Gabriel F. Dietary (n-6) and (n-3) fatty acids and energy restriction modulate mesenteric lymph node lymphocyte function in autoimmune-prone (NZB x NZW) f1 mice. *J Nutr.* 2000; 130(7): 1657- 64.
15. Ballerstadt R, Evans C, McNichols R, Gowda A. Concanavalin A for *in vivo* glucose sensing: A biotoxicity review. *Bio Sens Bioelectron.* 2006; 22:275- 84.
16. Berenice K, Francis L. Activity of host-derived T-cells which differentiate in nude mice grafted with co-isogenic or allogenic thymuses. *J Exp Med.* 1974; 139: 1215-27.
17. Garman RD, Raulet DH. Characterization of a novel murine T cell-activating factor. *J Immunol.* 1987; 138(4): 1121-29.
18. Sambuddho M. CTLA4-CD80/CD86 interactions on Primary mouse CD4⁺ T cells integrate signals- strength information to modulate activation with Con A. *J Leucocyte Biol.* 2005; 78: 144- 57.
19. Kemp A, Kakizoe T, Komatsu H, Nijjima J, Kawachi T, Sugimura T. Increased agglutinability of bladder cells by concanavalin A after administration of carcinogens. *Cancer Res.* 1980; 40 (6): 2006- 9.
20. Wand JR, Podolsky DK, Isselbacher KJ. Mechanism of human lymphocyte stimulation by Concanavalin A: role of valance and surface binding sites. *P Natl Acad Sci USA.* 1976; 73 (6): 2118- 22.
21. Betel I, Knor J. Interaction of concanavalin A with rat lymphocytes. *Eur J Biochem.* 1972; 30:571-78.
22. Benjamin B. Concanavalin A-mediated activation of antigen-primed lymphocytes into secondary cytotoxic lymphocytes. *J Exp Med.* 1977; 145: 293-301.
23. Hsi L, Jeffrey DA, Bret MS, Joan SS, Lony L, Ronald FS. Role of L3T4⁺ and 38⁺T-cell Subsets in resistance against infection with

- Treponema pallidum* subspecies *pertenue* in hamsters. *Infect Immun.* 1991; 59(2):529- 36.
24. Theolis B, Sergio A, Benildo C, Thalles BG, Luiz Antonio RF, Manoel BN. *In vivo* lymphocyte activation and apoptosis by lectins of the *Diocleinae* *Subtribe*. *Mem Inst Oswaldo Cruz, Rio de Janeiro.* 2001. 96(5): 673-678.
25. Black CDV, Kroczeck RA, Barbet J, Weinstein JN, Shevach EM. Induction of IL-2 receptor expression *in vivo*: Response to Con A. *Cell immunol.* 1988; 111:420- 32.
26. Marco SL, Chobtar B, Barta JR. Cellular dynamics and cytokine response in Balb/c mice infected with *Eimeria Papillata* during primary and secondary infection. *J Parasitol.* 1998; 84(2): 328- 37.
27. Capsi RP. Th1 and Th2 responses in pathogenesis and regulation experimental autoimmune uveoretinitis. *Int. Rev Immunol.* 2002; 21: 199-208.
28. Chen X, Oppenheim JJ, Howard OM. BALB/c mice have more CD4⁺CD25⁺ T regulatory cells and show greater susceptibility to suppression of their CD4⁺CD25⁻ responder T cells than C57BL/6 mice. *J leukocyte biol.* 2005; 78(1):114-21.
29. Morokata T, Ishikawa J, Ida K, Yamadan T. C57BL/6 mice are more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses. *Immunology.* 1999; 98(3): 345-51.
30. Farrant J, Thorpe PE, Knight, SC. Optimal conditions for the preservation of mouse lymph node cells in liquid nitrogen using cooling rate techniques. *Cryobiology.* 2004; 13: (2): 126-33.
31. Einar H. Freezing of rat lymphocytes. The effects of dimethyl sulfoxide and freezing on the phytohemagglutinin and pokeweed mitogen-responding lymphocyte subpopulations. *Cryobiology.* 1976; 13 (2):134-41.
32. Grant CK. A simple method for the cryopreservation of lymphocytes. Retention of specific immune effectors functions by frozen-stored cells. *Clin exp immunol.* 1976; 23(1): 166-74.
33. Taylor MJ, Bank HL. Function of lymphocytes and macrophages after cryopreservation by procedures for pancreatic islets: potential for reducing tissue immunogenicity. *Cryobiology.* 1988; 25(1):1-17.
34. Brock MA. Seasonal changes in recovery of cryopreserved murine lymphocytes resemble endogenous rhythms of unfrozen cells. *Cryobiology.* 1987; 5: 412-21.