IN VITRO EVALUATION OF *PASTEURELLA MULTOCIDA* GHOSTS AS ADJUVANT FOR IMPROVING INACTIVATED FOOT AND MOUTH DISEASE VIRUS ANTIGEN IMMUNOGENICITY

THESIS

Submitted to the
DEEMED UNIVERSITY
INDIAN VETERINARY RESEARCH INSTITUTE
Izatnagar - 243122 (U.P) India

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Veterinary Immunology)
2018

Dedicated to my Family,
Friends and Teachers

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CERTIFICATE I

This is to certify that the research work embodied in this thesis entitled "***In vitro* evaluation of *Pasteurella multocida* ghosts as adjuvant for improving inactivated foot and mouth disease virus antigen immunogenicity"** submitted by **Dr Shashidhar.M.G, Roll No.M-5705,** for the award of **Master of Veterinary Science Degree** in **Veterinary Immunology** at ICAR-Indian Veterinary Research Institute, Izatnagar is the original work carried out by the candidate himself under my supervision and guidance

It is further certified that **Dr Shashidhar.M.G, Roll No.M-5705,** has worked for more than 21 months in the Institute and has put in more than 150 days of attendance under me from the date of registration for the **Master of Veterinary Science** Degree in this Deemed University, as required under the relevant ordinance.

(Ganesh Kondabattula)
Chairman
Student's Advisory Committee

CERTIFICATE -II

Certified that the thesis entitled "***In vitro* evaluation of *Pasteurella multocida* ghosts as adjuvant for improving inactivated foot and mouth disease virus antigen immunogenicity"** submitted by **Dr. Shashidhar. M. G,** Roll No.**M-5705,** in partial fulfillment of the requirements of Master of Veterinary Science degree in Veterinary Immunology at Indian Veterinary Research Institute, Izatnagar, embodies the original work carried out by the candidate. The candidate has carried out his work sincerely and methodically.

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of **Master of Veterinary Science** of this Institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master of Veterinary Science** of the Deemed University, Indian Veterinary Research Institute, Izatnagar.

Signature of the External Examiner

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Dated: 12/11/2018

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ABBREVIATIONS

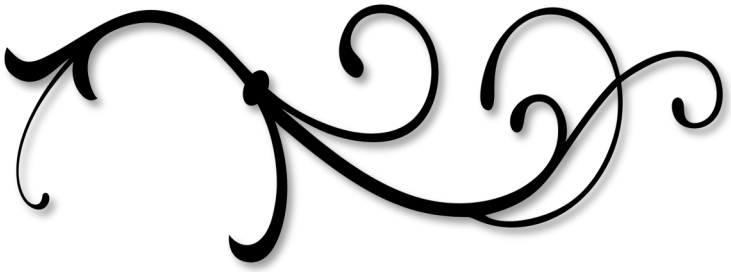
APS	:	Ammonium per-sulphate
BHK-21	:	Baby hamster kidney cell line
BSA	:	Bovine serum albumin
CBB	:	Coomassie brilliant Blue R 250
DDW	:	Double distilled water
DC	:	Dendritic Cells
EDTA	:	Ethylenediaminetetraacetic acid
FBS	:	Fetal bovine serum
Fig	:	Figure
FMD	:	Foot and mouth disease
FMDV	:	Foot and mouth disease virus
GM-CSF	:	Granulocyte Macrophage Colony Stimulating Factor
Ig	:	Immunoglobulin
IL-4	:	Interleukin 4
kDa	:	Kilo Dalton
MoDC	:	Monocyte derived Dendritic Cells
PAGE	:	Poly acrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
Pm	:	*Pasteurella multocida*
pH	:	Potential of hydrogen
rpm	:	Revolution per minute
SAT	:	South African Territories
SDS	:	Sodium dodecyl sulphate
SMP	:	Skimmed milk powder
TEMED	:	N,N,N,'N'-Tetra methyl ethylene diamine
Pm	:	*Pasteurella multocida*
pH	:	Potential of hydrogen
UV	:	Ultra violet
V	:	Volt
PMGs	:	*Pasteurella multocida* Ghosts

°C	:	Degree Celsius
%	:	Percentage
g	:	Gram
M	:	Molar
mL	:	Millilitre
mM	:	Millimolar
µg	:	Microgram
µl	:	Micro litre
µM	:	Micromolar
pM	:	Picomolar
ng	:	Nanogram
nm	:	Nanometre
v/v	:	Volume/volume
w/v	:	Weight/volume
x g	:	Centrifugal force equal to gravitational force

LIST OF FIGURES

CONTENTS

INTRODUCTION



1 INTRODUCTION

Foot and mouth disease (FMD) is caused by a virus of the genus *Aphthovirus*, family Picornaviridae. There are seven serotypes of FMD virus (FMDV) namely O, A, C, Asia 1, SAT 1, SAT 2, and SAT 3, that infect cloven hoofed animals. Infection with particular serotype will not provide protection against different serotype. Within serotypes, many strains can be identified by biochemical and immunological tests. Of the domesticated species, , sheep, goat, cattle, water buffalo (*Bubalus bubalis*) and pigs are susceptible to FMD. FMD is classified by Office International des Epizooties (OIE ,World Organisation for Animal Health) as an OIE List A disease, which, by definition, means that it has the potential for rapid and extensive spread within and between countries and can cause severe economic impact.

Dendritic cells (DCs) are the major antigen presenting cells in mammalian organisms and a subpopulation of DCs, plasmacytoid DCs (pDCs) produce significant amounts of IFNa early after virus infection. Moreover, DCs have been described as very important effectors of the host immune response. Studies in human and mouse systems have demonstrated their role in linking the innate and adaptive immune responses (Banchereau & Steinman, 1998). Multiple endogenous or exogenous stimuli can drive immature DCs into cells with a mature, costimulatory phenotype. These stimuli include the CD40 ligand (mainly expressed on activated CD4+ T-cells), cytokines such as GM-CSF, TNF-α and IL-1, prostaglandin E, bacterial lipopolysaccharide (LPS) (Sallusto *et al.,* 1995), whole bacteria (Winzler *et al.,* 1997), viral double-strand RNAs (Cella *et al.,*1999), CpG oligonucleotides or monocyte conditioned medium (Sallusto and Lanzavecchia, 1994; Romani *et al*., 1996; Cella *et al.,* 1999). In contrast, IL-10 (Allavena *et al*., 1998), TGFß and vascular endothelial growth factor (VEGF) (Oyama *et al.,* 1998) inhibit this maturation process. In response to activating stimuli, DCs express high levels of the NF-κB family of transcriptional control proteins for the synthesis of immune and

inflammatory proteins (Granelli-Piperno, *et al.,*1995). Moreover, upon maturation DCs begin to load newly synthesized MHC molecules (e.g., MHC class II) with antigenic peptides that are derived from phagocytosised or pinocytosis, and display these complexes on the cell surface. DCs also begin to express co-stimulatory molecules required for T-cell activation, as well as CD83, a maturation marker for human DCs (Zhou *et al.,* 1995**).**

Bacterial ghosts (BGs) are empty cell derived envelopes obtained from Gram-negative bacteria. They contain many innate immunostimulatory agonists, and are potent activators of a broad range of cell types involved in innate and adaptive immunity. Many landmark studies have shown the effectiveness of BG as adjuvants as well as their capacity to induce proinflammatory cytokine production. These proinflammatory cytokines trigger a generalized recruitment of T and B lymphocytes to lymph nodes that maximize the chances of encounter with their cognate antigen, and subsequent elicitation of potent immune responses. Production of empty envelope-bound antigens in immunological active forms that have proven to be effective vaccines in several animal studies. In addition to their adjuvant behaviour, BGs also considerably deliver DNA-encoded antigens to dendritic cells, there by leading to high transfection efficiencies, which subsequently result in higher gene expressions and improved immunogenicity of DNA-based vaccines (Tabrizi *et al*., 2004).

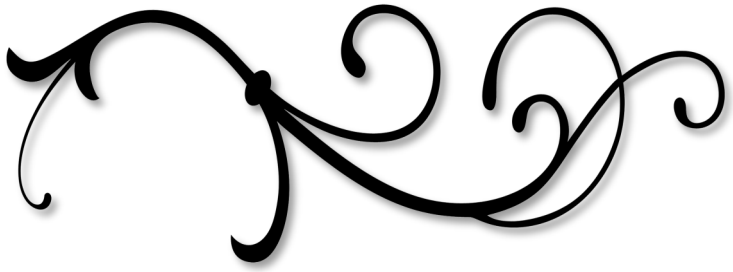
Literature also reveal very little information about bacterial ghost as adjuvants for inactivated viral antigens. BGs can be prepared chemically by NaOH method other than gene E lysis method. Hence, we have planned to use NaOH for the first time to produce *Pasteurella multocida* Ghosts (PMGs) as chemical methods are also reported for other bacteria (Hajam *et al*., 2017); In view of these studies, we intended to see whether any immune enhancement (upregulation of costimulatory molecules) of dendritic cell antigen processing and presentation occurs keeping FMDV inactivated antigen along with PMGs as adjuvant using flow cytometry. Present proposal was aimed at with following objectives.

Objectives;

1)To prepare and characterize *Pasteurella multocida* Ghosts (PMGs) using standardised chemical method

2)To study the effect of *Pasteurella multocida* Ghosts (PMGs) on immunogenicity of in-activated Foot and mouth disease virus Antigen (FMDV Ag) with *Invitro* bovine den-dritic cell culture

REVIEW OF LITERATURE



2 REVIEW OF LITERATURE

2.1. Foot and mouth disease (FMD)

Foot-and-mouth disease (FMD) is a highly infectious disease of domestic and wild cloven-hoofed animals including sheep, goats, cattle and swine. The disease is characterized by oral and pedal vesicles that result in high morbidity but, except for infection in young animals, FMD generally causes low mortality (Grubman and Baxt, 2004). The etiologic agent FMDV is a member of the Aphthovirus genus of the Picornaviridae family and contains a single-stranded positive-sense RNA genome of c. 8500 nucleotides surrounded by an icosahedral capsid composed of 60 copies each of four structural proteins VP1-4 (also termed 1D, 1B, 1C, and 1A) (Rueckert and Wimmer, 1984; Grubman and Baxt, 2004). FMD has been recognized as the most important constraint to international trade in animals and animal products by the World Organization for Animal Health (OIE). Countries which are free of FMD prohibit importation of susceptible animals or their products from infected countries. Thus, the economic consequences of an FMD outbreak can be devastating as demonstrated by the 1997 FMD outbreak in Taiwan and the 2001 outbreak in the United Kingdom. In these countries millions of animals were slaughtered and there was a cost of billions of dollars to their economies .

With the increasingly stringent requirement for the safety, purity and overall quality of vaccines, the quality control issues become more and more important in vaccine development and production (Metz *et al*., 2009). The complex structure and heterogeneous com-position of vaccine antigens (e.g., live-attenuated, inactivated virus, virus like particles and many others) make it a challenging task to develop reliable, rapid and stable analytical methods to monitor the product quality.

2.2. Role of dendritic cell in immune system

Dendritic cells have long been recognized as highly potent antigen-presenting cells. Upon activation, dendritic cells process and present antigens and express high levels of costimulatory and major histocompatibility complex (MHC) molecules, in addition to secreting various cytokines and chemokines which initiate and/or enhance many T and B lymphocyte responses (Wallet *et al*., 2005). These responses include: 1) induction of CD4+ T lymphocyte type 1 and type 2 subset differentiation (Constant and Bottomly, 1997). 2) CD8+ T lymphocyte activation and enhancement of cytotoxic T lymphocyte activity and 3) B lymphocyte maturation, Ig class-switching and antibody production (Macpherson *et al*., 1999). Only recently, however, has the tremendous versatility of dendritic cells become apparent. Besides routine antigen trapping, presentation and costimulation, dendritic cells play a substantial role in providing selective immune responses to specific classes of antigens (Lewis and Reizis, 2012). Additionally, dendritic cells are important mediators of peripheral immune tolerance and maintenance of immune homeostasis (Gad *et al*., 2003). In the past, these contrasting roles of dendritic cells have been described primarily as a function of maturation where immature dendritic cells were largely considered to be non-inflammatory or tolerogenic, but mature dendritic cells were considered capable of eliciting proinflammatory responses. Although generally correct, this paradigm is now proving to be too simple. Dendritic cells arising from myeloid or lymphoid-derived precursors exhibit an immature phenotype characterized by a high phagocytic capacity and low expression of costimulatory molecules such as CD40, CD80 and CD86 (Mahnke *et al*., 2002). The wide array phagocytic receptors of dendritic cells allows ingestion of a broad variety of antigens like microbial pathogens .Adhesion to, and phagocytosis of microbes by dendritic cells are dependent on several receptors including CD14, scavenger receptor-A (SR-A)11 and the Fc receptors (FcR): FcγR1, FcγR2b and FcγRIII (Takai, 2005). These pathways of phagocytosis lead to antigen processing mechanisms that load microbial peptides typically onto MHC class II for presentation to CD4+ T lymphocytes. Notably, dendritic cells defined by expression of CD8α are also efficient in shuttling phagocytosed antigens into the MHC class I pathway and presentation to CD8+ T lymphocytes (Blachère *et al.,* 2005). Upon phagocytic activity and further activation, dendritic cells found in peripheral tissues migrate to the draining lymph nodes, undergo further maturation, and stimulate T lymphocytes specific for the cognate peptide. Several factors can induce dendritic cell maturation and promote a proinflammatory phenotype. For example, pathogen-associated molecular patterns containing components of bacteria, viruses and parasites, such as lipopolysaccharides,

peptidoglycans, CpG motifs, flagella and viral nucleic acids, induce toll-like receptor signalling which results in dendritic cell maturation (Akira *et al.,* 2006). Maturation of dendritic cells is crucial for the induction of T lymphocyte immunity as demonstrated by Hugues *et al*., 2004. This group determined that dendritic cell maturation due to inflammatory stimuli, such as lipopolysaccharide, results in prolonged contacts between dendritic cells and T lymphocytes. The kinetics of this interaction differ from those observed for immature dendritic cells in which only short-term contacts with T lymphocytes are established. Interestingly, the extended contact between T lymphocytes and mature dendritic cells resulted in efficient T lymphocyte activation and proliferation not observed with immature dendritic cells. It is likely that the kinetics of dendritic cell/T lymphocyte interactions, along with dendritic cell phenotype (i.e., costimulatory molecule and cytokine expression) together determine whether immunity or tolerance is established. In addition to the kinetics of T lymphocyte interaction, dendritic cell maturation is crucial for appropriate migration to lymph nodes. Dendritic cells, which are matured by inflammatory stimuli in the periphery, become highly motile and readily migrate to local lymph nodes. Once in a lymph node, these mature dendritic cells settle within a network of non activated dendritic cells near the T lymphocyte rich regions. This selective migration allows the dendritic cells to interact with vast numbers of T lymphocytes, thus enhancing T lymphocyte priming and activation Toll-like receptor expression by dendritic cells serves multiple purposes. First, bodies that are phagocytosed in the presence of toll-like receptor signalling enter a specific phagosome maturation pathway. This pathway results in enhanced degradation of the engulfed material and efficient presentation of antigens (Blander and Medzhitov, 2004). Secondly, dendritic cell subsets express unique repertoires of toll-like receptors, allowing for specialized responses to each class of pathogen (Proietto *et al*., 2004). For instance, plasmacytoid dendritic cells uniquely express TLR9 and TLR7 which allow plasmacytoid dendritic cells to respond to viral CpG DNA (Krug *et al*., 2001) and viral single stranded RNA, respectively. However, plasmacytoid dendritic cells do not express TLR4 and therefore respond to lipopolysaccharide relatively weakly. In plasmacytoid dendritic cells, TLR9 ligation results in high production of type I interferons (IFN-α and IFN-β) promoting plasmacytoid dendritic cell survival and increased MHC expression by neighboring antigen-presenting cells, thus enhancing anti-viral immunity. Through selective toll-like receptor expression, plasmacytoid dendritic cells are able to mount appropriate anti-viral responses. Meanwhile, responses to bacterial infections may primarily be mediated by CD11c+CD11b+ myeloid dendritic cells that express TLR4. In the whole dendritic cells family, involvement of the several toll-like receptors results in

generation of various proinflammatory cytokines, including type I interferons, tumor necrosis factor (TNF)-α, IFN-γ, IL-12 and IL-1 (Proietto *et al*., 2004). These unique responses, tailored by each dendritic cell subset, serve to enhance innate immune responses at the site of inflammation and guide adaptive immunity. Currently, strategies are being developed whereby individual cell types, such as dendritic cells, can be manipulated *ex vivo* to induce immune tolerance in a more antigen-specific manner**.**

2.3. Bacterial ghosts as adjuvants: Mechanisms and potential

Bacterial ghosts (BGs) are hollow empty cell skeletons obtained from Gram-negative bacteria. They contain many innate immunostimulatory agonists, and are potent activators of a broad range of cell types involved in innate and adaptive immunity. Studies have depicted both adjuvant as well as their capacity to induce proinflammatory cytokine production properties of the BGs. These proinflammatory cytokines trigger a generalized recruitment of T and B lymphocytes to lymph nodes that maximize the chances of encounter with their cognate antigen, and subsequent elicitation of potent immune responses. Production of hollow empty enveloped antigens in immunologically active stage that have proven to be effective vaccines in several experimental animal studies. Including their adjuvant behavior, BGs also considerably present DNA-encoded antigens to dendritic cells, thereby leading to high antigen countering efficiencies, which subsequently result in higher gene expressions and enhanced immunogenicity of DNA-based vaccines (Tabrizi *et al*., 2004). Bacterial ghosts (BGs) provides a effective mechanism which not only acts as potent candidate vaccines but also acts as a tool for remarkable adjuvant and vaccine delivery systems. This novel approach has produced promising results to curb infectious diseases, tested both in natural hosts as well as in experimental animals. Scanning electron microscopic studies have shown that BG maintain cellular surface morphology similar to wild bacteria where entire cell surface components including LPS, outer membrane proteins, adhesins, and the peptidoglycan layer are well preserved (Huter *et al*., 1999). In addition, the foreign antigens have been loaded inside the cytoplasmic lumen or expressed both on the surface and in the periplasmic space of BGs (Huter *et al*., 1999; Mayr *et al*., 2005). These remarkable properties make BG an attractive tool for vaccine development and antigen delivery system for both humans and animals. The presence of LPS in the BG does not limit its use as an adjuvant or candidate vaccine due to minimal toxicity as compared to free LPS (Hajam *et al*., 2017). Considering the particulate nature of BG and the they possesses many well-known TLR agonists, BGs have the capacity to be effectively identifiable by

APC and to further elicitate potent immune responses against their own envelope structures or ghost-delivered foreign antigens (Jawale *et al*., 2014; Wen *et al*., 2012). With this consideration, BG have been successfully used as adjuvants and a delivery system for a number of viral and bacterial antigens.

2.4. Production of bacterial Ghosts (BGs)

BGs are empty envelopes of Gram-negative bacteria produced by the controlled expression of lysis gene *E* of bacteriophage phiX174 (Jawale and Lee, 2014; Henrich *et al.,* 1982). The role of gene *E* in the lysis of Gram-negative bacteria, *Escherichia coli*, was for the first time reported by Hutchison and Sinsheimer (Hutchison and Sinsheimer, 1966) and subsequently this gene was identified by Pollock *et al.,* (Pollok *et al.,* 1988); in heavily UV-irradiated *E. coli* cells. The gene *E* codes for 91 amino acids, possesses lytic action but no inherent enzymatic activity (Denhardt *et al*., 1965). It is a membrane protein with hydrophobic moieties at its N-termina region that oligomerizes into a transmembrane tunnel structure. The E-specific tunnel structure spans the inner and outer membrane and is located at the membrane adhesion sites within the host cell (Langeman *et al*., 2010).

Electron microscopic analysis has revealed that the tunnel formation is associated with the fusion of the inner and outer membrane, sealing the periplasmic space (Langeman *et al*., 2010); Due to high osmotic pressure, the cytoplasmic contents including DNA are expelled through the tunnel leaving behind empty cell envelopes known as BG (Hajam *et al*., 2015). A study by Witte *et al*., shows that tunnel formation on the surface of bacteria is not random but occurs at the potential cell division sites, indicating that cell division is mandatory for the formation of BG. Lytic activity of protein E is dependent on the physiological pH (autolytic system) and the growth phase since the non physiological pH and stationary phase of bacteria have inhibitory effects on the lysis effect (Hajam *et al*., 2015; Witte *et al*., 1990); BG which are produced must be free from any live bacteria and any viable bacteria must be further inactivated. BGs of a Gram-negative bacteria have been generated through gene E mediated lysis method and further used as candidate vaccines agents and adjuvants in a number of animal studies. Besides gene *E* mediated lysis, BGs of *E. coli* have also been prepared by the application of high hydrostatic pressure (HP) treatment (Vanlint *et al.,* 2008); In this approach, *E. coli* cells were first sensitized to a high pressure shock through over-expression of *E. coli* K12 Mrr protein. The Mrr protein is a cryptic type IV restriction endonuclease that is activated

by mild HP treatment and specifically targets methylated DNA (Vanlint *et al*., 2008);A study has demonstrated that BG retain their refractility, indicating that they are not lysed or permeabilized unlike ghosts prepared by the protein E mediated lysis. BG derived by this method can be efficiently exploited to deliver subunit or DNA vaccines and do not need to be artificially tethered to the membrane, as is the case with gene *E* mediated BG (Tabrizi *et al*., 2004). Recently, there was a novel method to prepare BG causing complete killing of the bacteria. In this method, they shown that, expression of holin-endolysins along with the gene *E lysis* and noticed that the lysis of *Salmonella* bacteria is much faster than the current BG production methods. The holinendolysins are small bacteriophage hydrophobic enzymes and when expressed, form oligomeric pores in the host cell membrane and lysis of the bacteria subsequently (Schmelcher *et al*., 2012). This new combination of endolysins with the present gene *E* mediated lysis provides a safer method to prepare genetically inactivated BG. The BG have also been prepared by the application of minimal concentrations of chemicals including NaOH, SDS and H_2O_2, resulting in the production of sponge like structures (Arnara *et al*., 2013). This method can be applied to both Gram-negative as well as Gram-positive bacteria; however, the efficacy and potential of these BG to act as adjuvants and a delivery system must be evaluated both in vitro and in vivo. Recently, BG of Gram-positive bacteria *Listeria monocytogenes* have been prepared using a chemical method (Wu *et al*., 2017). Suggesting that this method could be useful in future vaccine development against important Gram-positive food-borne pathogens. The expression of protein E in Gram-positive bacteria results in cell killing without lysis as the formation of BG depends on the fusion of inner and outer membranes of bacteria, which occurs only in Gram-negative bacteria (Witte *et al*., 1990).

Although the lysis E gene-induced BGs provided efficient protection against specific Infections (Panthel *et al*., 2003); A primary disadvantage of the method is a limitation to Gram-negative bacteria only. Secondly, it is very difficult to reach 100% lysis rate of BGs strain in a short time (Eko *et al.,* 2000); which may cause potential risks. Thirdly, it is a multi-step process that is cost expensive and time consuming. Alternatively, Amara et al developed the protocol for *Escherichia coli* BGs production using the minimum inhibition concentration (MIC) and the minimum growth concentration (MCG) of various chemicals. Recently, sodium hydroxide (NaOH)-induced BGs were generated from a Gram-negative bacterium (*Salmonella enteriditis*) (Vinod *et al*., 2014); and a Gram-positive bacterium (*Staphylococcus aureus*) Nevertheless, it has never been characterized as to why NaOH was chosen as the best chemical to produce

BGs. Effects of Chemicals on Bacterial Cell Envelopes which adversely effects on the integrity of bacterial cell envelopes that inhibit bacterial cell growth are known. Especially, acids and alkalis have strong bactericidal effects. The MICs of six acids (acetic acid, boric acid, citric acid, hydrochloric acid, maleic acid, sulphuric acid) and 1 alkali (NaOH) against *V. parahaemolyticus* were determined by the two-fold broth dilution method. Furthermore, no colony was formed on LB agar plates spread with *V. parahaemolyticus* bacteria treated with respective chemicals at their MICs (Park *et al*., 2016); This was consistent in all of the three replicates performed. Among those chemicals boric acid showed the highest MIC, while hydrocloric acid showed the lowest MIC. Previously, acetic acid, hydrochloric acid, and lactic acid inhibited Helicobacter pylori growth in a pH-dependent manner while butyric acid inhibited the bacterial growth in a pH-independent manner (Midolo *et al.*, 1995). The MICs of different chemicals changed the culture medium pH from pH 7.0 to 9.99 (NaOH), 4.28 (acetic acid), 3.11 (citric acid), 3.95 (hydrochloric acid), 3.56 (maleic acid), and 5.83 (sulfuric acid). The data indicated that the different chemicals above did not inhibit the *V. parahaemolyticus* growth in a pH-dependent manner but they inhibited the bacterial growth in a concentration-dependent manner. There is a complete lysis of the *V. parahaemolyticus* cells treated with different chemicals, respectively, at various time points. Except for acetic acid (99.99% at 60 min), other chemicals reached 100% lysis rate within 10 min. In the lysis E gene-mediated method, it took almost 8 h to produce non-living BGs with complete lysis and the lysis efficiency was commonly 99.9% (Kwon *et al.,* 2005); Thus, production of BGs by chemically-mediated lysis is a more simple and rapid process than that by the genetically engineered E gene-mediated lysis method.

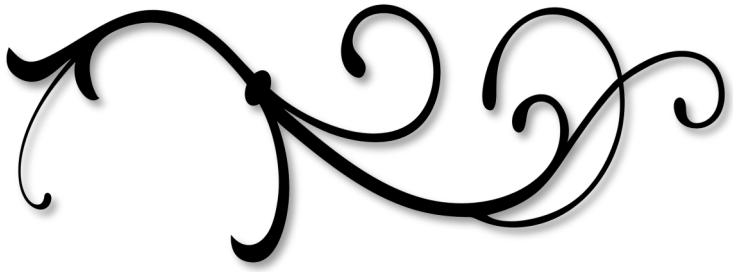
2.5. Adjuvant mechanisms of BGs

Elicitation of immune response not only depends on the molecular properties of the antigen or on the immunogenic susceptibility of the host but also on the formulation of the antigen. Thus, adjuvants are immunomodulatory components which enhance the immune responses against the weak immunogenic antigens. Adjuvants mostly potentiate the immunogenicity of vaccine antigens through the stimulation of innate immune receptors present on the cells of the host immune system (Hajam *et al.*, 2013; Akira *et al*., 2006; Honko *et al*., 2006); The cells of the innate immune system respond to a variety of stimuli including bacterial, viral, parasitic or fungal infections via members of structurally related receptors termed as Toll-like receptors (TLR). TLR are evolutionary conserved type I transmembrane receptors representing a critical link between innate and adaptive

immunity. TLR do not possess fine specificity like that of BCR or TCR, the adaptive immune receptors, but individually can respond to a limited but specific number of microbial pathogen associated molecular patterns (PAMP) (Akira *et al*., 2006); The interaction of PAMP with the TLR on the innate immune cells regulates the induction of more efficient adaptive immune responses. TLR sense bacterial cell wall components such as lipopolysaccharide (LPS) (TLR-2/4), lipoteichoic acids (TLR-2/4), CpG DNA (TLR-9), flagellin (TLR-5), and others.The pro-inflammatory cytokines provide augmentary signals, through up-regulation of co-stimulatory and adhesion molecules, essential for the activation of the adaptive immune cells, and in prevention of tolerance to infectious nonself antigens (Granucci et al.,2003); In recent years, a number of microbial molecules have been used as adjuvants to augment the immune responses of poor immunogenic vaccines. The use of TLR agonists as vaccine adjuvants have shown promising results in animal models and eventually some of them have paved their way into human clinical trials (Steinhagen *et al*., 2011); BG contain well-known innate immune stimulating components, and have thus tremendous potential to act as efficient adjuvants. An increasing number of studies have demonstrated that protein E mediated lysis preserves the antigenic nature of BG membrane components including LPS, peptidoglycan or flagella, and are thus identical to the components of native bacteria (Huter *et al*., 1999; Mayr *et al*., 2005); Therefore, these envelope structures are efficiently recognized and taken up by immune and non-immune cells (Ebensen *et al*., 2004); BG mostly stimulate cells through TLR2 and TLR4 pathways (Adam *et al*., 2010; Quevedo-Diaz *et al.,* 2010); DC activation and maturation, and their recruitment to T cell areas in lymph nodes (Benko *et al*., 2008); DC are unique APC with abilities to prime naïve T cells, and thus play an essential role in the initiation of primary immune responses (Hart, 1997). They are located at antigen capture sites where they take up antigen and subsequently migrate to lymph nodes for antigen presentation and development of immune responses. BG are efficiently taken up by DC and result in the induction of proinflammatory cytokines, which subsequently upregulate the costimulatory molecules on DC for efficient presentation of foreign antigens to naive T cells (Ebensen *et al.*, 2004; Hajam *et al.,* 2015); BG deliver efficient and early maturation signals to DC, and the induction of Th1 cytokines, especially IL-12, occurs many folds which is the main cytokine driving the stimulation of NK and Th1 cells (Ebensen *et al.,* 2004; Hajam *et al*., 2015; Hamza *et al.,* 2010). The MHCII levels are up-regulated after 12 h exposure to BG , indicating that they have the potential to induce early protective immune responses, which are very much required during emergency vaccination. BG also enhance MHC-I expression on DC and the presence of LPS effectively improves the cross presentation

and maturation of DC (Trombetta *et al*., 2003); These findings suggest that BG have the ability to stimulate both humoral and cell mediated immune responses. Besides DC, BG also effectively stimulate monocytes and macrophages and polarize the response toward Th1 (Tabrizi *et al*., 2004). All these factors contribute to the overall potency of BG adjuvated vaccines. Another factor that contributes to the adjuvant potential of BG is the presence of TLR on non-professional APC. BG are known to stimulate both professional and non-professional APC like conjunctival epithelial cells, fibroblasts, keratinocytes, melanoma cells etc. (Abtin *et al.,* 2010). These studies have demonstrated that BG are effectively recognized and internalized by non-professional APC, and induce the expression of antimicrobial psoriasin and pro-inflammatory cytokines, IL-6 and IL-8. The cytokine IL-6 helps in the development of effective and potent mucosal immune responses, and protects the host against viral and bacterial infections (Ramsay *et al.,* 1994; Deinz *et al.*, 2012); This indicates that BG have the potential to induce potent mucosal immune responses and could provide nonspecific protection against pathogenic organisms, as has been reported with the use of other TLR agonists. The elucidation of these activation pathways will result in the better understanding of BG and host cell interactions, and therefore will be helpful for the design of BG-based novel therapies. The presence of TLR on the cells of adaptive immune cells, B and T cells, further contributes to the adjuvant potential of BG. The presence of LPS is the major contributor factor in the adjuvant potential of BG. A study by Means *et al*., shows that flagellin-treated DCs have a slightly lower stimulatory T cell effect than LPS-treated DCs, and flagellin stimulation induces the expression of chemokines in DC. The induction of these chemokines is very rapid and results in the recruitment of lymphocytes to the secondary lymphoid sites. This indicates that BG have the potential to recruit not only innate immune cells but also adaptive immune cells at the site of immunization. All these factors lead to the efficient interaction of innate and adaptive immune cells, necessary for the induction of potent immune responses. BG increase the ICAM-1 expression on DC, providing the necessary costimulation for the efficient generation of CD8 + T cell responses (Lefor and Fabian, 1998); The effectiveness and adjuvant potential of BG can further be enhanced by expression of potent immunostimulatory molecules on their surfaces.

MATERIALS AND METHODS



3 MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Virus

Foot and Mouth disease virus type 'A' strain maintained at FMD research centre IVRI Bengaluru at different passage level propagated and used for preparation of killed viral antigen.

3.1.2. Cell line

Baby hamster kidney (BHK)-21 clone 13 (Glasgow) cell line monolayer maintained at FMD research centre IVRI Bengaluru was used for virus cultivation and virus titration and infectivity assay BEI inactivation.

3.1.3. Experimental animal

Blood collected from 9-12 month old healthy cattle calf in IVRI yelahanka animal experimental station Bengaluru.

3.1.4. Plastic ware and glassware .

1) Microcentrifuge tubes and Micropipette tips

2) Plastic wares for cell culture

3) BD vacutainer sodium heparinized

4) Falcon FACS tube

5) Six well culture plate

6)Glassware was available in FMD QC & QA unit and FMD research lab.Glassware was thoroughly washed and sterilized as per standard protocols before usage.

3.1.5. Equipments

1) Refrigerated Micro centrifuge (Eppendorf, 5417 R model)

2) Variable volume pipettes (Eppendorf, Germany)

3) Refrigerated high speed centrifuge (Sorvall RC5C, Dupont,USA)

4) Ultra-centrifuge Optima XL-80, (Beckman Inc., USA)

5) Refrigerated table top Centrifuge (Sorvall - RT7 DuPont, U.S.A)

6) Refrigerated high speed centrifuge (REMI)

7) Vacutainer (Becton Dickinson)

8) pH meter (ECIL, India)

9) -80°C ultra-low freezer (Sanyo electronic.co. Japan)

10) Electronic weighing balance (Avery, India)

11) MiniMACS separator (Miltenyi biotech)

12) LS column (Miltenyi biotech)

13) MACS multistand (Miltenyi biotech)

14) Vertical laminar air flow (Clean Air, India)

15) Horizontal laminar air flow (Klenzaids, India)

16) Electronic Shaking Incubator (Innova-4000, New Brunswick scie.USA)

17) Microwave oven (MX 2100Microwin Electronic India Ltd)

18) Water bath (Heto, Danmark)

3.1.6. Media and buffers

Roswell park memorial institute (RPMI) -1640 without phenol red was obtained from (Sigma- Aldrich USA), Cell culture media – Dulbecco modified eagles (DMEM),

FACS buffer, MACS buffer, PBS, Cell dissociation buffer (Sigma). Compositions of media, buffers and solutions used in the study are given in the appendix.

3.1.7. Biologicals and Enzymes

1) Mouse anti bovine – (CD 14 FITC ,AbD Serotec)

2) Mouse anti bovine – (CD 40 PE ,AbD Serotec)

3) Mouse anti bovine – (CD80 PE ,AbD Serotec)

4) Mouse anti bovine – (CD86 PE ,AbD Serotec)

5) Fetal Bovine Serum (Sigma Aldrich)

6) Bovine Serum Albumin (Sigma Aldrich)

7) Recombinant Bovine interleukin -4 protein (R and D system)

8) Recombinant bovine GM-CSF protein (AbD Serotec)

9) Histopaque 1077 (Sigma Aldrich)

10) Cesium chloride (Sigma Aldrich)

11) Benzyl penicillin (Sigma Aldrich)

12) Polyethylene glycol (PEG)6000 (Sigma Aldrich)

13) Streptomycin (Cell culture tested, Sigma Aldrich)

3.2. METHODS

3.2.1.1. Preparation of Inactivated FMD Virus

BHK -21 clone 13 monolayer cells obtained from FMD Research centre Bengaluru were grown in T25 flask in 5 ml of growth media. The cells were allowed to grow at 37 ^{O} C till attainment of 80% confluency (48hr). After 80% growth , the cells subcultured subsequently in T75 flasks,T175 flasks and roller bottles. Sub culturing was done by discarding the growth media from flasks and 1 ml of TVG for T25 flasks and 2 ml for T75 and T175 flasks was applied to monolayer cell surface. After 20 seconds, TVG was discarded and flasks were left undisturbed till cells get detached from the surface. Cells from the T25 and T75 were splitted in the ratio of 1:3 and further cultured into separate

flasks. Cells with 80% confluency T75 flasks are further used to grow cells in T175 flasks. Finally two T175 flasks were used to grow cells in each roller bottles. The amount of media used in T25, T75, T175 and in the roller to grow cells is 5ml, 20ml, 50ml and 200ml respectively. Cells were allowed to grow at 37 ^{o}C temperature till attainment of 80% confluency. The monolayer grown in batches of 20 roller bottles were infected with freshly passaged MP3 seed virus type A (strain). Infection was done by diluting the inoculums (MP3seed virus) with a MOI (0.01-0.1) about 3ml of in 10 ml of maintenance media and was applied on monolayer cells . The inoculum was adsorbed for 15 minutes at 37 ^{o}C temperature. The roller bottles were gently shaken every 10 min to ensure the inoculum was evenly distributed over monolayer. At the end of adsorption period, added 100 ml of maintenance media and continue incubation at 37^{o}C overnight. The virus was harvested after culture shows CPE affecting 90-95% of cell monolayer. The infected cell culture was collected and 1% (v/v) chloroform was added and kept for 1hr at 4^{o}C. The virus suspension was carefully decanted and clarified (Sorvall RC5C) at 4^{0} C as the virus will be used for BEI inactivation at 37^{o}C. After centrifugation the supernatant was collected and virus was inactivated by adding BEI (Binary-Ethyle Eimine) solution (10ml/liter) to achieve 3Mm final concentration) as per OIE standard. BEI was prepared by adding 0.62gm (per litre of virus) of BEI (2- bromoetyleamine hydrobromide) to 10 ml of 0.7% NaOH filtered using sterile PVDF membrane and kept at 37 ^{o}C for 1 hr with continuous stirring. After addition of BEI, sterile magnetic bar was added the virus was stirred for 24 hr on magnetic stirrer at 37^{o}C, after 24 hr inactivated virus was

centrifuged at 7700 rpm for 15 min to remove precipitates formed during inoculation. Supernatant was collected and the BEI was hydrolysed by adding sterile 20% sodium thiosulfate to final concentration of 20% (100ml/liter). BEI inactivation was checked by infectivity assay done on BHK cells. The inactivated virus was chilled on ice and added sterile 2% NaCl solution to a final concentration of 0.35M and proceed for PEG concentration.

3.2.1.2. Concentration of FMD virus antigen by PEG precipitation method

After adding NaCl solution inactivated virus was concentrated by 50% PEG 6000 to final concentration of 8% w/v and stirred overnight at 4^{0}C. PEG concentrated virus suspension was centrifuged at 7700 rpm for 30 minutes in RC-5C centrifuged at 4^{o}C. Supernatant was discarded, pellet was resuspended with Trish-NaCl buffer to 1/10th of original volume and then incubated for 2 hr at 4^{o}C, after incubation. Insoluble debris was removed by centrifugation at 10000g for 10 min at 4^{o}C and supernatant was discarded and the pellet was stored at -80^{0} C.

3.2.1.3. Purification of PEG concentrated clarified inactivated FMD virus

It is done by CsCl density gradient method described by Bachach et al 1964. 4 ml of 1.42 g CsCl/ml (5.678 g CsCl in10 ml of Trish –NaCl) was taken in 36 ml of polyallomer ultracentrifugation tube over which another 4 ml of 1.38 g CsCl/ml (5.095 g CsCl in 10 ml of Trish-NaCl buffer) (Appendix) was layered. The PEG concentrated clarified inactivated virus was layered on to 8 ml of CsCl gradient slowly to avoid mixing. Ultracentrifugation was done in swinging bucket rotor (AH- 629) OTD 75B sorvall ultracentrifuge at 27,000 rpm for 4 hours at 4^{o}C. The gradient was fractionated from the bottom of the tube using siphon tube. 6 fractions were collected and absorbance of fraction was measured at wavelength of 259 nm and 239 nm. The fraction with maximum absorbance 259nm and minimum at 239nm and giving a ratio of 1.3-1.4 at 259/239 were selected and 146 S concentration were calculated at extinction coefficient (E1%259) OF 76 the fraction containng146S virus particle were pooled together and dialysed extensively overnight against Trish-NaCl buffer at 40C to remove CsCl salt.

3.2.1.4. Assay for testing BEI inactivation

The infectivity assay was done on BHK-21 monolayer grown in 25cc tissue culture flask. BHK-21 monolayer grown in tissue culture flask was washed once with growth medium and 1 ml of BEI inactivated virus was adsorbed onto the monolayer for 1 hour. After 1houre added 6 ml maintenance media with 5% TPB and incubated for 24 hours. After this it was freeze thawed and taken for 2nd and 3rd passage following similar procedure as done for passage. The absence of visible CPE during three blind passages was considered for ascertaining inactivation of the virus.

3.2.1.5. Titration of virus

FMD virus serotype A propagated in BHK-21 cell line was titrated in micro culture system 50 µl of maintenance media was dispensed in micro titter well in triplicate prior to addition of diluted virus sample.Virus sample was diluted logarithmically (10^{-1} to 10^{-10}) in maintenance media and dispensed in 50 µl volume in microtiter well in triplicates which already contained maintenance media . 50 µl of BHK-21 cells (2×10^{6}) prepared in growth media supplemented with 2% (v/v) FCS

were added to all the well. Cell control was also kept which contained only 100 µl maintenance media and 50 µl BHK-21 cells. The plate was shaken gently to distribute

cells evenly and incubated at 37^{o}C with 5% CO2 for 48 hours. Cultures were observed for the presence or absences of CPE. Result was recorded and LOG 100 TCID 50 per ml of virus titrated then calculated according the Reed and Munch formula.

3.2.2. Production of bacterial Ghost of *Pasteurella multocida* P52 strain by chemical method

3.2.2.1. Procurement of *Pasteurella multocida* P52 strain

Pasteurella multocida P52 vaccine strain procured from Indian veterinary research institute izzatnagar, bareilly,uttarpradesh .Bacteria has been procured in the form of Pasteurella multocida P52 strain positive bovine blood.

3.2.2.2. Culturing of *Pasteurella multocida* P52 strain

A loop full of procured blood, cultured in the 5% sheep blood agar plate (composition given in appendix)by streak plate method. After streaking the the plates are incubated for 48 hrs in the humidified incubator in a sterile way. A single slight brownish dewdrop like colonies of *Pasteurella multocida* P52 strain were observed after 48 hrs.

3.2.2.3. Confirmation of *Pasteurella multocida* P52 strain by grams staining

A single colony from the plate was picked using sterile loop and streaked in a circular way on a clean grease free slide and the cells are heat fixed. Further Gram staining was done by following method.

1. Place slide with heat fixed smear on staining tray.

2. Gently flood smear with crystal violet and let stand for 1 minute.

3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.

4. Gently flood the smear with Gram's iodine and let stand for 1 minute.

5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.

6. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.

7. Immediately rinse with water.

8. Gently flood with safranin to counter-stain and let stand for 45 seconds.

9. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.

10. Blot dry the slide with bibulous paper.

11. View the smear using a light-microscope under oil-immersion.

3.2.2.4. Preparation of 1 litre of *Pasteurella multocida* P52 strain broth culture

Initially a single colony of *Pasteurella multocida* P52 strain was picked in a sterile way from the sheep blood agar plate and cultured in 5 ml pre inoculum test tubes containing brain heart infusion media broth (composition given in appendix) with cotton plugs for 12-18 hrs at 37^{o} incubation. After confirming the presence of bacteria in broth by gram staining, the 5 ml pre inoculam tranfered to 2 ltr capacity conical flask containing 1 ltr brain heart infusion media broth in a sterile way and kept for incubation at 37^{o} for another 24 hrs. Gram staining performed to confirm the presence of *Pasteurella multocida* P52 strain.

3.2.2.5. Measurement of the Bacterial biomass concentration by the spectrophotometer

2ml of 36-48 hrs grown bacterial biomass broth is taken in a sterile test tube. In one of the two cuvattes 2 ml of freshly prepared sterile broth was taken to set the reading of spectrophotometer to zero at 600 nm. In another cuvatte 2 ml of bacterial biomas was taken to measure the reading of bacterial biomass at 600nm. It is desired to have the OD value in the range of 6-8 to get good concentration of bacterial biomass in the culture

3.2.2.6. Measurement of Bacterial count by standard plate count method

1. Bottom of six BHI media petri plates from 1-6 and four tubes of saline 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} are labelled.

2. Using aseptic technique, the initial dilution was made by transferring 1 ml of cultured broth sample to a 9ml sterile saline blank. To get a 1/100 or 10^{2} dilution.

3. The 10^{-2} blank was then shaken by grasping the tube between the palms of both hands and rotating quickly to create a vortex. This serves to distribute the bacteria and break up any clumps.

4. Immediately after the 10^{-2} blank has been shaken, 1ml was aseptically transfered to a second 9 ml saline blank. Since this is a 10^{-2} dilution, this second blank represents a 10^{-4} dilution of the original sample.

5. The 10^{-4} blank shaked vigorously and transfered 1ml to the third 9ml blank. The third blank represents a 10^{-6} dilution of the original sample. Process was repeated once more to produce a 10^{-8} dilution.

6. 10^{-4} blank was shaken again and aseptically transferd 1.0 ml to one petri plate and 0.1 ml to another petriplate. Same procedure followed for the 10^{-6} and the 10^{-8} blanks.

7. Agar pour tube was removed from the 48 to 50^{o} C water bath. Carefully cover was removed from the 10^{-4} petri plate and aseptically agar was poured into it. The agar and sample are immediately mixed gently moving the plate in a figure-eight motion or a circular motion while it rests on the tabletop. The above process was repeated for the remaining five plates.

8. After the pour plates have cooled and the agar has hardened, they are inverted and incubated at 37^{o} C for 24 hours.

9. At the end of the incubation period, all of the petri plates containing between 30 and 300 colonies were selected. Plates with more than 300 colonies cannot be counted and are designated too many to count (TMTC). Plates with fewer than 30 colonies are designated too few to count (TFTC). Counted the colonies on each plate. A Quebec colony counter was used Calculated the number of bacteria (CFU) per millilitre of sample by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to liquified agar.

Number of bacteria per ml = number of colonies /dilution × amount plated

3.2.2.7. Determination of the MIC (Minimum inhibitory concentration) of NaOH for the *Pasteurella multocida* P52 strain.

MICs of the NaOH chemical agent were determined by using the two-fold broth dilution method. The *Pasteurella multocida* P52 strain culture was grown in BHI medium. Serially diluted solution of the NaOH (stock solution, 100 mg/mL) was added to the bacterial culture of volume 1 ml and then incubated at 37 °C for 18 hr. After incubation, the MICs of different chemicals were determined in triplicate. To confirm the MICs, the culture that showed no visible growth was verified by spreading 100 µL of the culture on to BHI agar plates and incubated at 37 °C for 24 h.

3.2.2.8. Production of Bacterial Ghost

The 36-48 hr cultured biomass of *Pasteurella multocida* P52 strain was treated with NaOH at the rate of 3.12 mg/ml of culture ie, 3120 mg contained in 31.2 ml of NaOH solution (stock solution, 100 mg/ml). The excess volume given by addition of 31.2 ml of NaOH was adjusted by addition of 1 ml of excess NaOH. Thereafter, the broth culture was incubated at 37 °C for 10 min. At different time points (2, 4 and 6 and 8 min), the lysis rates of the bacterial culture treated with the NaOH were determined by standard plating procedure. The viability assay for each time was carried out in triplicate. After lysis had been completed for the 10 minutes, the chemically-induced VPGs were harvested by centrifugation (5 min, 4 °C, 8000× *g*) and washed twice with PBS. The final cell pellets were resuspended in ice-cold PBS and stored at 4 °C until further use.

3.2.2.9. Assessment of sterility of the Ghosts

A 100 µl ghosts sample of *Pasteurella multocida* P52 strain was inoculated into the sterile test tubes containing the BHI broth, nutrient broth, thioglycolate broth and sabouraud dextrose agar broth 5 ml of each and kept 48 hrs of incubation at 37^{o} to determine the absence of any growth.

3.2.3. Characterisation of PMGs

3.2.3.1. Scanning electron microscopy

1)1 ml of freshly prepared bacterial ghosts at the concentration of 1mg/ml (on dry weight basis) were taken in a bullet and centrifuged at 12000 rpm for 5 minutes to get pellet.

2) Bacterial pellet was fixed in the 3% glutaraldehyde suspension in 0.1M phosphate buffer pH 7.2 (composition given at appendix) for 1 h, and after that 3 washes in PBS (pellet cells in between washes) was done

3) Further the ghosts were treated with the 2% PTA (phosphotungstic acid) solution overnight.

4) Ghosts were pelletted after treating with the PTA solution and resuspended in 1 ml of PBS.

5)A drop of ghosts sample was mounted on scanning electron microscopy instrument (NovananoSEM 600 fei) stage with the aluminium foil surface and the sample was allowed to dry for 15 mintes.

6) Ghosts sample was observed at the magnification of 16000X , 23000X ,and 60,000X magnification.

7) Morphology including shape, size, outer surface and structural integrity of the Ghosts structure were studied.

3.2.3.2. Dynamic light scattering

Average size and structural integrity of ghosts was determined by dynamic light scattering using a Malvern Mastersizer 3000 instrument . The measurement was carried out at fixed angle of 90 with the sample used 1:10 dilution. The particle size of the ghosts was determined by mean (Z-average) diameter and the size distribution was describes by polydispersity index (PDI), in Malvern Mastersizer 3000.

3.2.3.3. SDS- PAGE

The gel casting apparatus was assembled without leakage. 12% resolving gel buffer was prepared by adding the reagents (Appendix). The resolving gel buffer was added to the gel mould until there is enough space to add stacking gel. The gel solution was

overlayed with distilled water to make its upper end even and bubble free. The mould was set aside for polymerization and gel solidification.

5% Stacking gel buffer was prepared by adding the reagents (Appendix). The distilled water layer over the resolving gel was poured off, stacking gel buffer added to the gel mould, and comb applied. The mould set aside for polymerization. 25 µL of sample was mixed with 15 µL of sample buffer and boiled over water bath for 5 minutes. 20 µL of each sample was loaded into wells. 10 µL of protein marker (BLUeye Prestained Protein Ladder, #94964-500UL) was added into one of the wells. The gel was run at a voltage of 90 mV until stacking and 200 mV until the dye front reached the finishing point. The gel was placed in staining solution for 2 hours and destained by placing it in destaining solution overnight.

The molecular weights of different bands were determined using standard curve method. A standard curve was created by plotting molecular weights of bands in the protein marker lane against their migration distances. From this curve molecular weights of unknown bands in the test samples were calculated using the curve formula in Microsoft Excel based on their distance of migration.

3.2.4. *In vitro* dendritic cell culture

3.2.4.1. Preparation of RPMI-1640 media

RPMI-1640 medium was prepared by dissolving 10.4g of RPMI-1640 medium without phenol red (Sigma) in 900 ml of double distilled water. Along with, 2g of sodium bi carbonate was added and stirred until dissolved. Antibiotics like 100 mg of streptomycin and 60µg of penicillin-G were added to a final concentration of 100 µg /ml and 100 IU/ml of medium respectively. A 2.38gm of HEPES was added to a final concentration of 10mM ml-1 of culture medium. The pH was measured to be 7.1-7.2. Additional water was added to bring the final volume to 1L. The medium was sterilized immediately by filtering through a sterile membrane filter with a porosity of 20 micron, using vacuum. After filtering the medium, pH was measured and it was found to be 7.4. The medium was dispensed as a 200 ml of aliquot into five sterile containers. 10 ml of medium was transferred into a bacteriological sterile culture tube for sterility test. The liquid medium was stored at 4°C and in dark until used.

3.2.4.2. Isolation of peripheral blood mononuclear cell (PBMCs)

Peripheral venous blood was collected in the heparin coated vacutainer (BD, India) from healthy cattle calf aged between 9 - 12 month, old, at animal experimental house facility, IVRI, Yelahanka, Bangalore. Isolation of peripheral blood mononuclear cells was done by density gradient centrifugation as described by Romani *et al*., 1994 with minor modification. Blood collected in the Heparin coated BD vacutainer was dispensed into a 15 ml conical centrifuge tube. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood diluted 1:1 in PBS and overlaid by Histopaque(1077) with blood in ratio of 1:2 than centrifuged at 2500 rpm for 27 min at room temperature in Refrigerated Centrifuge Sorvall - RT7 DuPont. After centrifugation, collected PBMC layer at interface. When buffy coat fraction was collected at the interface between the lower red blood cell layer and upper plasma layer. (I.e.1 ml of buffy coat represents material from 10 ml of whole blood). Buffy coat was diluted with PBS at a ratio of 1:3. For example, for 1ml of buffy coat 3ml of PBS was added. It was overlaid on Histopaque 1077 (Sigma) at a ratio of 2:1 in 15 ml conical centrifuge tube. This gradient was then centrifuged at 2500 rpm for 30 minutes at 15˚C. After centrifugation, the cells at the Ficoll plasma interface containing mononuclear cells were collected carefully with a micropipette and were washed two times with PBS. First the PBMC was washed with 10 ml of PBS at 1200 rpm for 10 minutes at 4°C and then 7ml of RPMI-1640 medium at 900 rpm for 10 min at 4°C. After washing with RPMI-1640 medium, cell pellet was re-suspended in 1 ml of RPMI-1640 medium containing 1% FCS and antibiotics, penicillin 100 I.U per ml and streptomycin 100 µg/ml. The cells were counted with counting chamber, Calculation was done as follows:

Cell per ml= Average cell count ×dilution factor×10^4

Viability of the cells was ascertained by staining with 0.4% trypan blue and counted in Neubauers's chamber using the following formula.

Viability= no of viable cells÷total no cells ×100

3.2.4.3. Freezing of PBMC

The PBMC that were collected from whole cattle blood were stored in -80^0c by suspending the PBMC in the freezing media containing 1:9 parts of DMSO and FBS with the concentration of cells having 15 million PBMC per ml of freezing media.The PBMC cell suspention was finally stored at -80^oc by consecutively tranfering the cryovials from 4^0c, 0^0c, -20^0c each one hour of duration.

3.2.4.4. Thawing and revival of freezed PBMC

1. The cryovials were taken to the 37^0c water bath in the icebox having the temperature 0^0c to 4^oc carefully.

2. The cryovials are thawed in the 37^0c water bath by partially immersing the cryovials for 45 to 60 seconds by frequent dip in and dip out method to avoid sudden shock to the cells.

3. Then the thawed freezing media containing PBMC was diluted by 10 times with the thawed RPMI-1640 media.

4. The final volume was centrifuged at 400g for 5 minutes to get PBMC pellet.

3.2.4.5. Monocyte isolation by (Magnetic activated cell sorting) MACS method

Approximately $1x10^7$ peripheral blood mononuclear cells (PBMC) were suspended per ml of RPMI-1640 medium supplemented with 10% FCS. It centrifuged at 1200 rpm for 10 min. The supernatant was discarded completely than cell pellet was resuspended with anti human CD14+ (2.5 µl) beads (Miltenyi Biotech) and incubated in room temperature for 10 min in dark, removed residual fluid from pellet prior to adding beads. After incubation, cells were washed once with 1 ml of PBS to remove the unbound CD14 anti-human paramagnetic microbeads, centrifuged at 1200 rpm for 10 min. The supernatant was completely aspirated Than resuspended pellet in 5 ml cold MACS buffer (0.5%BSA+ 2Mm EDTA+PBS) ,after fixed the magnet on stand and placed column with in magnet and MiniMACS separator was selected along with MS column for separation of CD14 antihuman magnetic beads labelled cells. Mini MACS separator and Mini MACS stand (Miltenyi Biotech) were assembled properly according to manufacture protocol. Approximately $1x10^8$ PBMC applied on column . Washed column with 2.5 ml of MACS buffer (Appendix) into top of column allowed to pass than slowly drop wise collected in collection vial as waste, Once the MACS buffer has passed through completely, add the cells to top of the column and allow to pass through. Unbound cells were collected in waste tube , add the flow through back to column and collect the second flow through. Column was washed two times with 2.5 ml MACS buffer collecting the flow through Finally eluted all the cells by adding 5ml of medium on top of column removed column from magnet used plunger, eject the medium and collected the cells in clean tube. The cells were counted with counting chamber, Calculation was done as follows: No of cells/ml = Av. no of cells × dil.factor $\times 10^4$ × cell suspension volume

3.2.4.6. Reconstitution and aliquoting of recombinant cytokine GM-CSF and IL-4

3.2.4.6.1. Reconstitution and aliquotion of GM-CSF protein

Recombinant bovine GM-CSF protein vial (Abd serotech) briefly centrifuged at 800-900 rpm for 25 second before opening to dislodge any lyophilised material that may be dispersed on the wall or cap of vial .the protein reconstituted with 1ml of distilled water. It dispensed as 50 µl aliquot. The protein supplied as crude cell culture supernatant. The protein was supplied on dilution basis rather than concentration basis. According to company instruction, it stimulated cell growth from ½-1/10 dilution. So the protein was diluted at ratio of 1:6 dilution with distilled water. The required volume of protein was determined by monocyte differentiation test . It was determined as following. PBMCs about 7×10^6 /ml in RPMI medium supplemented with 1% FCS ,1ml of PBMC suspension was added in 6 well tissue culture plate . After 2 hr incubation , nonadherent cells were removed and the adherent cells layer were washed twice with PBS before addition of 1ml of culture medium supplemented with 35 ng of IL4 cytokine. Finally the 1:6 diluted GM-CSF protein was added like 5µl,10µl,25µl respectively in each well. Monocyte differentiated very well in culture which supplemented with 5µl 1:6 diluted GM-CSF protein.

3.2.4.6.2. Reconstitution and aliquotion of IL-4 protein

Bovine IL-4 protein vial (R& D system) was briefly centrifuged at 800-1000 rpm for 30 second before to dislodge any lyophilised material that may be dispersed on the wall or cap of the viol. A 250 µl of 1x PBS 0.1% BSA was added to dissolve lyophilised protein in the vial. After adding the buffer, the viol was recapped and was inverted gently by hand .this was allowed the reconstitution buffer to coat all the surface inside the vial. Vortexing or pipetting the material up and down was avoided. The vial was kept at room temperature with gentle agitation for 15 min before aliquoting. It dispensed as a 10 µl aliquot. The reconstituted protein was stored at -80^{o}C.

3.2.4.6. Reconstitution and aliquoting of recombinant cytokine GM-CSF and IL-4

4.2.4.6.1. Reconstitution and aliquotion of GM-CSF protein

Recombinant bovine GM-CSF protein vial (Abd serotech) briefly centrifuged at 800-900 rpm for 25 second before opening to dislodge any lyophilised material that may

be dispersed on the wall or cap of vial .the protein reconstituted with 1ml of distilled water. It dispensed as 50 µl aliquot. The protein supplied as crude cell culture supernatant. The protein was supplied on dilution basis rather than concentration basis. According to company instruction, it stimulated cell growth from ½-1/10 dilution. So the protein was diluted at ratio of 1:6 dilution with distilled water. The required volume of protein was determined by monocyte differentiation test . It was determined as following. PBMCs about 7×10^6 /ml in RPMI medium supplemented with 1% FCS ,1ml of PBMC suspension was added in 6 well tissue culture plate . After 2 hr incubation , non-adherent cells were removed and the adherent cells layer were washed twice with PBS before addition of 1ml of culture medium supplemented with 35 ng of IL4 cytokine. Finally the 1:6 diluted GM-CSF protein was added like 5µl,10µl,25µl respectively in each well. Monocyte differentiated very well in culture which supplemented with 5µl 1:6 diluted GM-CSF protein.

3.2.4.6.2. Reconstitution and aliquotion of IL-4 protein

Bovine IL-4 protein vial (R& D system) was briefly centrifuged at 800-1000 rpm for 30 second before to dislodge any lyophilised material that may be dispersed on the wall or cap of the viol. A 250 µl of 1x PBS 0.1% BSA was added to dissolve lyophilised protein in the vial. After adding the buffer, the viol was recapped and was inverted gently by hand .this was allowed the reconstitution buffer to coat all the surface inside the vial. Vortexing or pipetting the material up and down was avoided. The vial was kept at room temperature with gentle agitation for 15 min before aliquoting. It dispensed as a 10 µl aliquot. The reconstituted protein was stored at -80^{o}C.

3.2.5. Generation of MoDC from the $CD14^+$ monocytes

3.2.5.1. Culturing of MACS sorted $CD14^+$ monocytes

MACS sorted $CD14^+$ monocytes plated in six well culture plate at the concentration of 2 million cells per well and provided with 2 ml of RPMI-1640 media. The cytokines GMCSF and IL-4 were added at the concentration of 5µl and 20ng (Ashutosh, 2016) per well respectively.

Half of the media (1ml) was changed on third, sixth day of culture along with cytokines addition.

3.2.5.2. Antigen pulsing of MoDC

Sixth day the MoDC were pulsed with antigen in the standard concentration . The wells were pulsed with different antigen combination viz, PMGs alone, FMDV alone, FMDV+PMGs seperately as per the requirement of the study and observed for 24 hrs.

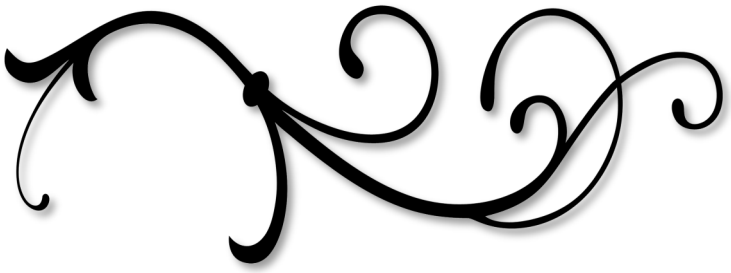
Antigens were added as follows;

- FMDV(A serotype) : 2µg/mL (Ashutosh, 2016)
- PMGs : 40 cfu/MoDC (Hajam et al., 2015)

3.2.5.3. Costimulatory molecule analysis of MoDC by flow cytometry

Media from each well were removed and cells were washed 2 times with 1mL PBS. The washed PBS was collected in a 15mL centrifuge tube. Further, 2mL of 1x Cell Dissociation Solution Non-enzymatic (Sigma, C5914) was added to each well and incubated at 37^{o}C for 30 minutes. After incubation the cells were detached by vigorous pipetting and transferred to 15mL centrifuge tube used earlier for the collection of washings. The cells were pelleted at 400xg for 5 minutes and resuspended in appropriate volume of FACS buffer. For staining anti-bovine CD40:PE, CD80:PE and CD86:PE antibodies were used at a dilution recommended by the manufacturer at the rate of 10uL per million cells in 100uL suspension. After adding conjugate antibodies, the suspension was incubated at 4^{o}C for 15 minutes, protected from light. The stained cells were washed twice with FACS buffer (500xg, 5 minutes) and read in Beckman Coulter FC 500 flow cytometer on channel FL2. The results were analysed using software FCS express 6.

RESULTS



4 RESULTS

4.1. Preparation of inactivated FMD virus type 'A' antigen

4.1.1. Growth and harvesting of virus

FMDV antigen in large scale was prepared by infecting BHK-21 cell monolayers grown in batches of 10 Roller bottles as described in the material method. Virus was harvested in 1 litre batch after development of 90% CPE.

4.1.2. Virus inactivation and concentration

After harvesting of virus, inactivation was done by BEI for 24 hours at 37^0 C and neutralization of BEI was done by sodium thiosulphate. Inactivation was checked by three successive blind passages on BHK-21 monolayer and complete CPE was absent in all the three passages, showing inactivation was successful. Inactivated virus suspension was concentrated by PEG as described in the methods(4.2.1.1&4.2.1.2). After concentration, supernatant 1 and 2 were taken and pooled and 146 S was estimated.

4.1.3. Quantification and purification of inactivated concentrated virus

Purification was done by CsCl density gradient method described by Bacharach, 1968. Six fractions were collected and absorbances of the fraction were measured at wavelength of 259 nm and 239 nm. The fraction with maximum at 259 and minimum at 239 and giving a ratio of 259/239 were selected and 146 S concentrated were calculated at extinction coefficient (E%259) of 76 as given below. The density gradient centrifugation yielded pure and highly concentrated virus in 1^{st} to 4^{th} fractional monitored by A259/239. The fraction containing 146 S were pooled and dialysed extensively overnight Tris-NaCl buffer at 4^oC to remove CsCl salt. Concentration was calculated by multiplying 131 to OD value at 259nm. Finally the quantity of the dialysed virus was

determined from A 259nm values and OD at A 259 after dialysis was 2.427, therefore the concentration of purified inactivated virus was 318µg/ml.

4.2. Production of PMGs by Chemical method

4.2.1. Culturing of *Pasteurella multocida* P52 strain

A single convex, slight brownish, smooth, and translucent dewdrop like colonies of *Pasteurella multocida* P52 strain were observed after 48 hrs of incubation at 37^{o} C in a humidified chamber(Figure 1).

4.2.2. Confirmation of *Pasteurella multocida* P52 strain by Grams staining

Spherical, ovoid, rod-shaped cells, occuring singly and also in pairs and short chains with characteristic bipolar gram negative staining are observed under microscope with 100X oil immersion technique. (Figure 2)

4.2.3. Preparation of 1 litre of *Pasteurella multocida* P52 strain broth culture

1 ltr of *Pasteurella multocida* P52 strain broth culture was prepared by culturing the broth at 37^{o}C for 48 hrs. Gram negative bipolar organisms were observed under 100X oil immersion microscope to confirm the presence of organisms.

4.2.4. Measurement of the bacterial biomass concentration by spectrophotometer

48 hrs of *Pasteurella multocida* P52 strain broth culture at 600nm under spectrophotometer gave 0.8 OD value.

4.2.5. Measurement of bacterial count by standard plate count method

A total of $3.16x10^{8}$ cfu/ml of bacterial culture was determined by counting the colonies at the dilution rate of 10^{-5} by plating 100 µl of the culture (Figure 3).

Calculation

$316x10^{5}/0.1$ ml=$3.16x10^{8}$ cfu/ml

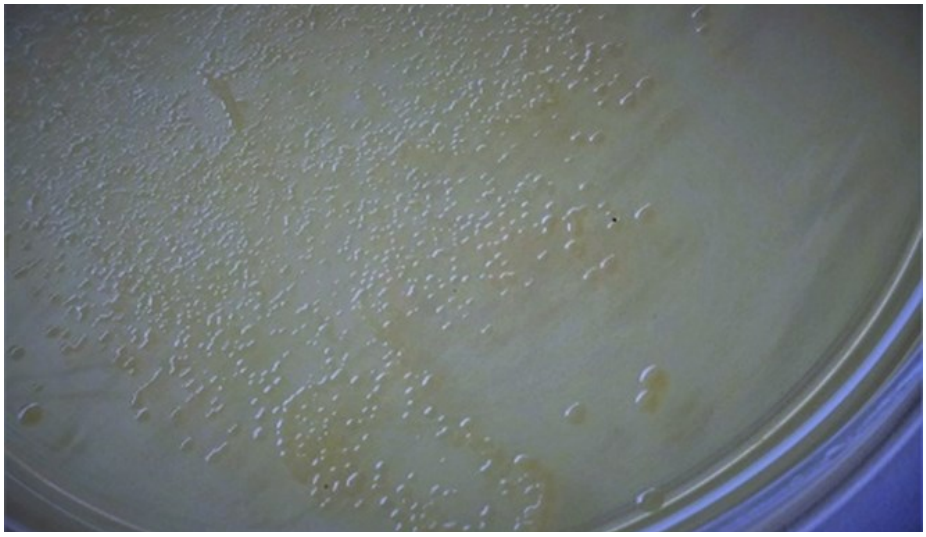


Figure 1: BHI agar plate showing single convex, slight brownish, smooth, and translucent dewdrop like colonies of *Pasteurella multocida* P52 strain

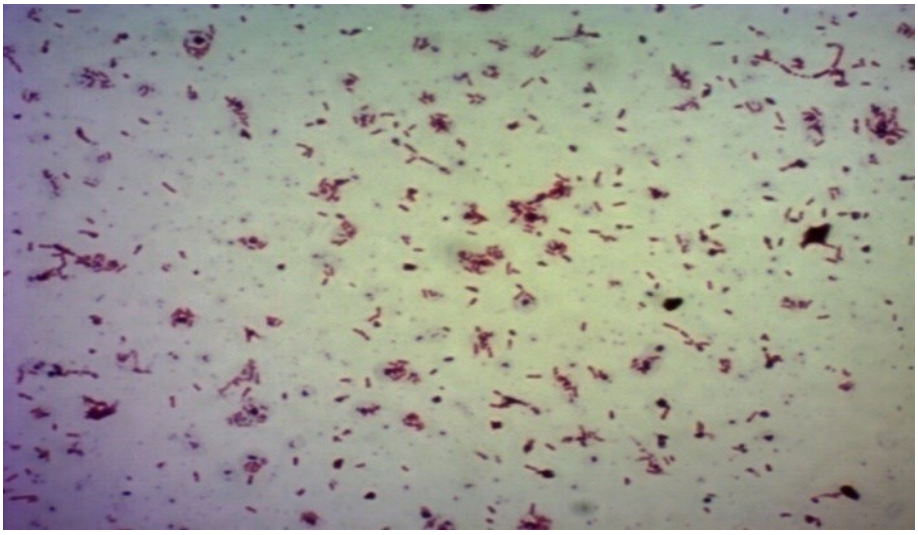


Figure 2 : Spherical, ovoid, rod-shaped cells, occurring singly and also in pairs and short chains with characteristic bipolar Gram negative staining organisms. Magnification 1000x

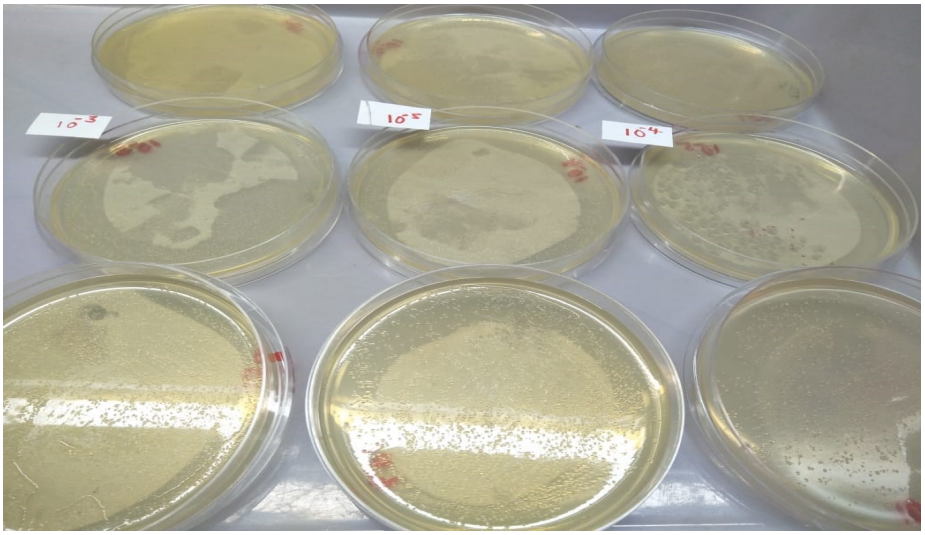


Figure 3: Standard plate count technique

4.2.6. Determination of the MIC (Minimum inhibitory concentration) of NaOH for *Pasteurella multocida* P52 strain.

The bacterial culture treated with the NaOH at the concentration of 3.15mg/ml and incubated for 18 hrs, and plated on the BHI agar plate showing absence of colony growth, but the next dilution ie,1.575mg/ml shows some colony growth on the agar plate. So the MIC needed to inhibit the growth of bacteria to produce bacterial ghost is 3.15mg/ml (Figure 4).

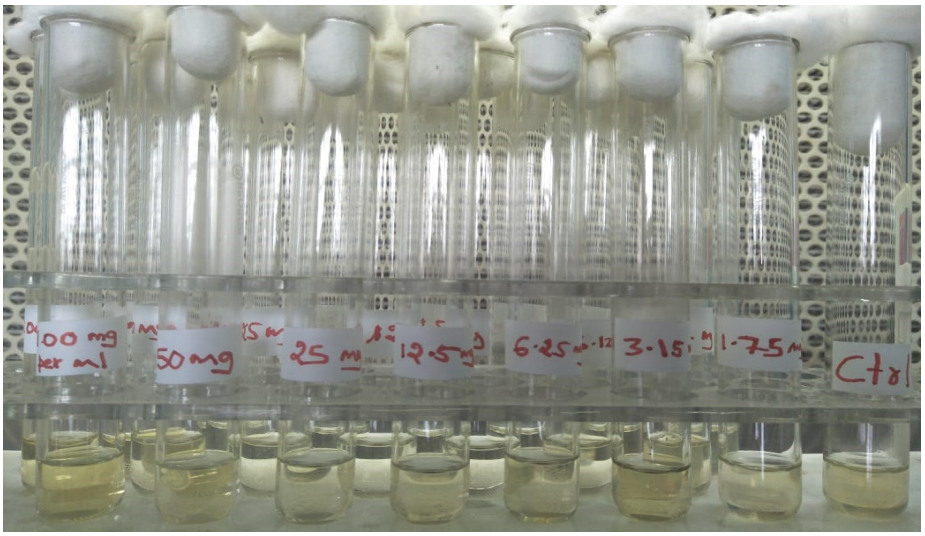


Figure 4: Determination of Minimum inhibitory concentration (MIC). Serially diluted solution of the NaOH (stock solution, 100 mg/mL) was added to the bacterial culture of 1 ml in volume and then incubated at 37 ° C for 18 hr. The lowest dilution (3.15mg/ml) successfully inhibited the bacterial growth.

4.2.7. *Pasteurella multocida* Ghost (PMGs) production

31.2 ml of NaOH (stock solution-100mg/ml) at the rate of 3.15mg/ml was used to treat 1 ltr of bacterial culture for 8 minutes and centrifuged at 8000g for 10 min at 4^0c to get pellet of bacterial ghosts, which was washed thrice with ice cold PBS and resuspended in 15ml of ice cold PBS.

4.2.8. Sterility assessment

There is no visible growth (lack of turbidity) noticed after inoculating 100µl freshly prepared PMGs suspension in different test tubes containing the selenite casein broth, nutrient broth, thioglycolate broth and sabouraud dextrose agar broth 5 ml of each, (Figure 5) which were incubated at 37°C for 24 hrs and subsequently absence of colony growth noticed by further plating on BHI agar plates.

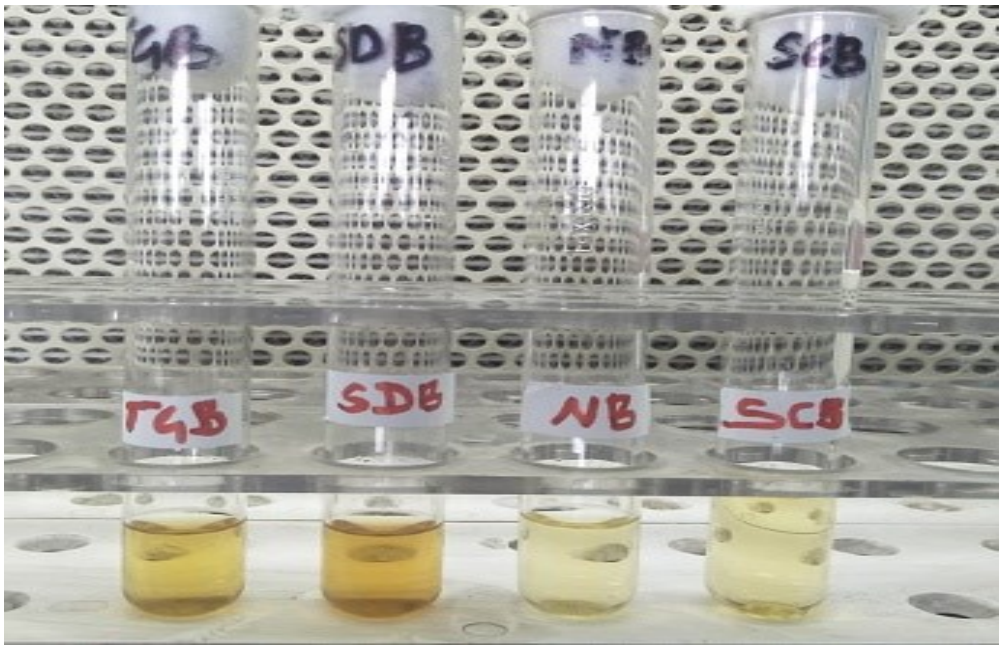


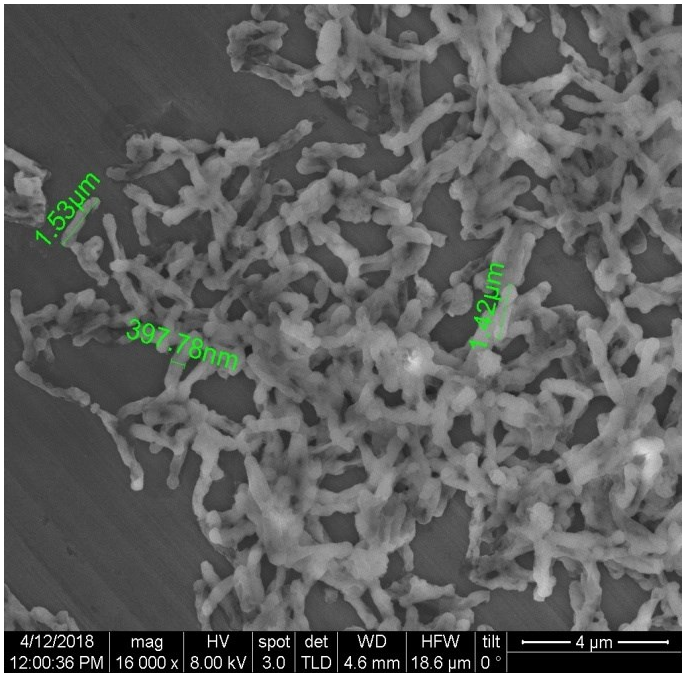
Figure 5: Test tubes showing no visible turbidity after 24 hrs of incubation (37° C). Sterility further determined by plating 100µl of the growth medium on the respective agar plates.

4.3. Characterization of PMGs

4.3.1. Scanning electron microscopy

The scanning electron microscopic observation of PMGs revealed the presence of transmembrane tunnels with altered morphology due to expelling of cytoplasmic contents of the bacteria. The length and breadth of the PMGs varies between 1µm -2µm and 0.3µm -0.5µm respectively (Figure 6)

A)



B)

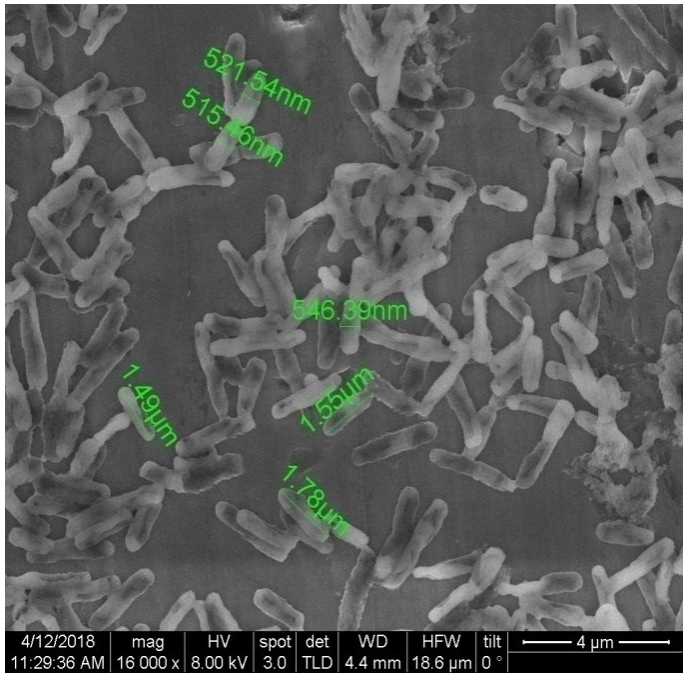


Figure 6: Scanning electron microscopic analysis of PMGs. (A) NaOH induced PMGs. Showing Presence of transmembrane tunnels (arrows), collapsed cell membrane and altered morphology(16000X); and (B) formalin inactivated *P.multocida* bacterial cells with intact stable cell morphology(16000X).

4.3.2. Characterization by dynamic light scattering

The average size of the PMGs in suspension was found to be 1.214µm (Figure 7) which falls in the normal bacteria (*Pm*) size(1µm to 2µm). So we can infer that, the integrity of the bacterial skeleton was preserved during the production of PMGs .

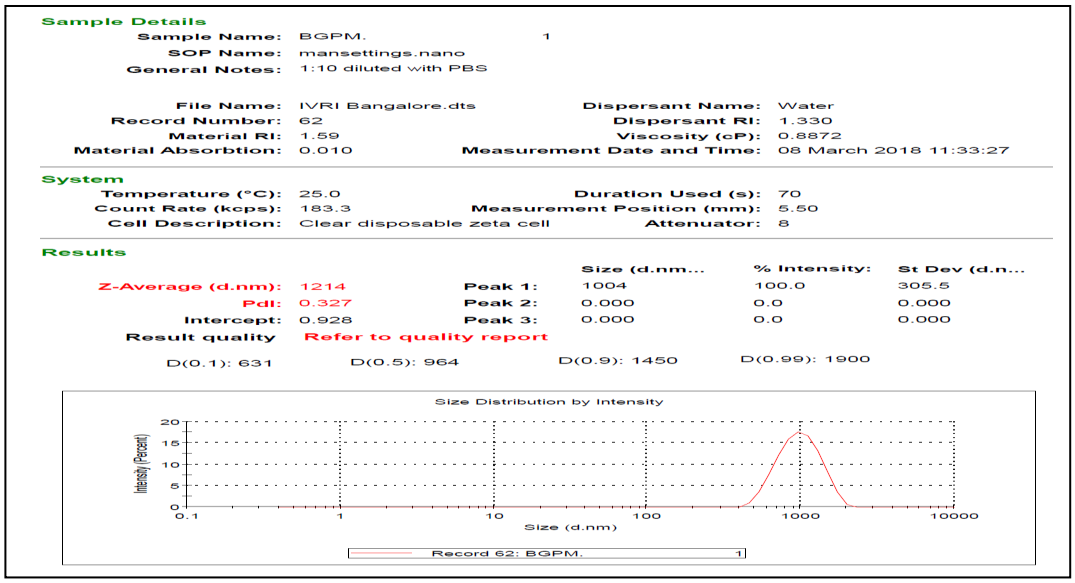


Figure 7: Dynamic light scattering of PMGs showing average size as 1.214 µm.

4.3.3. Determination of zeta potential of the PMGs suspension

The zeta potential of the PMGs suspension was found to be -15.6 mv (Figure 8), which shows that the suspension was less stable because the suspension is said to be stable when it possesses zeta potential above +30 or -30 mv (Muller *et al.,* 2000).

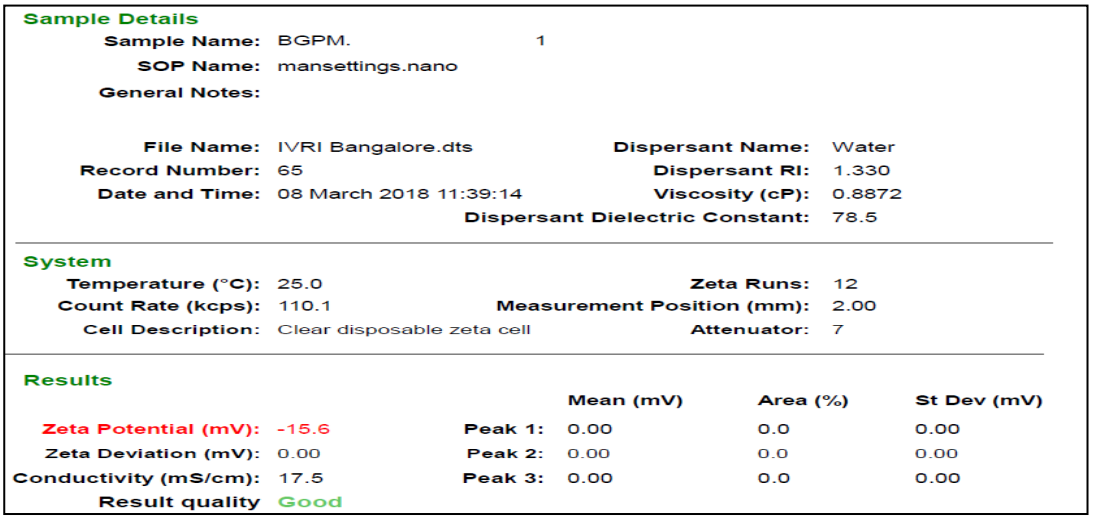


Figure 8: Zeta potential analysis of PMGs suspension

4.3.4. SDS- PAGE

A total of 9-14 different polypeptide bands were observed with approximate molecular weight ranging from 16-123 kDa (Kang et al., 2002) of the PMGs. On the basis of stain intensity, 32 kDa protein appeared to be the major protein band. Thus by comparing all the three lanes, it can be interpretable that the major protein composing 32 kDa protein is conserved in all the three samples *(Pasteurella multocida* P52 strain, formalin inactivated *Pasteurella multocida* P52 strain, BGs of *Pasteurella multocida* P52 strain)(Kang et al., 2002). So the major proteins are conserved while production of bacterial ghost without any substantial changes (Figure 9)

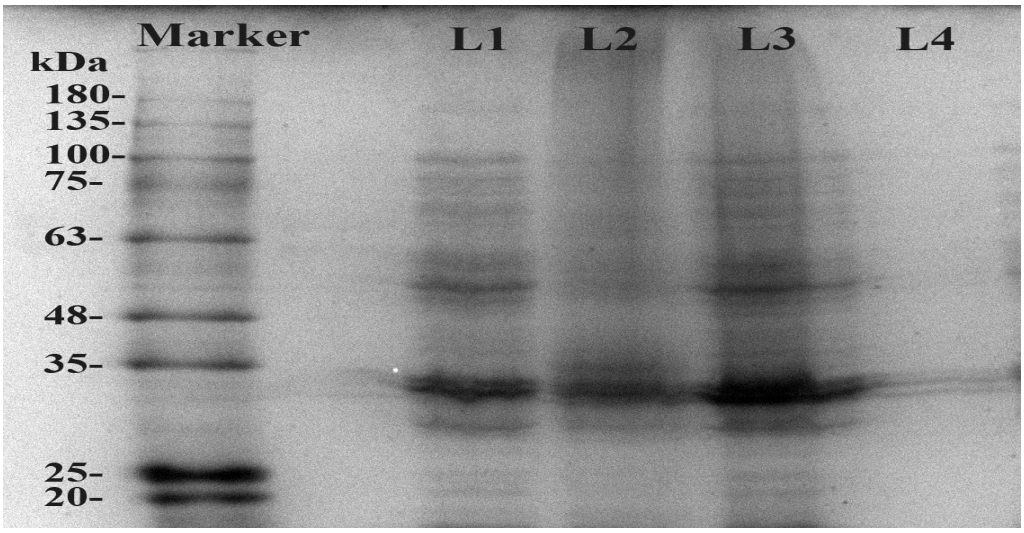


Figure 9: SDS-PAGE showing protein profiles of *Pasteurella multocida* P52 strain (lane 1), formalin inactivated *Pasteurella multocida* P52 strain (Lane 2), BGs of *Pasteurella multocida* P52 strain (Lane 3)

4.4. *Invitro* culture of MoDC

4.4.1. Isolation of peripheral blood mononuclear cells

With the help of Histopaque 1077 (Figure 10) the cattle peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation method. After the process of centrifugation blood showing PBMC layer at interphase. Variation between samples was reflected on the absolute numbers of cells in PBMC. For each experiment, we collected different volume of cattle blood for culturing MoDC and calculated the yield of monocytes and PBMC. On the average $4.26x10^6$ PBMC cells were separated from 1ml of blood.

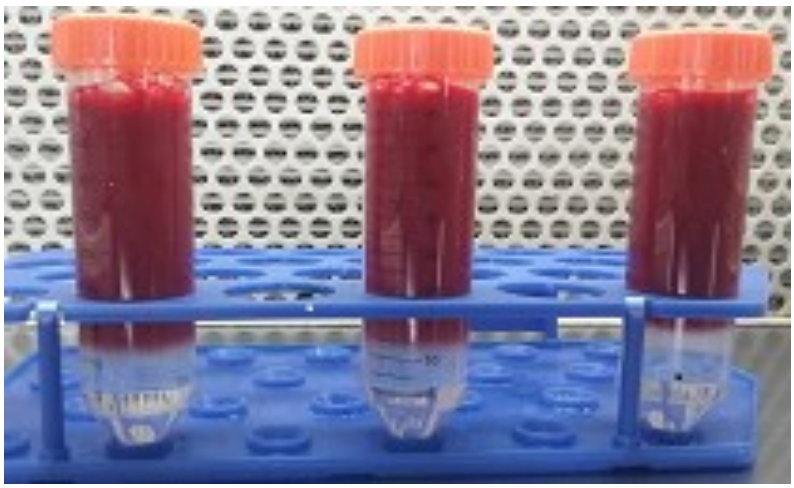


Figure 10: Histopaque 1077 and blood interphase for density gradient centrifugation method. histopaque and the PBS diluted blood (1:1) were added in the ratio of 1:3 for the density gradient centrifugation to collect PBMC

4.4.2. Thawing of freezed PBMC

After thawing the frozen PBMC which were stored in -80^{0}C, after one week the revival rate was found to be 93%.

4.4.3. Isolation of CD14$^+$ monocytes by MACS method

Monocytes were isolated by Magnetic Activating Cell sorting method (Fig 11) by using 2.5 µl antihuman CD14 microbeads supra paramagnetic particles, which has the ability to label 1×10^7 PBMC cells. PBMCs were adjusted to 1×10^7 per 2.5µl of antihuman CD14 paramagnetic beads which were incubated for 10 minutes at room temperature. For separating labelled cells, we used miniMACS- high gradient magnetic cell separator with miniMACS column, according to the manufacture instruction (MiltenyiBiotech). Labelled and positively enriched cells were eluted from miniMACS magnetic columns by removal of columns from the magnetic device. The isolated cells were counted by Neubauer's counting chamber. We had isolated pure population of monocytes by using MACS method from collection of different volume of blood.

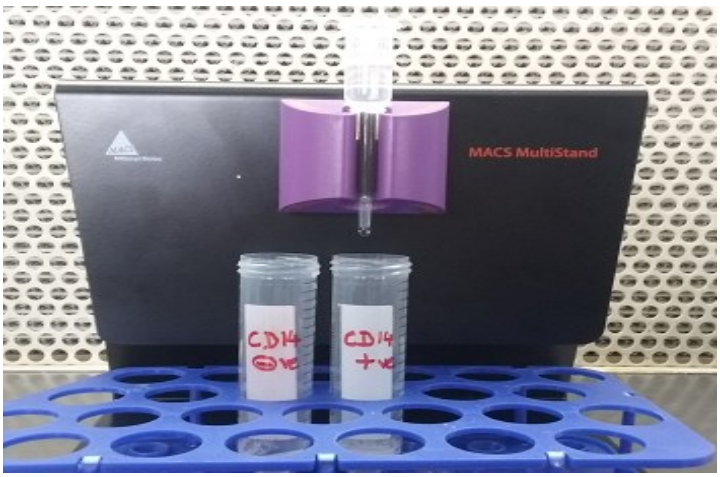


Figure 11: Magnetic activated cell sorter (MACS).

4.4.4. Confirmation of purity of $CD14^+$ monocytes

The $CD14^+$ monocyte cell fraction (cells collected from the magnetic column sorting) and the the $CD14^-$ cell fraction (the remaining cells of PBMC after sorting the CD^{14+} fraction) were separately stained with Mouse anti bovine CD 14 FITC and analysed by the flow cytometry. The purity of separation was found to be 92.48% (Figure 12) .

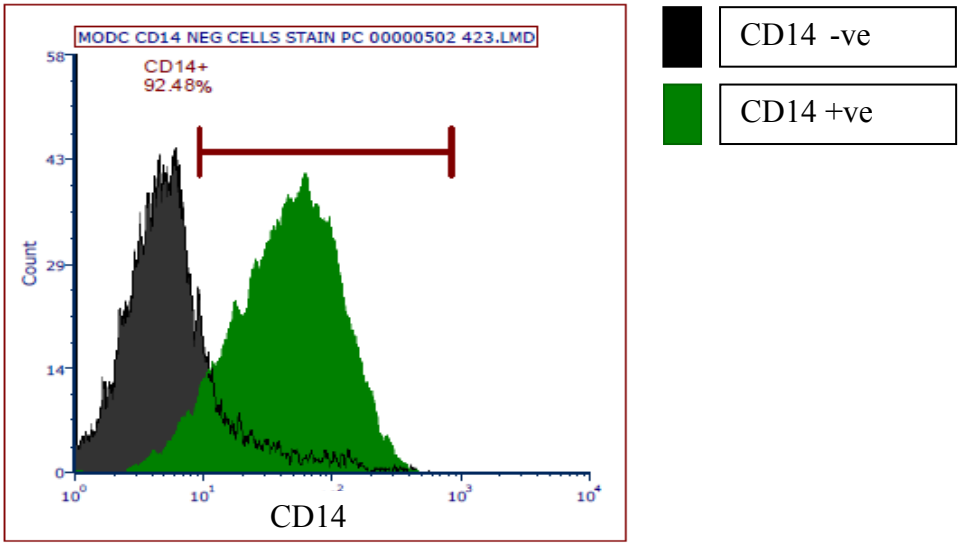


Figure 12: Histogram FCS express(area under the curve shaded by the green colour shows that, over 92.48% of the cells were CD14+ve

4.4.5. Generation of MoDC

Monocytes were isolated by MACS method and cultured in RPMI-1640 media

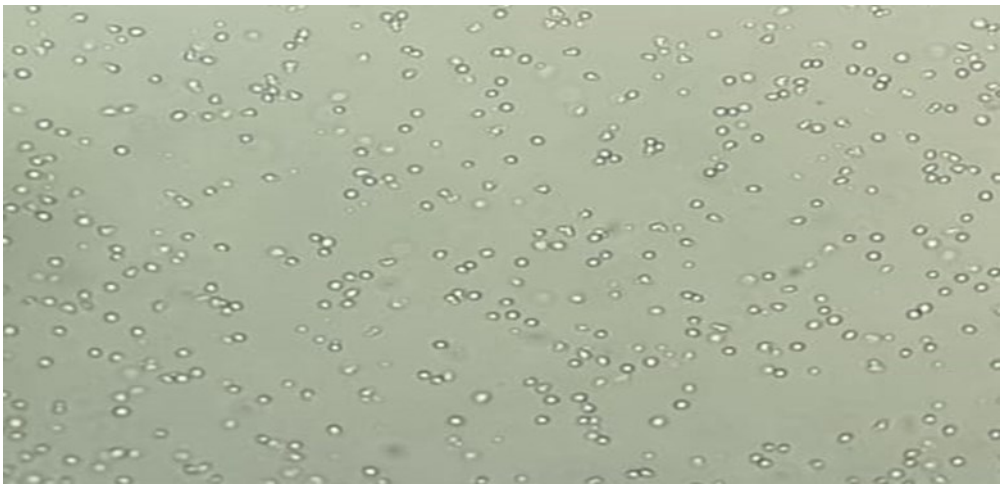


Figure 13: $CD14^+$ monocytes (transparent and floating cells in RPMI-1640 medium) on the first day of culture

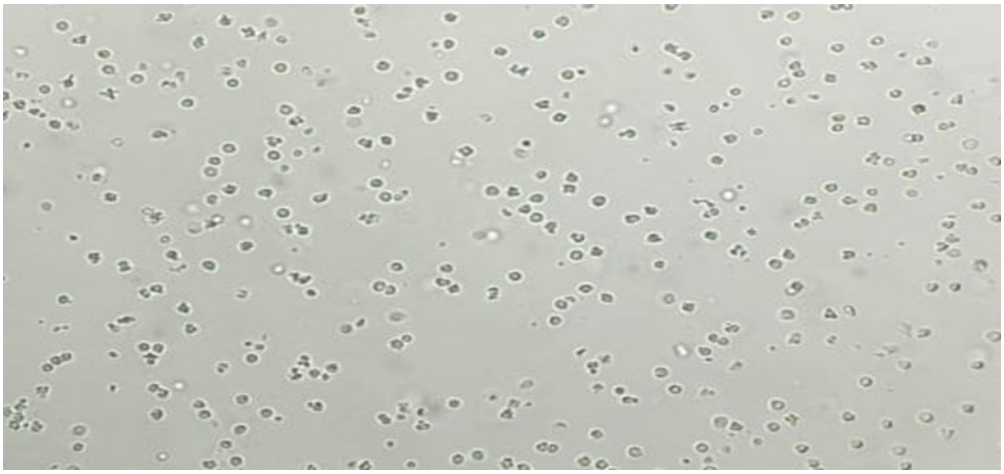


Figure 14: $CD14^+$ monocytes attached to plates surface on the 2^{nd} day of culture

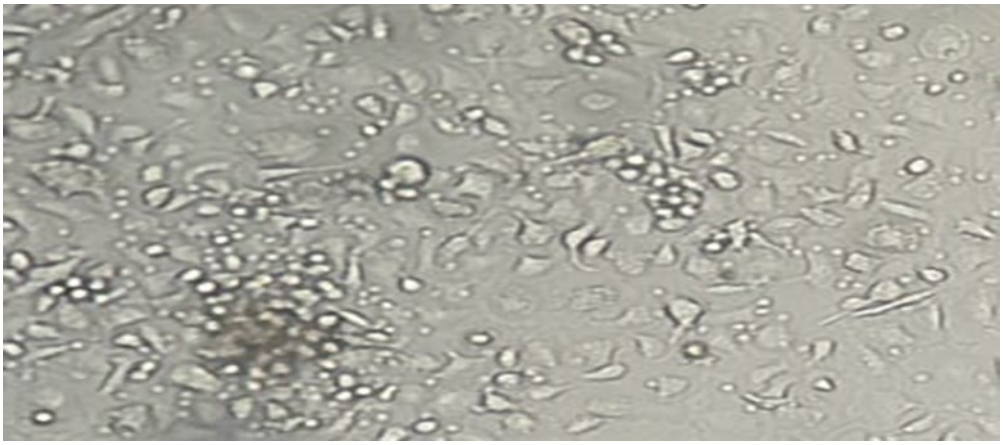


Figure 15: Immature petal shaped MoDC at the third day of culture

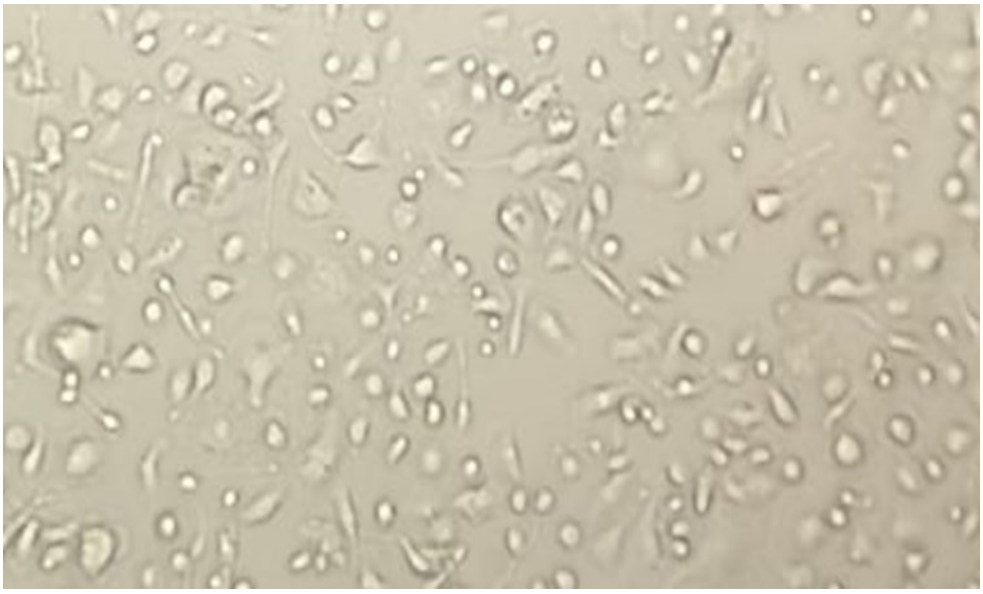


Figure 16: Immature MoDC at the 6th day of culture with veils elongated and dendritic processes

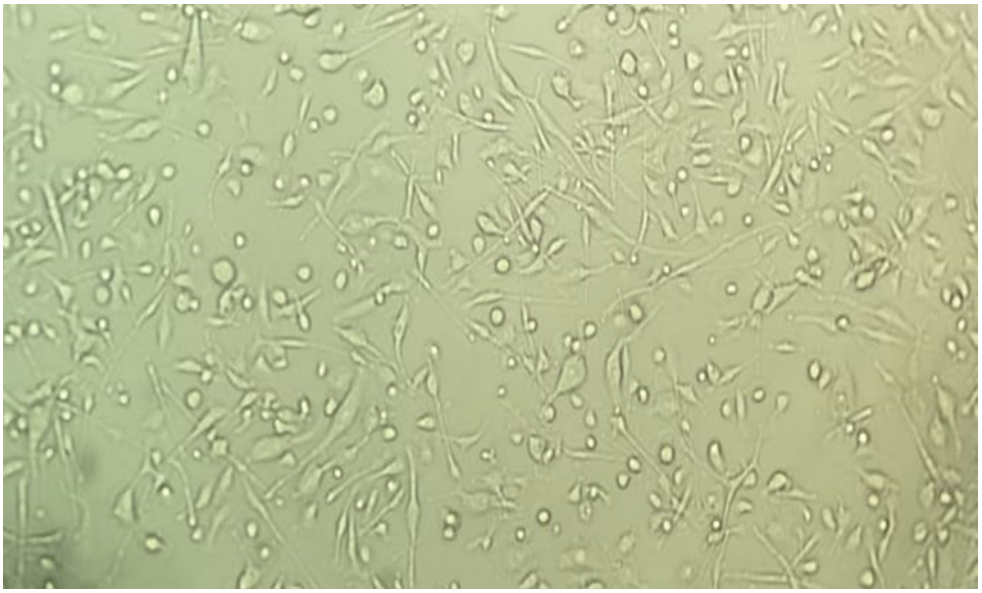


Figure 17: Mature dendritic cells after antigen exposure with well developed dendrites

along with recombinant cytokine GM-CSF and IL4 for 5-6 days. At day 0 pure monocytes were uniform in shape without contamination of other cells (Figure 13) . At day 2 cells were attached to the bottom surface of the plates (Figure 14). At day 3 round and petal shaped immature cells were developed (Figure 15), and at day 6, veils elongated cells and dendrites were developed (Figure 16). Dendritic cells were showing large cytoplasmic processes in day 7 (Figure 17) after pulsing with antigen on 6th day. Cells were loosely attached with the culture surface and collected and counted in counting chamber. The cells were phenotypically analysed using flow cytometry.

4.5. Cell surface costimulatory molecules analysis of MoDCs

Mouse anti bovine CD80-PE, CD86-PE and CD40-PE monoclonal antibodies were used for the analysis of costimulatory molecules CD80, CD86 and CD40 respectively of MoDC and monocytes using flow cytometry.

4.5.1. Phenotypic characterization of CD40 molecule

The CD40 costimulatory molecule expression on MoDC in control wells and antigen treated wells were analysed using flow cytometry. There was increased upregulation of CD40 costimulatory molecules on MoDC treated with FMDV+BGs (70.79%) as compared to the MoDC treated with FMDV (62.51%), BG alone (13.58%) and the control group (4.51%) which shows that the MoDC which were treated with the FMDV antigen along with adjuvant like PMGs helps in the enhanced upregulation of CD40 costimulatory molecules. (Figure 18 & 19)

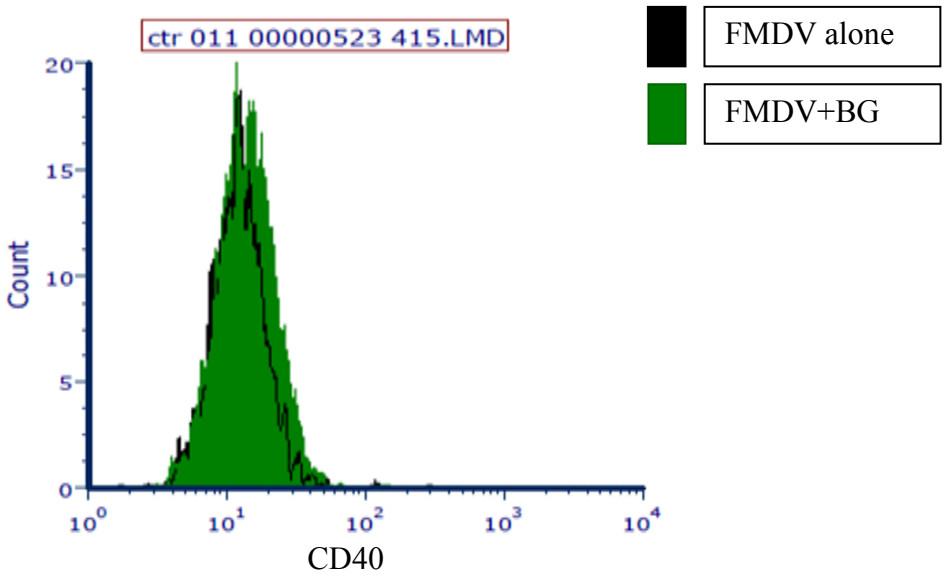


Figure 18: Histogram showing the upregulation of CD 40 molecules in FMDV+PMGs group

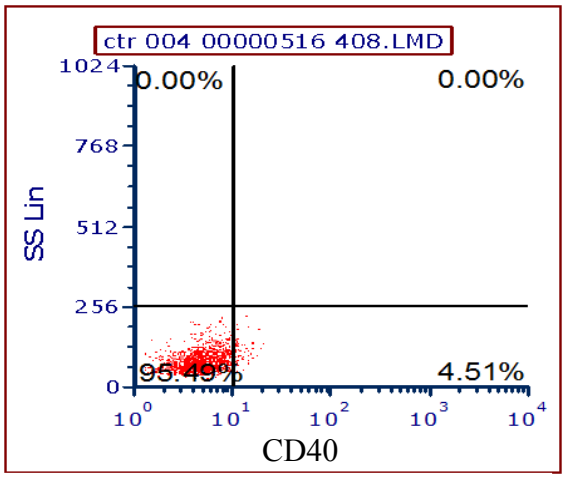


Figure 19 A : Dot-plot showing the expression of CD40 molecules in Control group

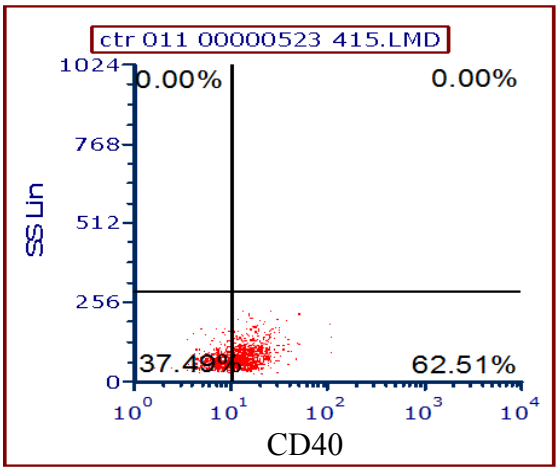


Figure 19 B : Dot-plot showing the expression of CD40 molecules in FMDV alone group.

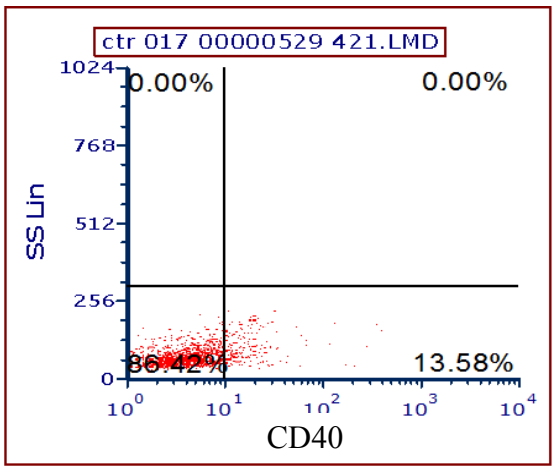


Figure 19 C : Dot-plot showing the expression of CD40 molecules in PMGs alone group.

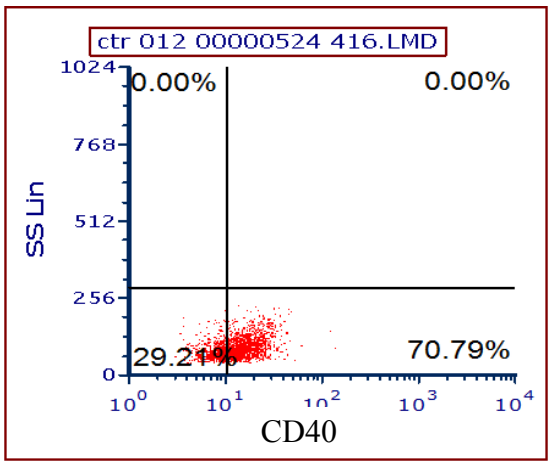


Figure 19 D : Dot-plot showing the expression of CD40 molecules in FMDV+PMGs group.

4.5.2. Phenotypic characterization of CD80 molecule

The CD80 costimulatory molecule expression on MoDC in control wells and antigen treated wells were analysed using flow cytometry. There was increased upregulation of CD80 costimulatory molecules on MoDC treated with FMDV+PMGs (95.26%) as compared to the MoDC treated with FMDV (61.65%), PMG alone (68.68%) and the control group (38.18%) which shows that the MoDC which were treated with the FMDV antigen along with adjuvant like PMGs helps in the enhanced upregulation of CD80 costimulatory molecules. (Figure 20&21).

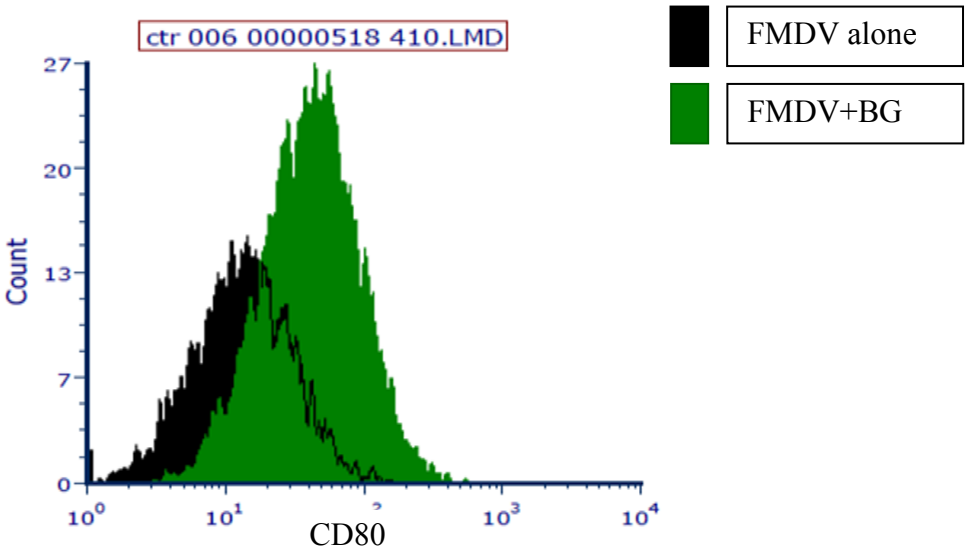


Figure 20: Histogram showing the upregulation of CD80 molecules in FMDV+PMGs group

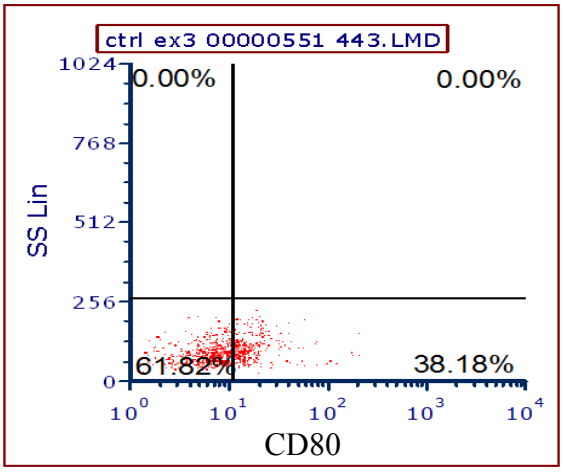


Figure 21 A : Dot-plot showing the expression of CD80 molecules in Control group

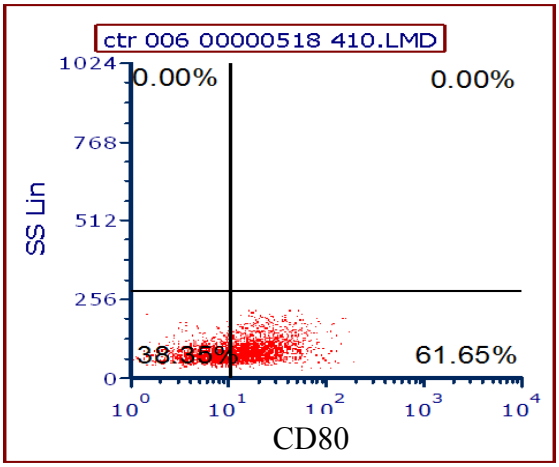


Figure 21 B : Dot-plot showing the expression of CD80 molecules in FMDV alone group.

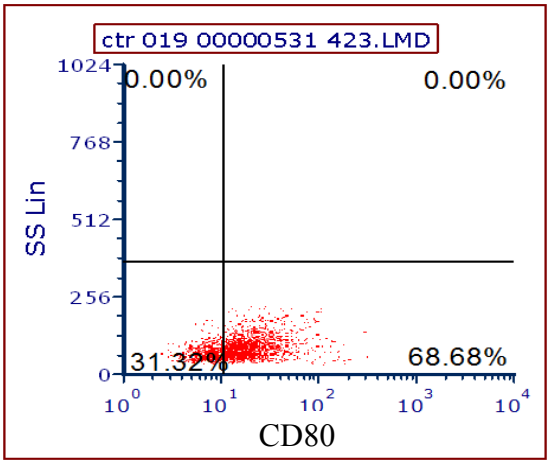


Figure 21 C : Dot-plot showing the expression of CD80 molecules in PMGs alone group.

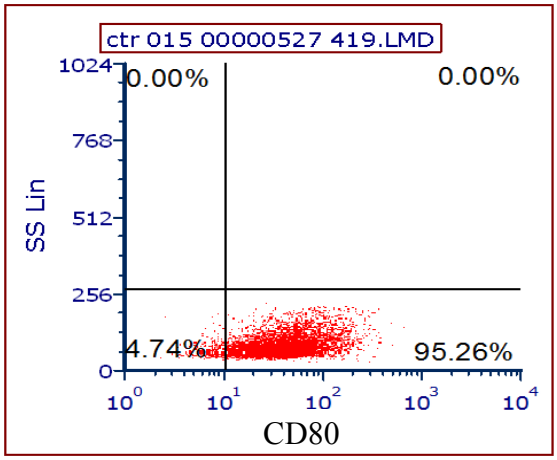


Figure 21 D : Dot-plot showing the expression of CD80 molecules in FMDV+PMGs group.

4.5.3. Phenotypic characterization of CD86 molecule

The CD86 costimulatory molecule expression on MoDC in control wells and antigen treated wells were analysed using flow cytometry. There was increased upregulation of CD86 costimulatory molecules on MoDC treated with FMDV+PMGs (95.90%) as compared to the MoDC treated with FMDV (45.11%), PMGs alone (61.41%) and the control group (25.21%) which shows that the MoDC which were treated with the FMDV antigen along with adjuvant like PMGs helps in the enhanced upregulation of CD80 costimulatory molecules. (Figure 22 & 23).

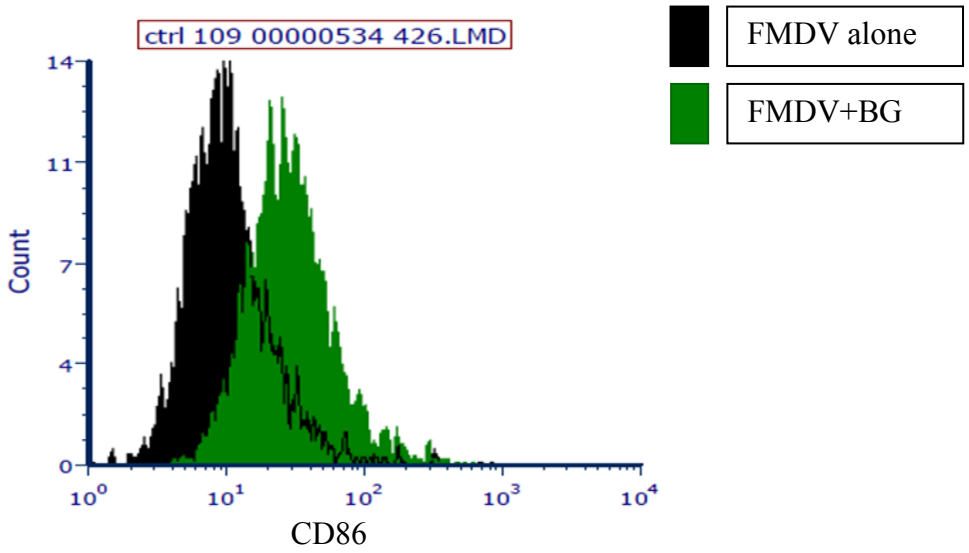


Figure 22: Histogram showing the upregulation of CD 86 molecules in FMD+PMGs group

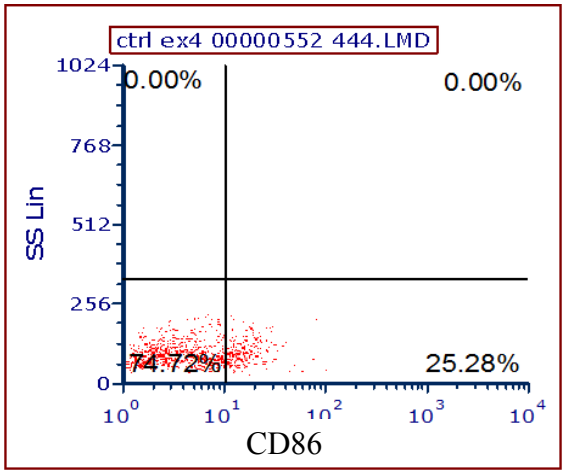


Figure 23 A : Dot-plot showing the expression of CD86 molecules in Control group

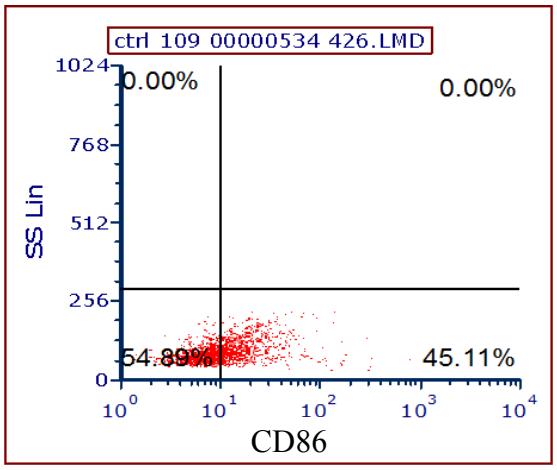


Figure 23 B : Dot-plot showing the expression of CD86 molecules in FMDV alone group.

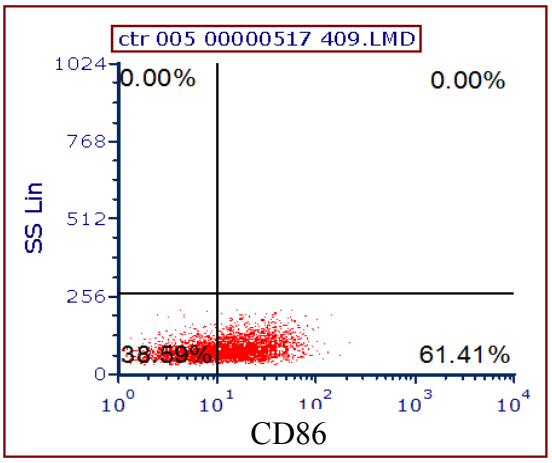


Figure 23 C : Dot-plot showing the expression of CD86 molecules in PMGs alone group.

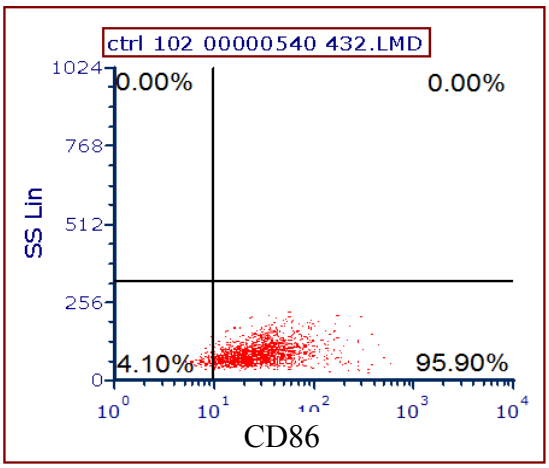
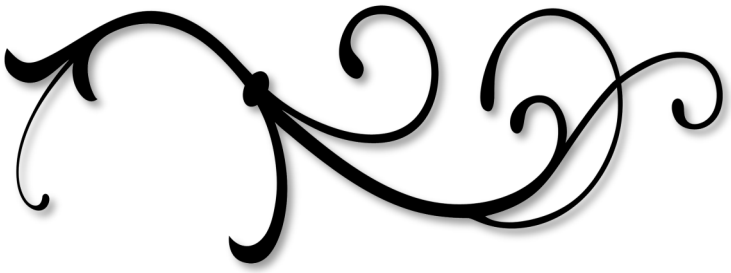


Figure 23 D : Dot-plot showing the expression of CD86 molecules in FMDV+PMGs group.

DISCUSSION



5 DISCUSSION

Dendritic cells play central role in production of innate and adaptive immune responses. Dendritic cells are cultured from its precursor cells, monocytes using GM-CSF and IL-4. *In vitro* generated model of cattle MoDCs are useful to study interaction with FMD virus and bacterial ghosts, the effects can be studied through the phenotypic changes or expression of surface molecule of bovine DCs. Exposure of DCs to microbial stimuli resulted in the generation of fully activated DC that able to sustain the differentiation of helper T cell responses (Sporri and Reis e Sousa, 2005) Activated or matured DCs, upregulation of the expression of the CC-chemokine receptor CCR7, which results in more DCs recruitment at the draining lymph nodes (Weninger and von Andrian, 2003).

5.1. Preparation of PMGs and its characterization

BGs are empty envelopes of Gram-negative bacteria produced by the controlled expression of lysis gene *E* of bacteriophage phiX174 (Jawale and Lee, 2014). The role of gene *E* in the lysis of Gram-negative bacteria, *Escherichia coli*, was for the first time reported by Hutchison and Sinsheimer (Hutchison and Sinsheimer, 1966); and subsequently this gene was identified by Pollock *et al*., (Pollok *et al.,* 1988); in heavily UV-irradiated *E. coli* cells. The BGs have also been prepared by the application of minimal concentrations of chemicals including NaOH, SDS and H_2O_2, resulting in the production of sponge like structures (Arnara *et al*., 2013); Recently, BGs of Gram-positive bacteria *Listeria monocytogenes* have been prepared using a chemical method (Wu *et al*., 2017); Suggesting that this method could be useful in future vaccine development against important Gram-positive food-borne pathogens.

In the present study, the PMGs were prepared by chemical method using NaOH (3.15mg/ml) . The observation on the prepared PMGs shows good stability at different time intervals, desirable size between 1-2µ (Izquierdo *et al*., 2001). Presence of Transmembrane tunnels on the outer cell membrane of the Ghosts, collapsed nature of cytoskeleton due to expulsion of the cytoplasm during NaOH treatment. The normal size of the Ghosts by DLS analysis indicates that the bacteria is able to maintain its overall integrity and morphology during the process of NaOH treatment and the cytoskeleton of the Ghosts is said to be intact. The SDS-PAGE analysis of outer membrane protein of the ghosts shows that a total of 9-14 different polypeptide bands were observed with approximate molecular weight ranging from 16-123 kDa (Kang et al., 2002). On the basis of stain intensity, 32 kDa protein appeared to be the major protein band. So it can be interpretable that the major protein composing 32 kDa protein is conserved in PMGs (Kang et al., 2002). So the major proteins are conserved while production of bacterial ghost without any substantial changes .They contain many innate immunostimulatory agonists, and are potent activators of a broad range of cell types involved in innate and adaptive immunity. Studies have depicted both adjuvant as well as their capacity to induce proinflammatory cytokine production properties of the BGs. Bacterial ghosts (BGs) provides a effective mechanism which not only acts as potent candidate vaccines but also acts as a tool for remarkable adjuvant and vaccine delivery systems. This novel approach has produced promising results to curb infectious diseases, tested both in natural hosts as well as in experimental animals. In addition, the foreign antigens have been loaded inside the cytoplasmic lumen or expressed both on the surface and in the periplasmic space of BGs (Huter *et al.*, 1999; Mayr *et al.,* 2005)

5.2. Generation of dendritic cells from monocytes

We had done the culturing of monocytes with IL-4 and GM-CSF at different concentration for 4-5 times to optimize the proper dose which will gives the better monocyte derived dendritic cells. From these studies we found that the 20ng/ml of IL- 4 (R&D System) and 5 µl GM-CSF (AbD Serotech) gives the better MoDCs from bovine peripheral blood mononuclear cells. The in vitro experiments were carried out in six well culture plates with combination of both the IL-4 and GM-CSF cytokines. We compared our work with many other works which has been done with dendritic

cells isolated from monocytes and we found that our culture growth of MoDCs is optimum and developed good dendrtic cells morphology. Several authors have used 10 ng

bovine IL-4 and 100 ng bovine rGM-CSF per ml of medium,which was showed to be optimum for generating bovine DCs. (Zhuang *et al.,* 2006) reported 200 ng bovine IL-4 and 100ng bovine rGM-CSF per ml of medium was optimum for generating bovine DCs. It was found that over the time, the cytokines levels were depleted, which resulted in sub optimum DCs generation. The half of the spent medium was removed every third day and placed with fresh medium containing same level of cytokine. Monocytes stimulated *in vitro* with cytokines acquired dendritic cells characteristic without any sign of proliferation. This fact reveals that major population of isolated monocytes is retaining their properties to acquire dendritic cells morphology when stimulated with optimum concentration of required cytokines. Pickl *et al*., reported that all the monocytes have the capacity to differentiate into DCs. In contrast to the above report our studies with purified blood monocytes, demonstrated that monocytes can be driven to acquire DCs characteristics. But it is difficult to observe all the monocytes differentiated into DCs. This differentiation capacity seems to be a feature of most but not all CDl4+ blood monocytes. According to (Romani *et al.,* 1996) significant proliferation was noticed when culturing monocytes from human PBMC with GM-CSF plus IL-4. Cell proliferation within the first week of *in vitro* culture of monocyte enriched PBMC fractions was mentioned in the report by Sallusto and Lanzavecchia (1994). Inspite of the above mentioned observation; in this study any signs of cell proliferation were not observed. It was found that cattle peripheral blood monocytes can be differentiated into DCs under *in vitro*. Monocytes were showed rounded uniform shape morphology and all monocytes were not converted into DCs, it depends on monocytes population on culture, it required good number of cells and adhesion capacity in plate that helps to cells on converting to dendritic cells.

5.3. Changes of cell morphology and growth during culture

Isolated population of peripheral blood monocytes in MACS method represented a homogenous population of uniform sized round cells without any contamination with other cells and artifacts. At day 3, cells were seen as spindle shaped cytoplasmic expansion mixed with population of monocytes after adding cytokines at day zero. Complete differentiation with a morphological appearance and cytoplasmic expansion required 5-6 days. The veiled surface (immature DCs) was very prominent and cellular morphology imposing dendritic cells. Large amount of dendritic cells can be obtained after *in vitro* exposure of peripheral blood monocytes to IL-4, GM-CSF, but the ability of monocytes to convert dendritic cells *in vivo* has not been so far established (Cella *et*

al;1999, Chapius *et al.,* 1997). The cells were remained loosely attached with culture surface (Mauel *et al*., 2006). After exposure of FMDV, PMGs, the DCs formed extensive cytoplasmic projections and were anastomosed with each other, along with some heterogeneous cells of various size. Size was bigger than parent monocyte cells. This study support the hypothesis that monocyte can be generated as DCs, it represent monocytes act as an immediate precursors of DCs

5.4. Surface marker characterization of dendritic cells

Dendritic cells were critical to increase immune response after stimulation with various antigen. In our study we had used FMDV antigen, and PMGs to stimulate immature MoDCs. It was observed that PMGs along with FMDV antigen increases higher immune response as compared to FMDV antigen, PMGs alone. Myloid derived and lymphoid dendritic cells lineages have been described (Reddy *et al.,* 1997) through their ability to determine the differentiations of either TH1 or TH2 cells distinct dendritic cell subset may present antigen *in vivo* in an immunogenic or tolerogenic fashion. It is important that the immunostimulatory properties of DCs are dependent on their maturity state; immature DCs have been shown to be capable of inducing antigen specific inhibition of *in vivo* T cells function in human (Dhodapkar *et al*., 2001)

In present study we had used monoclonal antibody against CD40, CD80, and CD86. The up-regulation of these molecules indicate the maturation of DCs and increasing co-stimulatory capacity of DCs. CD40,CD80 and CD86 are the basic co-stimulatory molecules of DCs which are only up-regulate after ingestion of antigen when immature DCs converted towards mature one. These molecules are base to check DCs maturation. Immature DCs have low level expression of co-stimulatory molecules (CD40, CD80, and CD86) and MHC molecules and are not capable of producing high level of pro-inflammatory cytokines like IL-2, TNFα, IL-1ß, IL-6 (Lei and Hostetter, 2007). Cool *et al.,* 2011, noted that fully differentiated, mature DCs are characterized by low levels of CD14 expression. Co-stimulatory molecules including CD80 and MHC antigen complex are up regulated during DC activation, and provide necessary signals to activate naïve lymphocyte and generate antigen specific responses. Most vaccine adjuvants are known to target innate signalling pathways that control the development of adaptive humoral and cellular immune responses, but the molecular mechanism of their action is still being characterized (Getz, 2005; Guy, 2007).

5.5. Phenotypic characterization of un-stimulated MoDCs

Unstimulated MoDCs were characterized by higher expression of CD14 molecule. In flow cytometric analysis we found low expression of CD40, CD80, and CD86 markers expression on immature dendritic cells. Immature DCs are characterised by intermediate surface expression of MHC-II and low levels of co-stimulatory molecules including, CD80, CD86 and CD40 (Cella *et al.,* 1999). We had found increase expression of CD40, CD80 CD86 molecule in unstimulated MoDC in cattle MoDCs, CD40 molecule was more expressed. Morphologically the levels of maturation were noticed in different MoDC cell population and some cells seem less mature. DCs identified by peculiar morphology and specific function such as ability to activate naïve T- cell (Sapp *et al*., 1999). In terms of their molecular characteristics and their morphologic features, the GM-CSF plus IL-4 cultured monocytes as they represent a highly heterogeneous population. On basis of these criteria we conclude that our GM-CSF plus IL-4-treated monocyte populations showing good differentiation of dendritic cells if population of monocytes is in optimum number.

5.6. Phenotypic characterization of PMGs stimulated MoDCs

After culturing of monocytes for five days with the major numbers of cells are already acquired DC morphology, MoDC were stimulated on sixth day with 40 PMGs/ MoDC and control cells were left untreated. During phenotypic analysis of surface markers with CD40-PE, CD80-PE, CD86-PE on the 4th day we found expression of these markers are increases among which CD80-PE are more prominent. Various co-stimulatory molecules and maturation of dendritic cells (DCs), are critical for the initiation of adaptive immunity. DCs maturation involves upregulation of costimulatory CD40, CD80, CD86, and MHC class II surface molecules and IL-12 cytokine expression (Reis e Sousa, 2004.). We had compared expressions of markers with control, inactivated FMDV stimulated MoDC and with PMGs + inactivated FMDV stimulated MoDC. We found that the expression of co-stimulatory molecule with PMGs was more as compared to control but lower as compared to PMGs+inactivated FMDV.

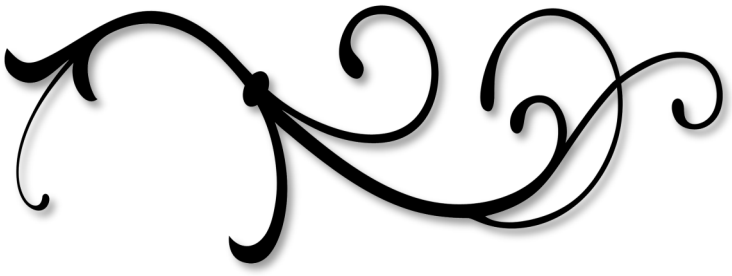
5.7. Phenotypic characterization of inactivated FMDV antigen stimulated MoDCs

Virus stimulated MoDCs showed more expression of CD80,CD86 and CD40 molecules as compared to control . Flow cytometric analysis revealed that the upregulation of surface markers CD80, and CD86 molecules in PMGs stimulated DCs as compared to the BEI inactivated FMDV stimulated DCs. But in case of CD40 molecule, the upregulation is found to be more in FMDV treated DC than PMGs treated DCs. Recent studies reported that, In UV-FMDV-loaded DCs, the expression of CD80, CD86 and MHC-II molecules were greatly up-regulated than mock-infected FMDV and DCs (Ostrowaski *et al.,* 2005).

5.8. Phenotypic characterization of PMGs and inactivated FMD virus stimulated MoDCs.

PMGs alone increases the surface markers expressions on dendritic cells like CD80, CD86, and CD40 as compared to control. Among them the expression of CD40 molecule is less as compared to other molecules. We found that the expression of CD40 molecule was less in PMGs treated DCs as compared to PMGs alone also. But the DCs treated with both PMGs and FMDV shows higher upregulation of all the three costimulatory molecules (CD80, CD86, and CD40) as compared to control DCs, PMGs treated DCs and FMDV treated DCs. This shows that the PMGs acted as adjuvants or stimulants of DCs when used along with the FMDV. As we and many other researchers showed that BGs when mixed with antigen, it enhances the immunogenicity of the antigen. Recent studies have documented that BGs can also be used as a mucosal adjuvant when mixed with antigens. In addition, the foreign antigens have been loaded inside the cytoplasmic lumen or expressed both on the surface and in the periplasmic space of BGs (Huter *et al*., 1999; Mayr *et al*., 2005). These remarkable properties make BG an attractive tool for vaccine development and antigen delivery system for both humans and animals. The presence of LPS in the BGs does not limit its use as an adjuvant or candidate vaccine due to minimal toxicity as compared to free LPS (Mader *et al*., 1997). Considering the particulate nature of BG and the they possesses many well-known TLR agonists, BGs have the capacity to be effectively identifiable by APC and to further elicit potent immune responses against their own envelope structures or ghost-delivered foreign antigens (Jawale *et al.,* 2014). With this consideration, BGs have been successfully used as adjuvants and a delivery system for a number of viral and bacterial antigens.

SUMMARY AND CONCLUSIONS



6 SUMMARY AND CONCLUSIONS

Bovine dendritic cells (DCs) are a heterogeneous population of cells that play a critical role in initiation and progression of the innate and adaptive immune response. Extensive knowledge of the phenotype and function of DC has been derived from mouse and human studies. In cattle, the role of DCs has been investigated by assessing the function of immature DCs *in vitro*. Now generation of bovine MoDC from monocyte precursors isolated from peripheral blood to assess their function is developed. MoDCs are currently the preferred *in vitro* study system for dendritic cells. They allow the investigation of the impact of FMDV maturation stimuli on dendritic cell system. We established MoDCs cultures platform in routine and simplified method and assessed MoDC maturation and expression of surface markers of antigen stimulated MoDC and the ability of these MoDC to take up antigen and induce phenotypic changes. The maturation markers CD40 and CD80, CD86 expression was measured in flow cytometry. MoDC stimulated with FMDV are functionally active and able to induce surface markers. Flow cytometry studies applied for the expression of antigen presenting and co-stimulatory molecules and finally to study the FMDV antigen

Specific to PMGs adjuvanted vaccine. Overall conclusions are

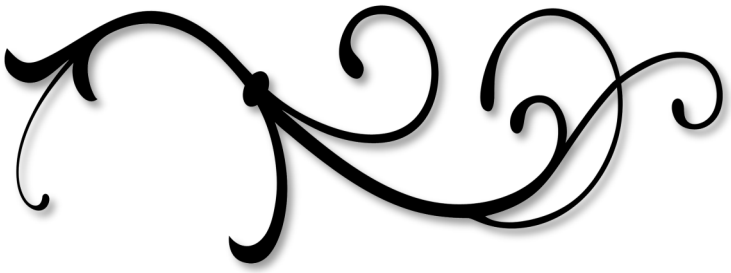
- Density gradient purification of bovine PBMCs and purification of $CD14^+$ Monocytes achieved.
- PMGs, FMDV antigen and PMGs with FMDV antigen interaction to MoDCs study to know if any increased FMDV antigen presentation and surface co-stimulatory molecules in cultures.
- Perfected Culture of $CD14^+$ cells using GM-CSF and IL4, differentiation into MoDC and identify the maturation by cell surface staining of MoDC.

- PMGs+FMDV and PMGs alone are giving approximately three time higher up regulation of CD80 and CD86 as compare to control.
- CD40 marker in MoDCs treated with PMGs+FMDV giving lesser up regulation as compared to other molecules
- CD40 marker upregulation also lower in the DC treated with PMGs alone as compared to other molecules
- On the basis of this study we can say that PMGs may activates either CD80 or CD86 signalling and CD80 activation which is sufficient for the B cell – dependent IgG response but not for Th1 cell immunity.

The present work can be taken up as model to study dendritic cell platforms in bovines, which can be applied to investigations of infection, vaccination, and therapeutic intervention in cattle and thus inform the rational design of vaccines for bovine pathogens including FMDV

- Further, antibody titre for the humoral response of BGs adjuvant FMDV vaccines can be assessed using ELISA
- The use of TLR agonist as vaccine adjuvants have shown promising results in animal models (Steinhagen *et al.,* 2011)
- Protection against parent bacteria can be assessed by chemically produced ghosts vaccines with animal challenge experimental studies

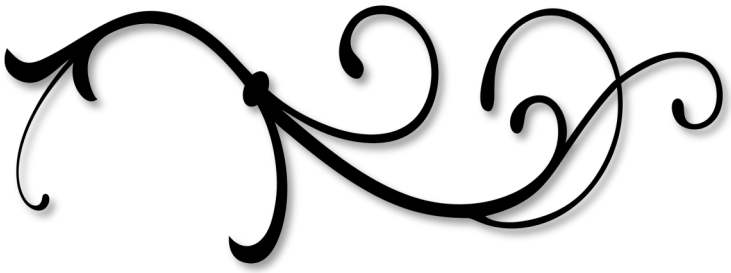
MINI ABSTRACT



7 MINI ABSTRACT

Foot and mouth disease is an acute, febrile, and contagious vesicular disease affecting cloven-hoofed animals. Elicitation of immune response not only depends on molecular properties of the antigen or on the immunogenic susceptibility of host but also on the formulation of the antigen. In our study we have used Bacterial Ghost of *Pasteurella multocida* P52 strain as adjuvants which were produced by chemical treatment using NaOH (3.125mg/ml) by determining the Minimum inhibitory concentration (MIC). BGs used along with inactivated Foot-and mouth disease virus antigen (serotype A) to study the upregulation of co-stimulatory molecules namely CD80, CD86, CD40 in *in vitro* cultured MoDC. We have also characterized the ghosts for their structural morphology (trans membrane tunnel, collapsed cell membrane), size(reduced diameter of ghost as compared to parent bacteria) and integrity and protein profile (outer membrane protein) using scanning electron microscopy(SEM), dynamic light scattering (DLS) and SDS-PAGE respectively. The study of DC function has been restricted by their less abundance in blood.This study was targeted to generate dendritic cells from $CD14^{+}$ monocytes under the influence of IL-4 and GM-CSF and compare surface costimulatory marker expression between immature and mature DC. The results showed that generating monocyte-derived dendritic cells required 5-6 days to complete differentiation of monocyte with a typical DC morphological character. In our study FMDV antigen along with ghosts of *Pasteurella multocida* stimulated MoDCs and showed enhanced up-regulation of CD40 (8.28%), CD80 (33.61%) and CD86 (50.79%) molecules as compared to FMDV antigen alone stimulated cells which were analysed by flow cytometry. So from above results we hypothesised that Upregulation of costimulatory molecules further helps in the efficient maturation of DC, induction of Th1/Th2 cytokines, increased capacity of T cells to proliferate and also helps in better humoral response against the antigen.

HINDI ABSTRACT



8 लघु-सार

खुरपका एवं मुंहपका रोग (FMD), एक तीव्र, febrile और संक्रामक vesicular रोग है जोकि जुडवां-खुर वाले पशुओं (cloven-hoofed) जानवरों को प्रभावित करता है। प्रतिरक्षा प्रतिक्रिया का आकर्षण, न केवल एंटीजन के आणविक गुणों या मेजबान की इम्यूनोजेनिक संवेदनशीलता पर, बल्कि एंटीजन के निर्माण पर भी निर्भर करता है। प्रस्तुत अध्ययन में, हमने न्यूनतम अवरोधक एकाग्रता (MIC) निर्धारित करके, NaOH (3.125 मिलीग्राम / मिली) का उपयोग करके, रासायनिक उपचार विधि द्वारा उत्पादित किए गए *Pasteurella multocida (Pm)* P52 तनाव के Bacterial Ghost (BG) का उपयोग किया है। ज्ञातव्य है कि (BG) का उपयोग निष्क्रिय एफएमडी वायरस एंटीजन (सीरोटाइप ए) के साथ किया जाता है, ताकि CD80, CD86, CD40 इनवेक्ट्रो सुसंस्कृत MoDC में, कम लागत वाले अणुओं के अपग्रेड का अध्ययन किया जा सके। तत्पश्चात, स्कैनिंग इलेक्ट्रॉन माइक्रोस्कोपी विधि (SEM) और dynamic light scattering(DLS) एवं SDS-PAGE विधियों द्वारा, गतिशीलता का उपयोग करके, (BG) को उनके संरचनात्मक रूपरेखा (ट्रांसमेम्ब्रेन सुरंग, ध्वस्त कोशिका झिल्ली), आकार (पैरेंट बैक्टीरिया की तुलना में ghost का कम व्यास करके) और अखंडता एवं प्रोटीन प्रोफाइल (बाहरी झिल्ली प्रोटीन) के लिए (BG) की विशेषताओं प्रदर्शित किया गया ।

DC कार्यों का अध्ययन करने के लिए, रक्त में उनकी कम प्रचुरता से प्रतिबंधित किया गया। प्रस्तुत अध्ययन में, CD14+ monocytes के प्रभाव से, डेंडराइटिक कोशिकाओं (DC) को उत्पन्न करना लक्ष्य बनाया गया तथा अपरिपक्व एवं परिपक्व DC के बीच, सतह परिसंचरण मार्कर अभिव्यक्ति की तुलना की गई। तुलनात्मक

परिणामों से यह पाया गया कि मोनोसाइट-व्युत्पन्न डेंडराइटिक कोशिकाओं (DC) को एक सामान्य DC मॉर्फोलॉजिकल चरित्रांकन के साथ, मोनोसाइट के भेदभाव को पूरा करने के लिए, 5-6 दिनों की आवश्यकता पडती है। प्रस्तुत अध्ययन में, एफएमडीवी एंटीजन ने *Pasteurella multocida* के ghosts के साथ MoDCs को उत्तेजित करते हुए पाया गया और एफएमडीवी एंटीजन अकेले उत्तेजित कोशिकाओं की तुलना में, CD40 (8.28%), CD80 (33.61%) and CD86 (50.79%) अणुओं का बढ़ता हुआ अप-विनियमन (upregulation) पाया गया। Cytometry Costimulatory अणुओं का यह अपग्रेडेशन्, DC की कुशल परिपक्वता में मदद करता है। साथ ही, Th1 / Th2 साइटोकाइन्स को शामिल करने के लिए, T-कोशिकाओं की क्षमता बढाने के लिए और एंटीजन के विरुद्ध, बेहतर विनम्र प्रतिक्रिया में भी मदद करता है।

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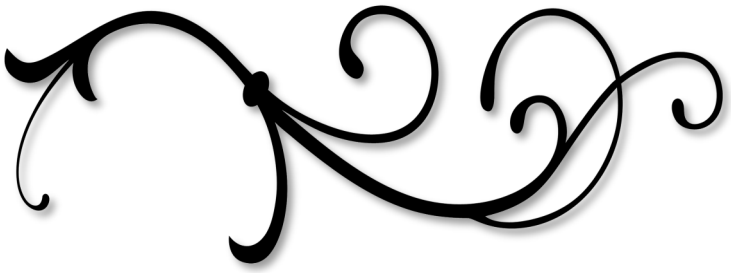

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10 APPENDIX

1) Media and Buffers for Cell Culture and Virus Preparation

1.1. MODIFIED EAGLE'S MEDIUM (GLASGOW MODIFICATION)

Ingredients required preparing 1000 ml of the medium.

NaCl	6.40 g
KCl	0.40 g
CaCl2.2H2O	0.26 g
MgSO4 .7H2O	0.20 g
NaH2PO4.2H2O	0.14 g
Dextrose	4.50 g
Ferric Nitrate	0.0001 g
L. Glutamine	0.292 g
Penicillin	10, 000 I.U
Streptomycin	0.10 g
Phenol red 1%	1.50 g
Distilled water	500 ml
NaHCO3	2.75 g
Amino acid stock solution	50.00 g
Vitamin stock solution	4.00 ml
Distilled water added up to	1000 ml

The ingredients were dissolved in order and CO2 was passed through the solution to adjust pH in between 7.2 to 7.4. The medium was sterilized by filtrating through Seitz-EKS pads and stored at 4°C.

1.2. STOCK SOLUTION OF AMINO ACIDS (1000 ML)

L. Arginine	0.84 g
L. Cystine	0.48 g
L. Histidine	0.38 g
L. Isoleucine	1g
L. Leucine	1.048 g
L. Lysine	1.462 g
L. Phenylalanine	1.600 g
L. Threonine	0.952 g
L. Tryptophan	0.160 g
L. Valine	0.936 g
Phenol red 1%	0.04 ml
L. Tyrosine	0.936 g

Dissolved separately in 25 ml of N/1 NaOH solution. The ingredients were dissolved by heating to 56°C and distributed in 250 ml screw capped bottles and stored at –20°C.

1.3. STOCK SOLUTION OF VITAMINS (1000ML)

Choline chloride	500 mg
Folic acid	500 mg
Nicotinamide	500 mg
Pantothenic acid	500 mg
Riboflavin	500 mg
Inisitol 350 mg	
Pyridoxal HCl	500 mg

The ingredients were dissolved properly and distributed and stored at –20°C.

1.4. RPMI-1640 MEDIUM

(With L-Glutamine, without Glucose and sodium bicarbonate, product number R1383)

Preparation as per the instructions given by the manufacturer.

1.5. TRYPTOSE PHOSPHATE BROTH (TBP): (PH 7.3)

Tryptose	20 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium Phosphate	2.5 g

Suspended 29.5 g in 1000 ml distilled water. Boil to dissolve the medium completely. Sterilized by autoclaving at 15 Psi for 15 minutes.

1.6. TRYPSIN VERSENE GLUCOSE: (TVG)

Trypsin (Difco; 1:250)	2 g
Distilled water to make	100 ml

The trypsin was dissolved by agitating the solution at 4°C for 15 minutes and then filtered through Seitz KES pads and stored at 4°C.

Versene (AR)	0.2 g
Distilled water to make	100 ml

The solution was sterilized by autoclaving at 10 lb pressure for 10 min and stored at 4°C.

Glucose (AR)	10 g
Distilled water to make	100 ml

The solution was sterilized by autoclaving at 10 lb pressure for 10 minutes and stored at 4°C.

1.7. TRYPSIN VERSENE GLUCOSE (TVG)

PBS	840 ml
0.2% Versene	100 ml
2% Trypsin	50 ml
10% Glucose	5 ml
1% Phenol red	1 ml

Mix the above aseptically in laminar flow, put for bacteriological sterility test. Stored at 4°C till further

1.8. NORMAL SALINE SOLUTION (NSS)

Sodium chloride	8.5 g
Distilled water	1000 ml

1.9. 1X PHOSPHATE BUFFERED SALINE (PBS) (0.15 M, PH 7.2)

Sodium chloride	8.0 g
$Na_2HPO_4.2H2O$	1.44 g
Potassium chloride	0.2 g
KH2PO4	0.2 g

Distilled water to make 100 ml.

1.10. TRYPAN BLUE SOLUTION (0.4%)

Trypan blue	0.4 g
Phosphate buffered saline (pH 7.2)	100 ml

Filtered and stored at 4°C.

1.11. GROWTH MEDIUM

EMEM	900ml
TPB	100ml
Fetal Calf Serum	50ml

1.12. MAINTENANCE MEDIUM

EMEM	950ml
TPB	50ml
Fetal Calf Serum	20ml

1.13. CESIUM CHLORIDE SOLUTIONS FOR ULTRACENTRIFUGATION

- **1.38g/ml density solution**

CsC l2	5.095g

Tris Nacl (pH 7.6) made up to 10.0 ml ,

- **1.42g/ml density solution**

CsCl2	5.678g

Tris Nacl, pH 7.6 (made up to) 10.0 ml

1.14. Tris–NaCl Buffer (pH 7.6)

Trizma HCl	1.212 g
Trizma Base	0.278g
NaCl	4.384 g
Distilled water up to	500 ml

1.15. TE Buffer

Tris HCl (pH 8.0)	10mM
EDTA (pH 8.0)	1mM

Use molecular grade water. Sterilized by autoclaving for 20 minutes at 15 psi (1.05 kg/cm2) on liquid cycle. Stored at 4˚C.

1.16. MACS BUFFER

1x PBS (pH 7.2)	100ml
Bovine serum albumin	0.5g
EDTA	2mM

1.17. FACS BUFFER

1x PBS (pH 7.2)	100ml
Bovine serum albumin	0.5g
EDTA	2mM

1.18. BUFFER FOR RECONSTITUTING IL-4

1x PBS (pH 7.4)	100ml
Bovine serum albumin	0.1g

1.19. NON ENZYMATIC CELL DISSOCIATION SOLUTION

1x PBS (pH-7.2)	100ml
EDTA	2mM
EDTA	0.5M
EDTA	18.6g
Distilled water	100ml

EDTA added in distilled water. The solution pH adjusted to 8.0 to completely dissolve the EDTA.

2. SOLUTIONS AND BUFFERS FOR SDS-PAGE

5X Tris-glycine electrophoresis buffer

25 mMTris base	15.1 g
250 mM glycine	94 g
SDS	5.0 g
DDW	upto 1000 ml

Acrylamide Solution (30% w/v)

Acrylamide	29.2 g
N, N'-methylene bisacrylamide	0.8 g
DDW	up to 100 ml

The solution filtered through a nitrocellulose filter(0.45 μm pore size). Filtered solution was stored in dark bottles at 4°C.

Ammonium Persulfate (10% w/v)

Ammonium Persulfate	1.0 g
DDW	up to 10 ml

Filter the solution through a nitrocellulose filter (0.45µm pore size) and store the filtered solution in dark bottles at room temperature. Prepare just before use.

SDS(10% w/v)

SDS	10 g
DDW	up to 100 ml

Heated up to 68°C and stirred with a magnetic stirrer to assist solubilization. [Do not autoclave]

1.5M Tris-Buffer(pH8.8)

Tris base	18.17 g
DDW	up to 100 ml
Adjust pH to 8.8 with HCl	

0.5 M Tris-Buffer (pH6.8)

Tris base	6.05 g
DDW	up to 100 ml
Adjust pH to 6.8 with HCl	

Resolving gel (12%), 9 ml

DDW	2.964 ml
30% Acrylamide Bis-Acrylamide	3.6 ml
1.5M TrisHCl (pH 8.8)	2.25 ml
10% SDS	90µl
10% Ammonium Persulfate	90µl
TEMED	6.0 µl

Stacking gel (5%), 3 ml

DDW	2.0 ml
30% Acrylamide Bis-Acrylamide	0.51 ml
0.5 M TrisHCl (pH 6.8)	0.375 ml

10%SDS	30 µl
10% Ammonium Persulfate	30µl
TEMED	3.0 µl

Glycerol (10% v/v)

Glycerol	1ml
DDW	9 ml

Sterilize the solution by passing it through a prerinsed 0.22µm filter. Store at 4 ^{0}C.

3X SDS-PAGE sample buffer

0.5 M Tris-HCl (pH 6.8)	240 µl
β -Mercaptoethanol	l20 µl
10%(w/v)SDS	1.2 ml
Bromophenol blue	40 µl
20%(v/v)glycerol	2.4 ml
DDW	up to 10 ml

Note:Store 3X SDS gel loading buffer lacking β-mercaptoethanol at room temperature. Add β-mercaptoethanol from stock just before the buffer is used.

Coomassie Staining Solution

Coomassie Brilliant Blue R-250	0.1 g
Methanol	45 ml
Glacial acetic acid	10 ml
DDW	up to 100 ml

The solution is filtered through WhatmanNo.1 filter paper to remove any particulate matter. The staining solution stored in the dark bottles at room temperature.

Destaining solution

Methanol	45 ml
Acetic acid	10 ml
DDW	up to 100 ml

VITAE

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