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PGEX2-T based β defensin primer designing and isolation of neutrophil, macrophage, bone marrow and mesenchymal stem cells

Jehangir Shah Syed Bukari [1], Mohd. Nadeem Shah Syed Bukhari [2], Mukhtar Ahmad Wani [3] and Promod Kumar Gautam [1,*]

[1] *Department of Biochemistry, All India Institute of Medical Science, New Delhi, India.*
[2] *TEMAG Lab, Istanbul Technical University, Istanbul, Turkey.*
[3] *Department of Chemistry, Govt. Degree College, Handwara, Jammu and Kashmir, India.*

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Abstract

Defensins are antimicrobial peptides that act mainly by disrupting the structure of bacterial cell membranes and are found in many compartments of the body. Defensins play a central role in defense against pathogens and they are considered part of the innate immune response.

Material and methods-Reagents: Animal cell culture media - Growth medium D-MEM, RPMI and alpha -MEM was purchased from gibco, CA, USA. Fetal bovine serum (FBS)was obtained from Invitrogen, CA, USA. DAPI, agarose was purchased from SRL, India. Penicillium streptomycin solution was obtained from himedia, Mumbai, India.

Animal modal: In bred populations of Swiss albino mice of either sex and at 6-8 weeks of age were used. Mice obtained from the Animal House, All India Institute of Medical Science, NewDelhi-110029 and India Standardization, Isolation and purification and characterization of bone marrow from Swiss albino mice. Bone marrow is consisting of multiple type of stem cells such as -Mesenchymal stem cells (MSCs) which are multipotent stem cells that have the potential to self-renew and differentiate into a variety of specialized cell types such as osteoblasts, chondrocytes, adipocytes, and neurons.

Result: Cells were harvested by adherence purification, the adherence of cells which is a symbol of macrophage and acentric nucleus is visible in DAPI stain. The extended cytoplasmic surface and CD14 positive staining show the purified macrophage as 98% purity. BM-MSC were harvested by adherence purification, the adherence of cells to the plastic culture dish surface is a symbol of Mesenchymal stem cell and nucleusis visible in DAPI stain.

Conclusion: This procedure is widely used to study macrophage biology since moderate numbers of resident, unstimulated macrophages can easily be obtained fromthe peritonealcavity.

Keywords: Bone Marrow; Swiss Albino; Mesenchymal stem; DAPI stains

1. Introduction

The discovery of human beta defensins has led to the recognition of the defense mechanism of mucosal surfaces to protect the host from potentially pathogenic organisms. In addition to their antimicrobial activity, the expression of beta defensins in various tissues and cell types has been linked with chemotaxis and innate immune signaling adaptive immunity, and carcinogenesis.[1] Defensins is a small amino acid cysteine rich cationic protein. They are found in both

*Corresponding author: Promod Kumar Gautam

vertebrates and invertebrates. They have also been reported in plants. defensins are active against bacteria, fungi and many enveloped and non-enveloped viruses.[2] The term Defensins was coined in 1985 after granule rich sediments were purified from human and rabbit neutrophils.[3] Defensins playarolein cell division, attraction and maturation of immune cells, differentiation and reorganization of epithelial tissues, wound healing and tumor suppression.

β-defensins are produced by phagocytic cells, leukocytes and epithelial cells within the gastrointestinal tract, liver, skin and lungs and constitute an important and versatile component of the innate immune system. Originally described as anti-microbial peptides (AMPs) because of their microbicidal activity, additional immuno regulatory and stimulatory functions has led to the term host defense peptide. It is now known that β- defensins link the innate and adaptive immune responses in higher organisms, by acting as signaling molecules in the immune system and chemo attractants for T-lymphocytes and immature dendritic cells more recently a role in fertility has been also demonstrated for these pleiotropic molecules.[4,5] It first became apparent in the early 2000s that beta defensins may play a part in the regulation of carcinogenesis when several studies reported altered expression of beta defensins in cancers. It was shown that many renal cell carcinomas, prostate cancers, basal cell carcinomas and oral squamous cell carcinomas either lacked or minimally expressed hBD1 protein.[6]

The evolutionary relationship between vertebrate and non-vertebrate defensins is still not clear, however phylogeny indicates that a primordial β-defensin is the common ancestor of all vertebrate defensins and this gene family expanded throughout vertebrate evolution. Beta defensin genes are present on adjacent locion chromosom e8p22-p23. The organization of this cluster is consistent with a model of multiple rounds of duplication and divergence under positive selection from a common ancestral gene that produced a cluster of diversified paralogous.[7] The most studied function for beta defensin is their direct antimicrobial activity, through permeabilization of the pathogen membrane, their exact mechanism of action is incompletely understood, and two different models have been proposed.[8] It first became apparent in the early 2000s that beta defensins may play a part in the regulation of carcinogenesis when several studies reported altered expression of beta defensins in cancers. It was shown that many renal cell carcinomas, prostate cancers, basal cell carcinomas and oral squamous cell carcinomas either lacked or minimally expressed hBD1 protein.[9] There seems to be a link between chronic infection and carcinogenesis, as epidemiological studies showed an association between inflammatory markers (CCL2, IL-6, IL-8,) and tumor formation. In this context, interactions of the innate and adaptive immune system seem to play important role, especially macrophages and natural killer cells.[10]

Beta defensins appear to play specific roles in regulating tumor growth and metastasis by manipulating the tumor microenvironment to favor tumor development in oral carcinomas, acting astumor suppressor genes or exhibiting direct cytotoxic activity towards cancer cells. In addition, beta defensins link innate immune responses to adaptive immune responses and may activate anti-tumor immunity. All of these observations suggest that dysregulation of beta defensins may be associated with tumor development.[11]

Tissue regeneration is closely associated with wound healing beta defensins exhibit various stimulatory effects on the proliferation and maturation of different cell types. These observations indicate that the surgical treatment of extensive lesions could also benefit from a better understanding of AMPs. The application of beta defensins could be useful to assist wound healing and epithelial reorganization. By local applications, these antimicrobial peptides may be used as a first step in fighting bacterial and viral infections and then, secondly, individually designed defensins could be applied to accelerate wound healing in microbially-traumatized lesions. The treatment of extensive lesions in modern surgery involves delicate local flap and microvascular procedures.[12]

A role for defensins in pro-inflammatory responses and more recently immunosuppression has been delineated over the last two decades. An initial observation was that β-defensins can recruit immature dendritic cells and memory T cells to sites of infection or inflammation providing a link between the innate and adaptive arms of the immune system. A mechanism for this was provided by Oppenheim's group where they demonstrated that natural and recombinant hBD2 could chemo attract human immature dendritic cells and memory T cells in vitro in a dose-dependent manner. This response was inhibited with the GαI inhibitor pertussis toxin and suggested the possible involvement of a chemokine receptor (s) which was confirmed using anti CCR6 blocking antibodies.

TH 17 cells express CCR6 and respond to β-defensins chemo attractant action. Furthermore, TH17cytokines (i.e., IL-17 and IL-22) induce expression of defensins from relevant cell types including primary keratinocytes potentially resulting in an amplification of TH17 levels have been reported in different auto immune diseases, such as multiple sclerosis, rheumatoid arthritis, and psoriasis, implicating β-defensin expression in autoimmunity.[13]

2. Material and methods

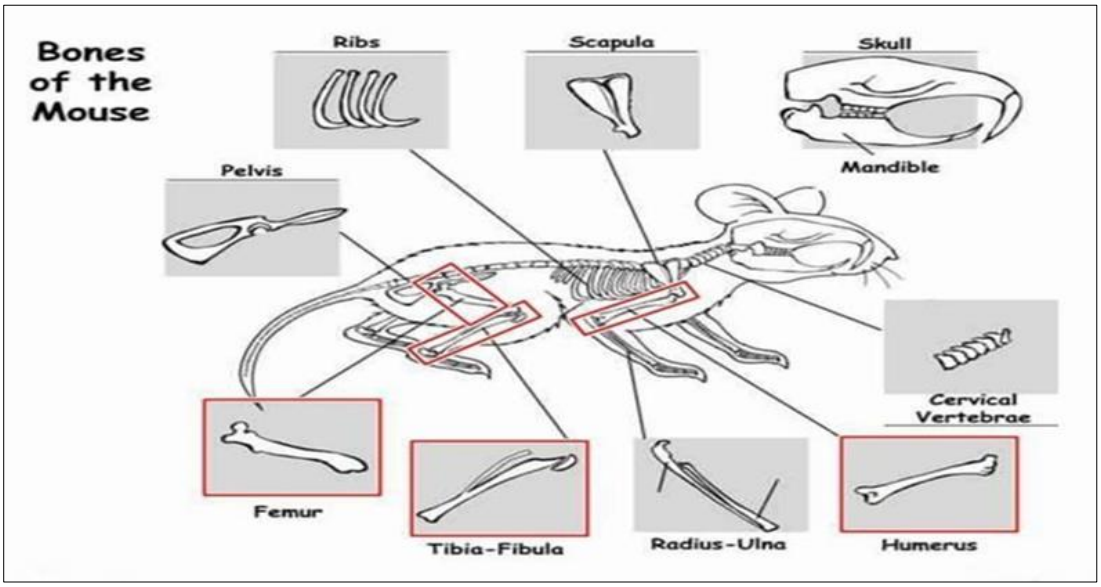
2.1. Reagents

Animal cell culture media - Growth medium D-MEM, RPMI and alpha -MEM was purchased from gibco, CA, USA. Fetal bovine serum (FBS) was obtained from Invitrogen, CA, USA. DAPI, agarose was purchased from SRL, India. Penicillium streptomycin solution was obtained from himedia, Mumbai, India. CD44 conjugated with PE from ebiosciences, san diego, CA, USA. Formaldehyde, Trypsin, and was obtained from Invitrogen CA, USA. GranuloSepTM GSM 1119(media) and LSM1077(media) were obtained from himedia, Mumbai, India All other chemicals otherwise stated were of Molecular biology grade and were from SRL, India. Breast cancer cell line MDAMB 231 was purchased from ATCC.

2.2. Animal modal

Inbred populations of Swiss albino mice of either sex and at 6-8 weeks of age were used. Mice obtained from the Animal House, All India Institute of Medical Science, New Delhi- 110029, India, and housed in a small animal facility, under pathogen-free specialized aseptic conditions with a 12-hour dark-light cycle. All the animals had free access to food, water and treated with the most care. For experiment purpose Mice were euthanized by cervical dislocation, a method authorized by Animal Ethical Committee, All India Institute of Medical Science, New Delhi, India, and Animals Ethical Committee, Indian Council of Medical Research(ICMR), New Delhi for the killing of experimental animals, and observed until all muscle activity and breathing has ceased for at least 120seconds. The ethic clearance has been taken from the Animal Ethical Committee, All India Institute of Medical Science with fileno-100/IAEC-1/2018 and 40 mice has been issue. Included, mouse dissection procedure, Isolation and purification, Immuno fluorescence Characterization, Standardization, Isolation and purification and characterization of bone marrow from Swiss albino mice.

Characterization of bone marrow was done by Microscopy slide and imaging



Complete α-MEM media: Complete α-MEM medium is MEM medium stock supplemented with10% fetal bovine serum (FBS) and 1% PSN. Phosphate-buffered saline (PBS): NaCl (8.0 g), KCl(0.2 g), KH2PO4(0.24 g), and Na2HPO4(1.44 g) in 1 L Mille-Q water (pH 7.4). 6- 8 weeks miceSwiss albino. Scalpel, blades, Mousedissection kit etc

Figure 1 Mouse bones dissected for bone marrow isolation hind limb long bones- Femur, Tibia-fibula and upper limb humerus

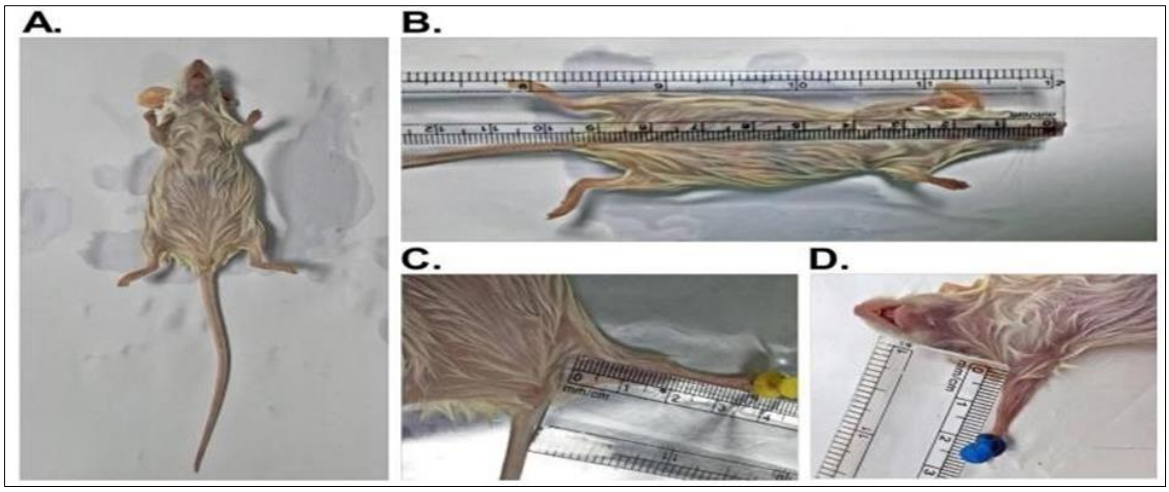


Figure 2 (a)Swiss albino mouse measurements; (B)Height of mouse 9.5cm;(C)Hindlimbs long bones-Femur,Tibia-fibula length 4cm;(D)Upper limb length 2cm

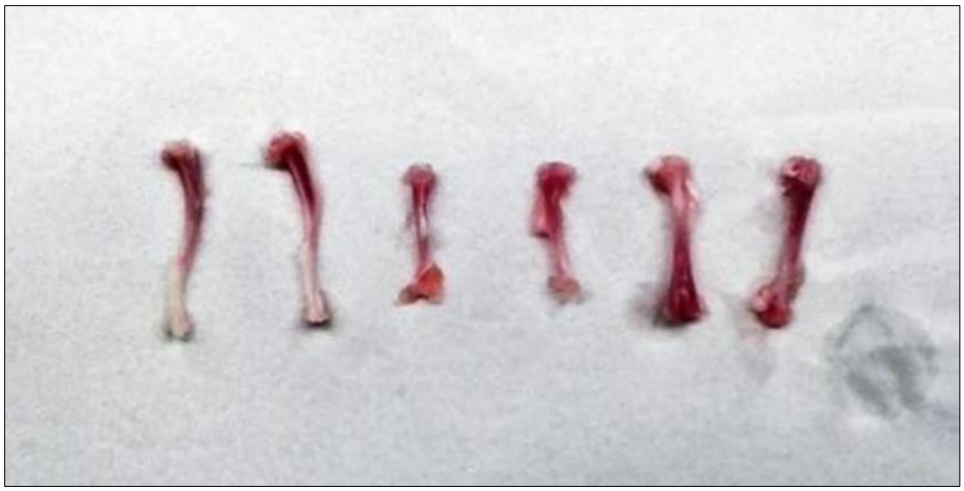


Figure 3 Tibias, femurs and humeri bones dissected for bone marrow isolationIsolation andpurification andcharacterization of Mesenchymalstem cell (CD44+)from Swiss albino mice, Isolation and Purification of Mesenchymal stem cell, Immunofluorescence Characterization of Mesenchymal stem cell, Isolation and purification and characterization of PBMC neutrophil from Swiss albino mice and Isolation and Purification of PBMC neutrophil

2.3. Reaction mixture

Table 1 cDNA synthesis reaction composition

S.no	Components	Volume added per reaction
1	5X I Script reaction mixture	4µl
2	I Script reverse transcriptase	1µl
3	Nucleus free water	11.7µl
4	RNA template	1µgor 3.3µl
	Total reaction volume	20µl

2.4. Reaction conditions

- Priming at 25°C for 5 minutes.
- Polymerization by reverse transcriptase at 46°C for 20 minutes.
- Reverse transcriptase inactivation at 95°C for 1 minute

Table 2 Features of PGEX2 plasmid

Type	Description
MCS	Multiple cloning site
Selectable marker	Ampicillin resistance gene
Bacterial origin	pBR322-type ColE1 origin of replication
Protease cleavage site	Thrombin cleavage site-leu Valpro argCUT glyser
Promoter	Ptac promoter
Tag	Glutathione S-transferase ORF
Repressor protein gene	lacI[q] lacoperon repressor protein ORF

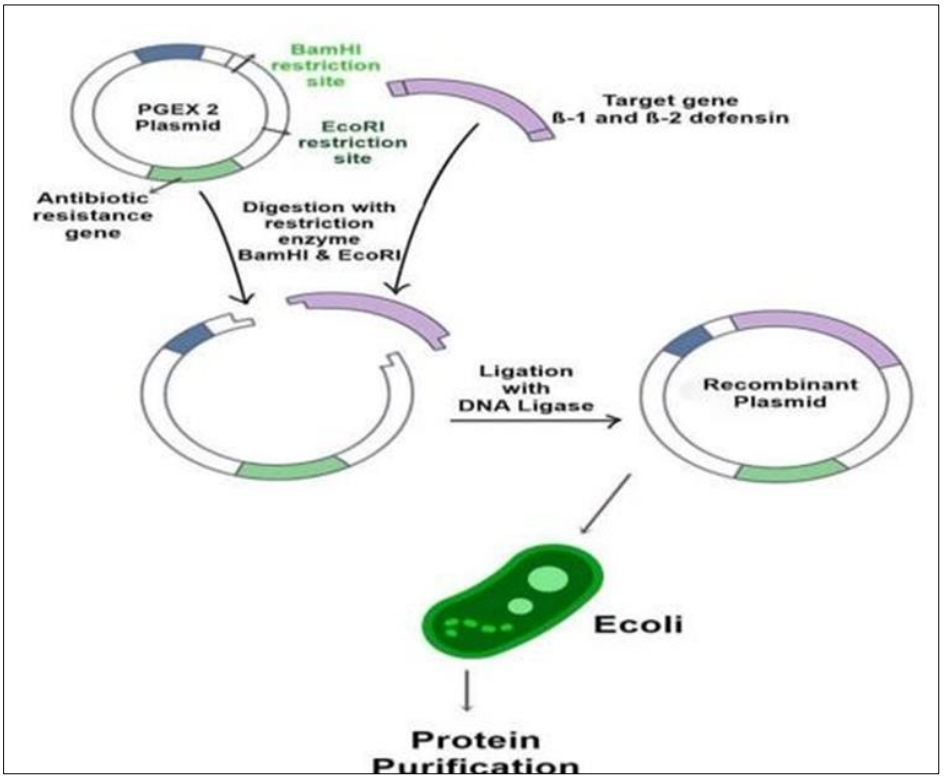


Figure 4 Diagram showing beta defensin gene cloning strategy using PGEX 2 plasmid

2.5. Primer designing

A primer is a short synthetic oligonucleotide which is used in many molecular techniques from PCRto DNA sequencing. These primers are designed to have a sequence which is the reverse complement of a region of template or target DNA to which we wish the primer to anneal or the target region to be amplified. β 1 and 2 defensin protein primers were designed using coding sequence of Mus musculus defensin beta1(Defb1)- NCBI reference sequence: NM_007843.3 and Mus musculus defensin beta 1(Defb2)-NCBIreference sequence: NM_010030.1 and synthesized by GCC biotech(India). The forward primer ofdefensin beta 1(Defb1): 5'- GCGAATTCATGAAAACTCATTACTT-3', sequence containing a restrictionsite for Eco RI (underlined) and codons of at the 5' end with additional guanine and cytosine. The reverseprimer: 5'-CGGGATCCTAGTCAGCTCGTTGGGC-3', sequence containing a restriction site for Bam HI(underlined)

at the 5 ends with additional guanine and cytosine. The forward primer of defensin beta1(Defb2): 5'-GCGAATTCATGAGGACTCTCTGCTC-3', sequence containing arestriction site for EcoRI (underlined) and codons of at the 5' end with additional guanine and cytosine. The reverse primer: 5'-CGGGATCCTAGTCATTTCATGTGCT-3', sequence containing a restriction site for Bam HI (underlined) at the 5'end with additional guanine and cytosine. Expression of β1 and 2 defensin protein is under process using expression vector PGEX2

Table 3 The primer sequence of Beta defensin 1 and 2 with restriction site, length GC content and melting temperature

S.no	Name	Sequence(5'-3')	Length	%GC	Tm(ºC)
1.	Beta defensin1(Defb1) Forward primer	GCGAATTCATGAAAACTCATTACTT	25	32.0	51.0
2.	Beta defensin1(Defb1)Reverse primer	CGGGATCCTAGTCAGCTCGTTGGGC	25	64.0	64.0
3.	Beta defensin 2(Defb2) Forward Primer	GCGAATTCATGAGGACTCTCTGCTC	25	52.0	52.0
4.	Beta defensin 2(Defb2)Reverse Primer	CGGGATCCTAGTCATTTCATGTGCT	25	48.0	58.0

3. Results

3.1. Characterization of Macrophage cells

Total 4 ml fluid out of 5 ml was recovered from the mouse peritoneal cavity. During the 2hrincubation time, the peritoneal macrophages adhere to the plastic surface and the floating non-macrophages discarded. After scraping adhere macrophages cell, 3.2x107resident peritoneal cells were recovered. Cell morphology in gray scale 3D DIC, DAPI merged shows the round morphology of macrophages Figure 5. Cells were harvested by adherence purification, the adherence of cells which is a symbol of macrophage and acentric nucleus is visible in DAPI stain. The extended cytoplasmic surface and CD14 positive staining show the purifiedmacrophage has 98% purity.

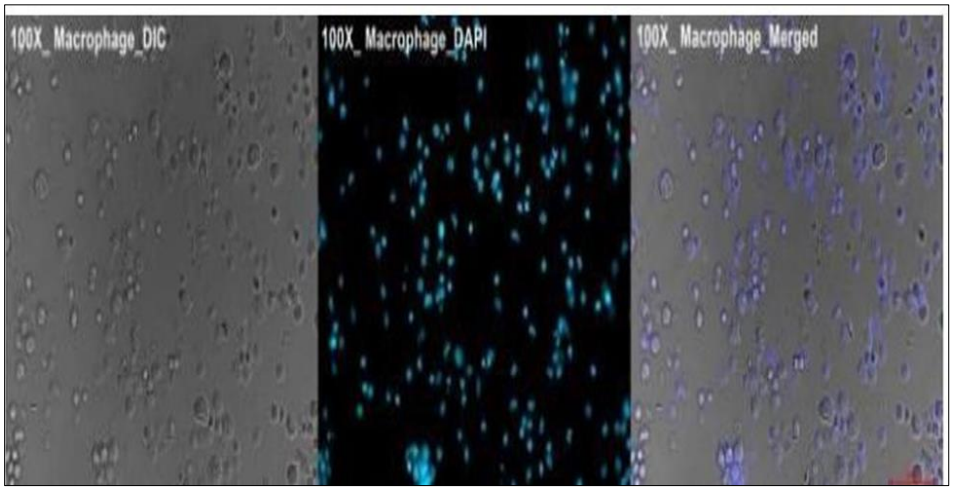


Figure 5 Fluorescent microscopic images of macrophage cell

3.2. Characterization of Bone marrow cells

Due to gravitational force the Bone Marrow got collected in the 1.5 ml MCT from the hole in the bottom of 0.5 ml MCT during the centrifugation. Total 5x107 bone marrow cell were isolated. Cell morphology in gray scale 3D DIC, DAPI merged showing cytoskeleton extensions morphology of BM-MSC.

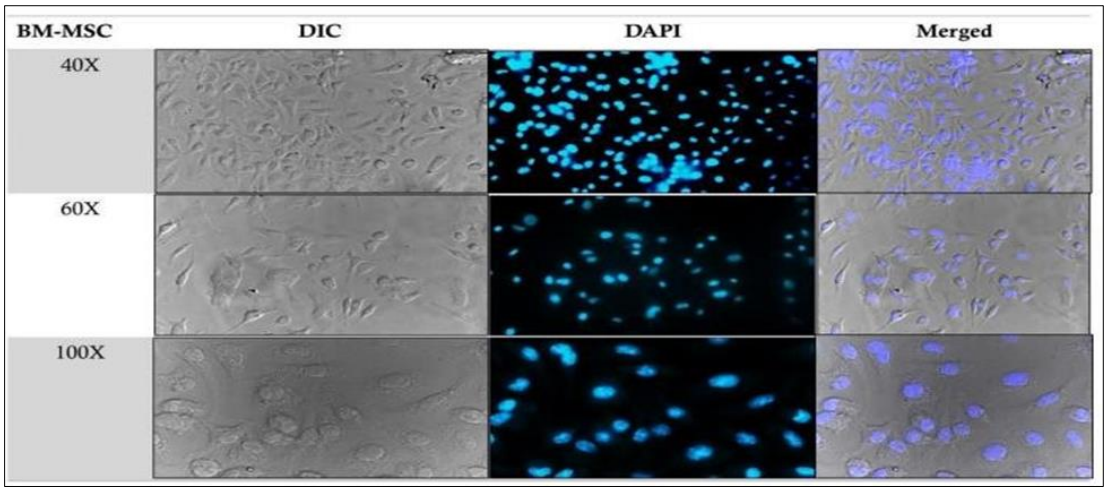


Figure 6 Cell morphology in gray scale 3D DIC, DAPI merged showing cytoskeleton extensions morphology of BM-MSC

3.3. Characterization of Mesenchymal stem cell (CD44+):

The initial spindle-shaped cytoskeleton extensions appear on Day 3/72 hours in phase-contrast microscopy, and culture becomes more confluent and reaches 70–90% confluence within only 3 days. Cell morphology in grayscale 3DDIC, DAP Imerged showing cytoskeleton extensions morphology of BM—MSC and staining with cell surface specific marker CD44+ confirms the cells to be Mesenchymal stem cell Figure7 BM—MSC were harvested by adherence purification, the adherence of cells to the plastic culture dish surface is a symbol of Mesenchymal stem cell and nucleusis visible in DAPI stain. The extended cytoplasmic surface and CD44+ staining show the adhere cells to be BM—MSC.

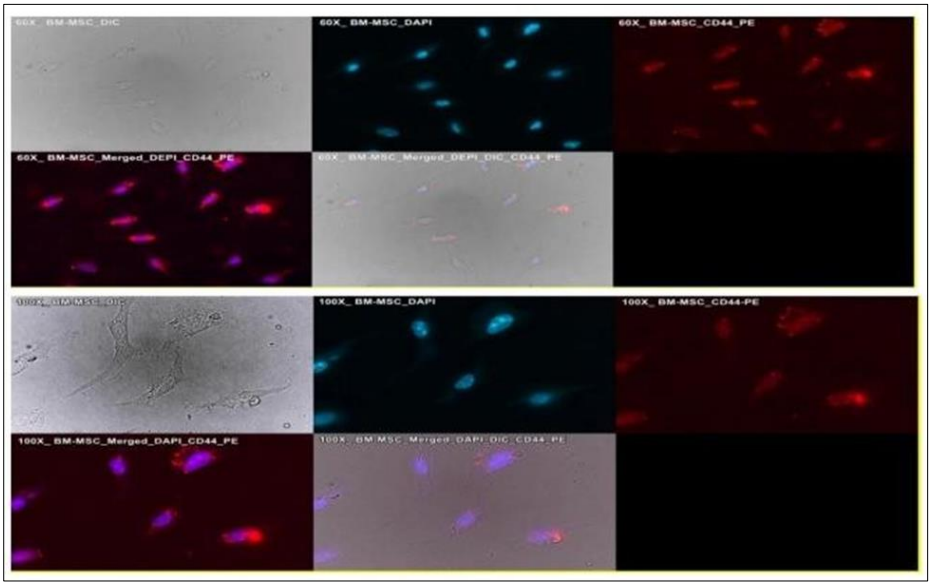


Figure 7 Cell morphology in gray scale 3 DDIC, DAPI merged showing cytoskeleton extensions morphology of BM-MSC and staining with cell surface specific marker CD44+ at 60x &100x

4. Discussion

We have standardized the isolation, purification and characterization of mouse perit on ealmacrophages, bonemarrow, and neutrophil isolation from blood and Mesenchymal stem cell. Characterization of mesenchymal stem cells has been done by their spindle-shaped cytoskeleton extensions appeared in phase contrast microscopy, staining with cell surface specific marker CD44. The characterization of macrophages has been done by their acentric nuclei and staining with cell surface specific marker CD14. Whereas, the PBMC neutrophil was characterized by their multi-lobed nuclear

morphology. Beta defensins are anti- microbial peptides and to modulate their effect on cancer cell we have isolated RNA from neutrophils followed by cDNA synthesis.

In contrast to human neutrophils, which can be easily harvested in large numbers from peripheral blood, isolation of mouse neutrophils from blood is hindered by the small volume of mouse blood that does not allow for the isolation of sufficient numbers of neutrophils. Therefore, mouse neutrophil isolation traditionally relied on eliciting granulocytes from the peritoneal cavity does not contain significant numbers of neutrophils at steady state that is why we preferred isolation of neutrophils from cardiac blood of mouse because cardiac blood is rich in neutrophils. In contrast to blood and the peritoneal cavity, the mouse bone marrow is a convenient reservoir for isolating large numbers of neutrophils both at study state under homeostatic conditions and during activation under a variety of infectious and non-infectious inflammatory conditions. It is the anatomical location of mice from where large numbers of neutrophils can be harvested at the steady state for immunological assays and adoptive transfer experiments. Bone marrow neutrophils have been shown to be functionally like blood neutrophils in mice and have been reported to survive for a longer period of time in culture, thus making them a very useful resource for research studies of neutrophil biology and physiology. Isolation of peritoneal cavity cells is an important technique for the study of different immune cells, primarily macrophages.

For characterization of bone marrow cells slides for microscopy was prepared of both adherent cells and cells in suspension. The bone BM-MSCs were characterize by their cellular morphology with cytoskeleton extensions and HSC by their non-adherent character and round morphology.

5. Conclusion

As components of the innate immune system, antimicrobial peptides in the form of defensins play an important role in host defense by serving as the epithelial layers biochemical barrier against local infections. Recent studies have shown these molecules to have far more additional cellular functions besides their antimicrobial activity. Defensins play a role in cell division, attraction and maturation of immune cells, differentiation and reorganization of epithelial tissues, wound healing and tumor suppression. Human defensins are more than just antimicrobial peptides. These molecules Compliance with ethical standards are involved in various cellular processes this multitude of function makes AMPs a promising tool for specific clinical application. Antimicrobial peptides have been successfully applied in cystic fibrosis. They were applied via aerosolization. Vector based mediated delivery of gene encoding for antimicrobial peptides were used in cancer therapy. However systemic treatment with intravenous applications of AMPs be arsan immense risk to healthy cells due to the possibility of adverse reactions to overcome this problem, diastereomers of antimicrobial peptides with D- configured amino acids were synthesized and successfully applied by topical and subcutaneous treatment. Hence many efforts have recently been made to focus on the pharmaceutical administration and modification of antimicrobial peptides as new leads for clinical therapeutic applications.

Compliance with ethical standards

Acknowledgments

We acknowledge the support from the Department of Clinical Biochemistry, University of Kashmir, Srinagar. We are thankful to the Department of Biochemistry, AIIMS, New Delhi for their permission to work in there.

Disclosure of conflict of interest

There are no conflicts to declare.

Statement of ethical approval

The ethic clearance has been taken from the Animal Ethical Committee, All India Institute of Medical Science with file no- 100/IAEC-1/2018 and 40 mice has been issued.

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