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A New Record on Inducement of Differentiation of Dendritic Cells by the Peri-vitelline Fluid of the Fertilized Eggs of Indian Horseshoe Crab (*Tachypleus gigas*, Müller)

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ABSTRACT The CRUDE-PVF was demonstrated to possess the ability to induce differentiation of dendritic cells from its bone marrow precursors. A number of studies have identified pathogen and host products such as proteins that induce the maturation of different DC subsets. Owing to their fully mature status these DCs are more programmed for antigen presentation and T-cell stimulation and have reduced capacity to capture antigens of pathogens. Immature DCs that are de novo differentiated from precursors offer better advantage than mature DCs because of their ability to scavenge pathogens followed by their uptake and processing and subsequent presentation to T lymphocytes. Our observation on the ability of CRUDE-PVF to induce differentiation of Dendritic Cells from mouse bone marrow leukocyte precursors, therefore, assumes paramount importance and is a significant step towards modulation of immune responses controlled by DCs. As the DCs differentiated by CRUDE-PVF are immature in nature and resemble very closely to the DCs differentiated by GM-CSF they will be efficient not only at antigen capture but also be effective T cell stimulators. Among the many applications, the two most important ones would include the use of 'the molecule' in vaccine formulations or in protection based studies that would promote more efficient and faster presentation of the antigens to T cells thereby initiate primary protective Th1 immune responses and help in the clearance of the pathogen. Further, 'the molecule' offers a cheap alternative to GM-CSF for commercial purposes to generate DCs *in vitro* for research-based studies

Keywords: Peri-vitelline fluid, fertilized eggs, horseshoe crab, dendritic cells

INTRODUCTION

Dendritic cells (DCs) constitute the most potent Antigen Presetting Cells (APCs) and act as a bridge between the innate and the acquired arm of the immune system (Stein *et al*., 1999, Takeshi *et al*., 2001, Morgensen, 2009). This is largely attributed to the ability of DCs to pick-up the antigen and stimulate primary naïve quiescent T-cells, thereby initiating a primary immune response. DCs exist in various states of activation that translates into distinct functions. For example, DCs arising from the Bone Marrow (BM) are essentially immature DCs. These DCs exhibit low levels of T-cell costimulatory molecules such as CD80, CD86, CD40 and CD54 and also low levels of the surface MHC class I and class II molecules (Coutant *et al*., 2002, O'Sullivan and Thomas, 2003). Further, these DCs express a range of phagocytic, endocytic and scavenger receptors.

Owing to the above features DCs are thus organized themselves tor capturing and up taking antigens. Upon contact with various inflammatory stimuli such as, TNF-α, bacterial endotoxins (e.g. LPS), CD40 ligand (CD40l), CpG containing bacterial DNA, double stranded viruses and certain (but not all) antigens, DCs endure a process of maturation, wherein they now up-regulate their surface levels of costimulatory molecules and MHC-peptide complexes. MHC-peptide complexes exported to the cell surface following degradation and loading onto MHC trimmers which are thus programmed for antigen presentation and T-lymphocyte stimulation (Caux *et al*., 1992 Steinman, 1999). The transformation from the immature to the mature stage is a tightly regulated process often requiring T-cell help. During maturation these DCs also secrete a range of cytokines such as TNF-α, IL-12, IFN-α and low levels of IL-10.

Owing to secretion of pro-inflammatory cytokines, DCs are thought to drive pro-inflammatory or Th1 responses and helped in the clearance of the pathogen. Therefore, factors that increase the population of immature DCs in the immune system offer added advantage for the host's fight against infectious pathogens (Banchereau *et al*., 1998). In this context efforts have been focused on finding and generating ways to increase the DC numbers in the immune system. These have included the use of recombinant DCs or even pathogens that have been transformed to express Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), the growth factor that is conventionally used to generate DCs *in vitro*.

The most potent of the APCs are the macrophages where different subsets of DCs, together generally regulate the antigen capturing and presenting the innate arm of the immune system. DCs in principle are APCs which are continuously produced in the hematopoeitic tissues by the stem cells. Generally DCs exist at various stages of activation those are primarily grouped as immature (iDCs) and mature (mDCs). Among DCs, iDCs are programmed to capture after receiving a particular stimulus like bacterial products, CD40 ligand, (TNF)-**α**, and certain antigens which undergo quickly a process of maturation. These DCs now are ready for antigen presentation and T-cell stimulation (Coutant *et al.,* 2002). There are some agents who promote the maturation of iDCs and play an important role determining the early immune responses elicited during outbreak of infection. DCs also activate significantly the effector cells like the various subsets of T-cells, Natural Killer (NK) cells and NK-T cells by secreting batteries of cytokines and finally they help in priming effector cells to carry out their functions, effectively (Frank *et al*., 1996, Morita *et al*., 2003). These functions are ranging from stimulating the adaptive arm of the immune system for the generation of antibody mediated responses and also stimulating the cytotoxic activity by the $CD8^+$ T-cells against the infected organs (Frank *et al*., 2001). In this regard an attempt has been made to use peri-vitelline fluid of the fertilized eggs of the Indian horseshoe crab (*Tachypleus gigas*, Müller) demonstrating a significant increase the number of DC *in vitro* experiment

Collection of peri-vitelline fluid form the fertilized eggs of the horseshoe crab

Fertilized eggs of the horseshoe crab were collected from the wild nests located on the sandy beach at Balramgari (Odisha) in India. The eggs were incubated at a constant temperature ($27+1^o$ C) in artificial incubators and the peri-vitelline fluid (PVF) was collected using a sterilized 22 gauze needle under highly aseptic condition. The PVF was collected at the time when the eggs became transparent showing fast movement of the trilobite larvae. The peri-vitelline fluid was aliquoted and freeze-dried using a tabletop freeze dryer (Edward: Micromodulo). When required the freeze-dried sample of PVF was dissolved (10 mg/ml) in HBSS (Hanks Balanced Saline Solution) wash buffer for experimentation (Alam, 2004).

Isolation* of *bone marrow

A group of 4 BALB/c mice were sacrificed by transferring animals into a chloroform chamber, for each set of experiment. This was followed by careful amputation of the hind limbs of the mouse. The limbs were then transferred to a Petri dish containing HBSS (Hanks Balanced Saline Solution) wash buffer. The tibias and femurs were cleared of all surrounding and attached tissues. A hypodermal syringe (No. 26 gauge) with HBSS solution was used to flush out the bone marrow from the limbs. The bone marrow was finally made into a fine suspension by syringing in and out of the fluid several times using 18-gauge syringe needle. All the experiments were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CSPCA), Government of India.

Differentiation* of *Dendritic cells

The fine suspension obtained above was transferred into a 50 ml sterile centrifuge tube and centrifuged for 10 minutes at 1200 rpm. The supernatant was then removed carefully and RBC lysis buffer was added. This was followed by mixing the solution thoroughly and then incubating it for 3-5 minutes at room temperature. HBSS wash buffer was used to wash the pellets which were followed by centrifuging the solution for 10 minutes at 1200 rpm. The same process was again repeated. The pellets were dissolved in 2 ml HBSS and passed through a pre-separation filter (Miltenyi Biotech # 130-041-407) to remove unwanted tissue. In the clear solution Microbeads (I-A, CD45R and CD90) were added and incubated for one hour at 4°C on a shaker platform to eliminate unwanted lymphocytes, macrophages and MHC positive cells. For getting complete leukocyte precursors, the suspension was then finally passed through a MACS (Magnetic Activated Cell Sorting) column. The cell pellets were suspended in the medium consisting RPMI 1640, 10% Foetal Calf Serum, Sodium pyruvate (1 mM) and 2-Mercaptoethanol (50 mM). About 2.5-3 x 10^6 leukocyte precursors were transferred into each well of a 24 well culture plate in 1 ml culture volume. Cells were stimulated with either GM-CSF (15 ng per ml) or 50 micro gms per ml of the CRUDE-PVF. The plate was incubated for 48 hours at 37°C in a CO_2 incubator.

Evaluation of cell surface marker expression

The evaluation of the up regulation of various cell surface markers conjugated with Dendritic cells, flow cytometry on the cells stimulated with GM-CSF or CRUDE-PVF was carried out using fluorescent labeled antibodies to various molecules (CD11c, CD80, H2-D^d). Figure 1 gives the fold increase in the levels of some of the molecules of cells stimulated with either 15 ng/ml of GM-CSF or 50 µg/ml of CRUDE-PVF for 48 h of incubation over non-stimulated control. The viability of the cultures was more then 99% at the end of the incubation period at this concentration of CRUDE-PVF. It was clearly observed that in presence of crude PVF, the cell viability was 150% whereas with GM-CSF, 180% which was only 16.6% less (Fig. 2). The difference in the increase in cells numbers were <24.2, >50 and <60% with the surface markers CD80, CD11c and H-$2D^d$ respectively when the cells were grown with crude PVF (Fig. 1). The data clearly suggested that CRUDE-PVF was found to induce the differentiation of DCs from bone marrow precursors.

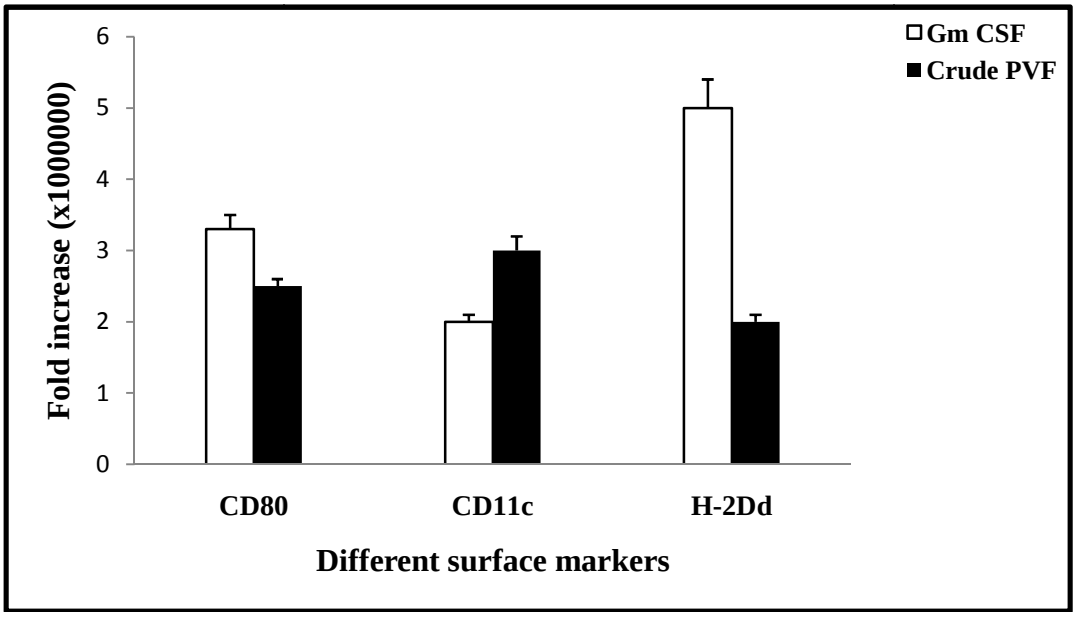

Fig. 1. Stimulation of BM precursors with CRUDE-PVF differentiates cells with DC specific markers (fold increase in relative MFI over un-stimulated control).

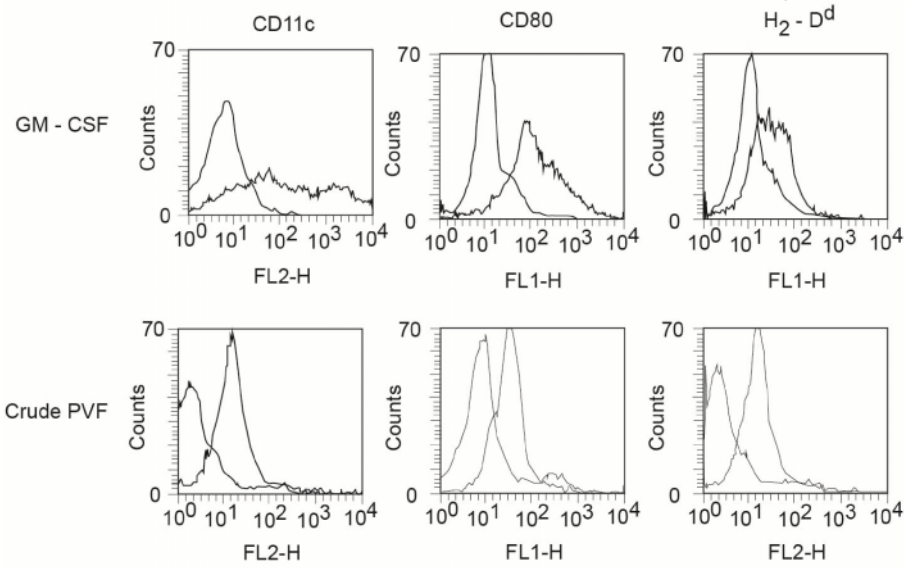

Fig. 2.

In many infectious diseases, immune suppression is a general clinical feature. Immune suppression generally cripples the ability of the immune systems as such infectious agent's results in the death of the host. It has been observed that the host recovering from diseases after the chemotherapy treatment shows a significant improvement of antigen-specific immune functions. The combined incidence of the diseases prevents the need for a drug which can restore the immune response of the affected individual to a normal stage (Jacques and Ralph, 1998, Derek**,** 2001).

The multicellular organisms have evolved a well developed defense mechanism to make their internal environment more hostile to invaders. All immune systems respond to infection by activating from a resting to an active state. It has been observed that the innate response always limited the infection and activated Antigen Presenting Cells (APCs) to trigger adaptive immunity. Subsequently, the immune responses that occur during an encounter with antigens of an infectious agent or allergens are primarily characterized by the plasticity of their nature and degree of the infection. This characteristic of the immune system provides an advantage which permits the immune system to develop its defensive strategy to counter antigens. The following of the immune responses, interactions between APCs like Dendritic cells (DCs), macrophages, different subsets of T-cells ($CD4^+$ and $CD8^+$) in the T-cell rich areas of the lymph nodes, and spleen start amplifying their activities. The T-helper (Th) cell precursors start differentiating into effectors which either secretes pro-inflammatory or suppressor/regulatory cytokines (*eq* Interferon (IFN)-α Tumor Necrosis Factor (TNF)-**α**, and Interleukin (IL)-1, or IL-4, IL-10 and Transforming Growth Factor (TGF)-**α**, respectively after following this interaction (Garaldine *et al*., 1996, Hemmi, *et al*., 2002, Kabashima *et al*., 2003). In our studies, we have not observed any toxicity to other cells in crude PVF. As such this extract can effectively be used for therapeutic applications. Moreover, this extract offers a cheap alternative to GM-CSF for commercial purposes to accelerate production of DCs *in vitro* (Caux *et al*., 1992).

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