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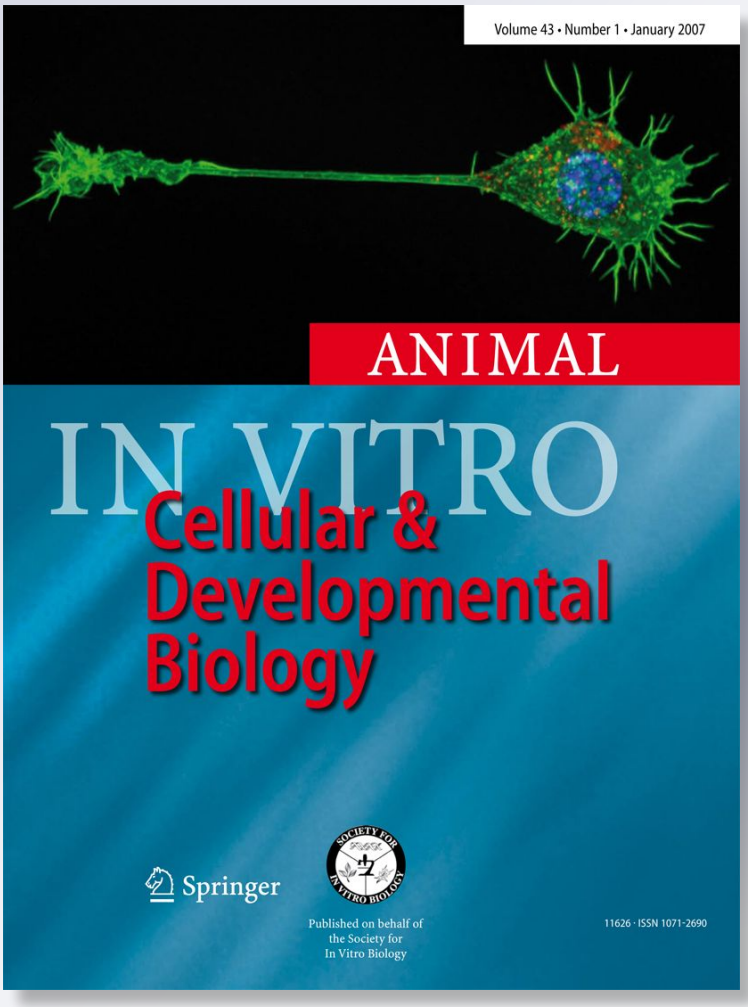

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REPORT

Establishment of a macrophage cell line from adherent peripheral blood mononuclear cells of *Catla catla*

Dharmendra K. Chaudhary · Neeraj Sood · P. K. Pradhan · Akhilesh Singh · Peyush Punia · N. K. Agarwal · Gaurav Rathore

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Catla catla, commonly known as catla, is endemic to the riverine system in northern India, Indus plain and adjoining hills of Pakistan, Bangladesh, Nepal and Myanmar. This fish grows best at water temperatures between 25°C and 32°C. Catla, an important culture species, has the highest growth rate amongst Indian major carps. A number of cell lines have been reported from catla (Ishaq Ahmad et al. 2008, 2009a, b). However, cell lines of leucocytic origin have not been reported from this species to the best of our knowledge. Such a cell line would provide useful information regarding functions of leucocytes in teleost species.

Macrophages are the multipotent cells of the immune system that play a central role in innate and adaptive immune response in teleosts. In the innate immune system, macrophages are believed to be the principal phagocytic cells in fish (Blazer 1991) and are considered to be important cells in disease resistance. Fish macrophages have a scavenging function (Ganassin and Bols 1998), bactericidal activity (Honda et al. 1986), larvicidal activity (Whyte et al. 1989) and tumouricidal activity (Mulero et al. 1994). These cells also act as professional antigen presenting cells and therefore, are required for eliciting specific immune response (Guidotti and Chisari 2001). In addition, macrophages are capable of producing a large array of proinflammatory, procoagulatory and immune-regulatory products (Ellsaesser and Clem 1994). These cells are distributed in most of the organs of the body, some of which are specialised, fully differentiated cells specific for their resident organ.

The macrophage cell lines provide a useful tool for studying functions of these cells. Moreover, such a cell line may contribute to the development and characterisation of macrophage cell markers and may be used as a source of macrophage signal peptides such as cytokines and other factors influencing the growth and maturation of leucocytes. These cell lines can be used for preliminary screening of immunomodulatory substances and therefore can provide an alternative to animal usage for such experiments. A number of fish macrophage cell lines have been developed namely from peripheral blood (Faisal and Ahne 1990; Vallejo et al. 1991; Weyts et al. 1997), spleen (Ganassin and Bols 1998) and kidney (Wang et al. 1995; Dannevig et al. 1997) as well as peritoneal washings (Watanabe et al. 1997). To date, no macrophage cell line is available from any of the commercially important fishes of the country. In the present study, we report the establishment and characterization of a macrophage cell line designated as *C. catla* macrophage (CCM) cell line from adherent blood mononuclear cells of *C. catla*.

The heparinised blood was collected from caudal vein of a healthy catla weighing 550 g, layered over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) and centrifuged at 400×*g* for 30 min. Mononuclear cells (MNCs) were collected, diluted with phosphate-buffered saline (PBS) and centrifuged at 250×*g* for 10 min. The pellet was suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 % fetal bovine serum (FBS). The cells were then seeded in 25 cm^2 flasks (Nunc, Roskilde, Denmark) at a concentration of 1×10^6 cells ml^{-1} and the flasks were incubated at 28°C. After 24 h, the non-adherent cells were removed and fresh medium was added. The adherent cells showed aggregation and multiplication at several places in the flask (Fig. 1*A*, *B*). A

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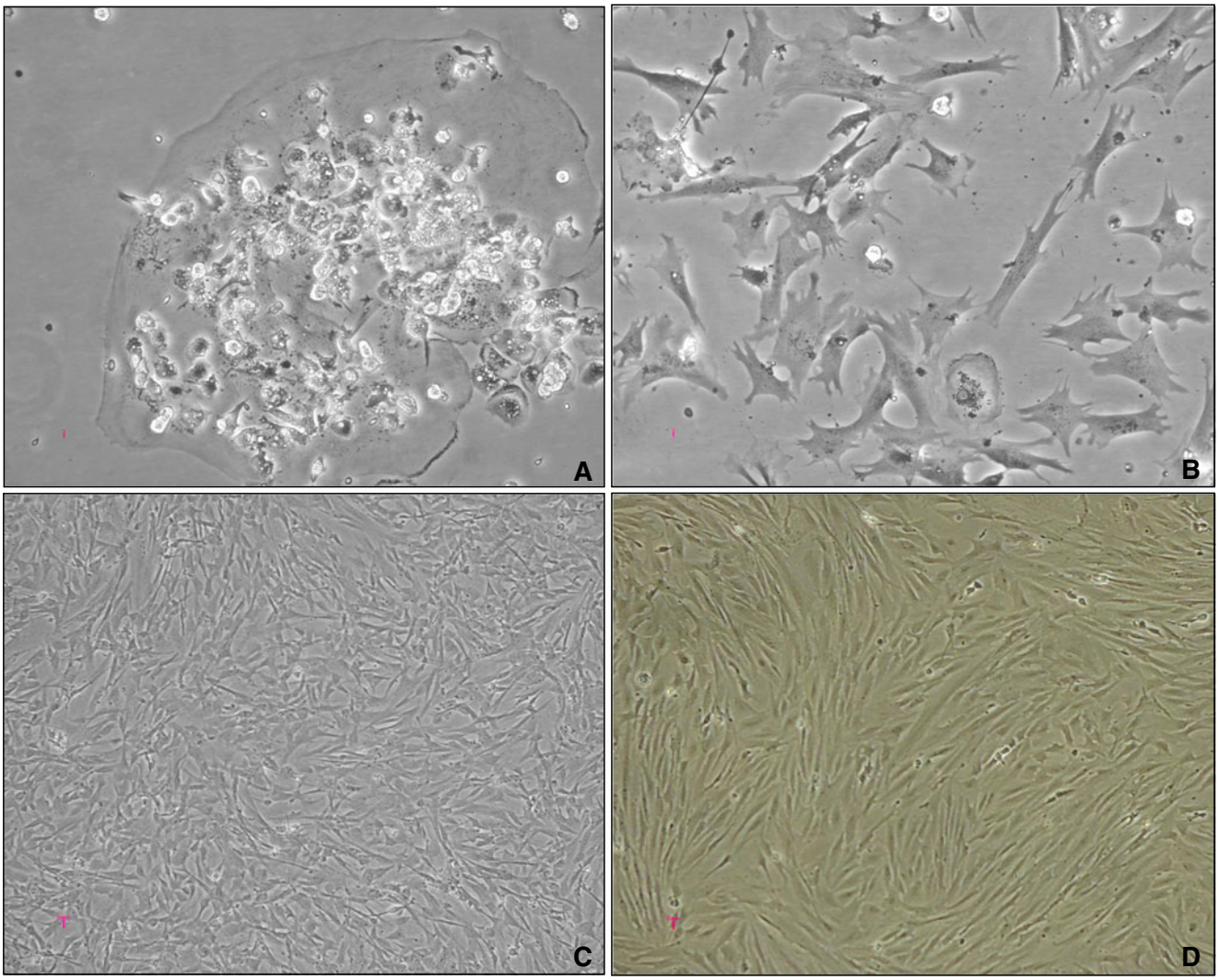

Figure 1. Photomicrograph of CCM cell line derived from adherent peripheral blood leukocytes of *C. catla*. *A*, After 3 d of plating, adherent cells were seen aggregating at some places in the flask; *B*, multiplying cells as observed after 10 d of plating; *C*, monolayer of CCM cells at 15th passage; *D*, monolayer of the cells at 90th passage.

complete monolayer was formed in about 3 wk. The cells were subcultured with 0.25 % trypsin-EDTA solution (Invitrogen, Carlsbad, CA) at a split ratio of 1:2 and maintained in DMEM. These cells showed faster growth than primary culture and formed a monolayer in 8 d. Gradually, a monolayer was formed within 3–4 d (Fig. 1*C*) and after 60 passages, the monolayer was formed in 3 d at a split ratio of 1:3. The concentration of FBS in medium was gradually reduced from 20 to 10 % between 20th and 30th subculture. The cultured cells exhibited epithelioid-like morphology, abundant basophilic cytoplasm with an eccentric vesicular oval nucleus and mitotic figures were frequently observed in Giemsa-stained cells. The cell line has been maintained for more than 1 yr now and has been subcultured for more than 90 passages (Fig. 1*D*).

Density gradient centrifugation is commonly used technique for separation of cells including fish leukocytes by virtue of their differing buoyant densities. The density sedimentation method followed by selective adherence to glass surface has proved quite successful for obtaining highly purified macrophage cultures from head kidney (Wang et al. 1995; Sorensen et al. 1997) and blood (Faisal and Ahne 1990; Vallejo et al. 1991). It has been reported that the cultures of adherent cells obtained after density gradient centrifugation contain 95–98 % macrophage-like cells by 24 h in vitro (Braun-Nesje et al. 1981). In the present study, density gradient centrifugation of blood cells of *C. catla*, followed by adherence to the surface of flasks was used to enrich macrophage population and therefore, the cell line was designated CCM cell line. Furthermore, functional tests were carried out to confirm that the CCM cells were indeed macrophage cells. Previously, three cell lines with monocyte/macrophage characteristics have been reported to have originated from peripheral blood of channel catfish and

common carp (Faisal and Ahne 1990; Vallejo et al. 1991; Weyts et al. 1997).

The subcultures were stored in the liquid nitrogen after every 10th passage in the freezing medium, which consisted of DMEM supplemented with 20 % FBS and 10 % dimethyl sulphoxide (DMSO). The cells revived after 4 mo of storage in liquid nitrogen showed 85 % viability by trypan blue staining and grew to confluency within 4 d. There was no alteration in morphology of cells after freezing and thawing. Cryopreservation of cell lines is necessary for long-term storage and the feasibility of cryopreserving the CCM cell line was demonstrated by a high recovery rate following revival. The recovery rate is in accordance with that reported for other cell lines (Swaminathan et al. 2011).

To examine the effect of different temperatures on CCM cell growth at 30th passage, a total of 5×10^5 cells were inoculated into 25 cm^2 cell culture flasks and incubated at 28°C for 2 h for attachment of cells. Afterwards, the batches of culture flasks were incubated at selected temperatures of 25°C, 28°C, 32°C, 37°C and 40°C for growth studies. The study was performed using DMEM supplemented with 20 % FBS. Every day, three flasks at each temperature were trypsinized to measure cell density and this study was carried out for 5 d. Maximum growth of the cells was observed at 32°C and 37°C (Fig. 2*A*). The growth rate was moderate at 28°C whereas, no significant growth was observed at 25°C and 40°C. From 31st passage onwards, the CCM cells were cultured at 32°C. It has been reported that cell lines growing over a wide range of temperature are suitable for isolating viruses from freshwater and cold water fishes (Nicholson et al. 1987). Another experiment was carried out to study the effect of different concentrations of FBS (5, 10, 15 and 20 %) on cell growth at 32°C at passage 31. The growth rate increased with increase in FBS concentration from 5 to 20 % (Fig. 2*B*). The cells exhibited good growth at 10 % FBS concentration though higher growth was observed at 15 and 20 % FBS concentration. Therefore,

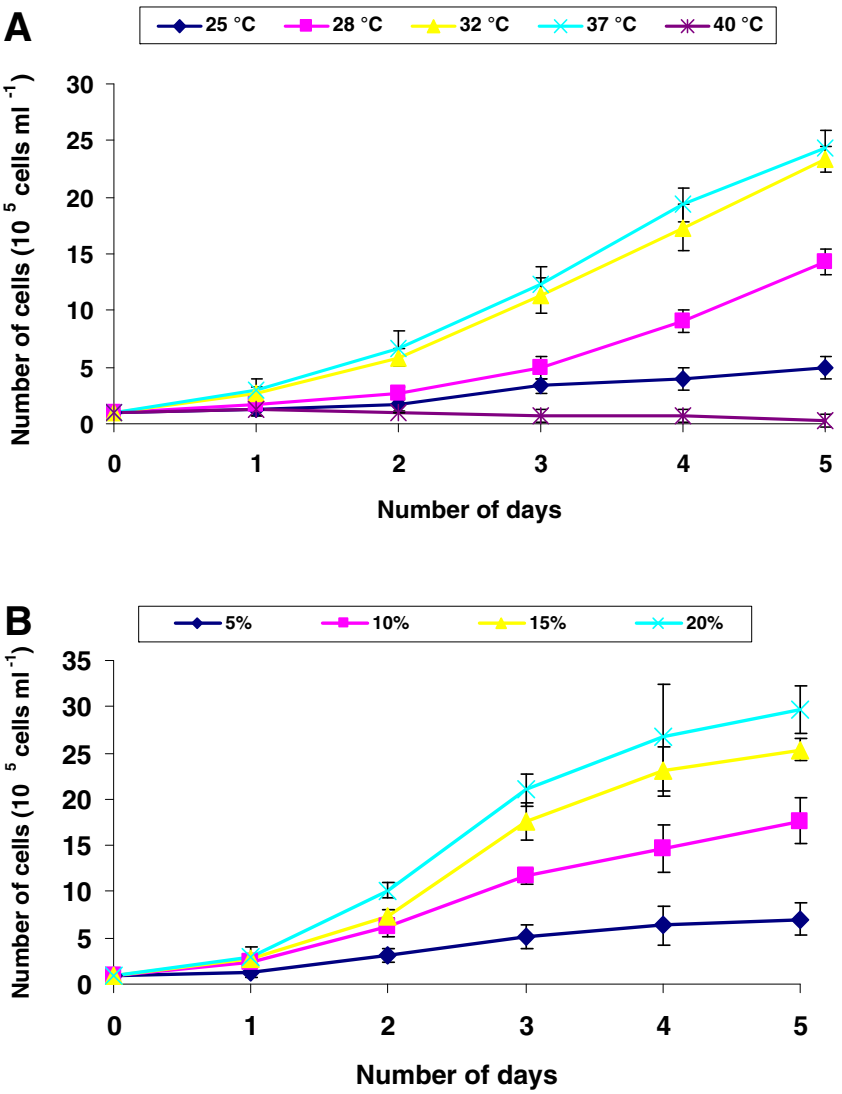

Figure 2. Growth response of CCM cells at different temperatures at 30th passage (*A*) and at different concentrations of fetal bovine serum at 32°C at 31st passage (*B*).

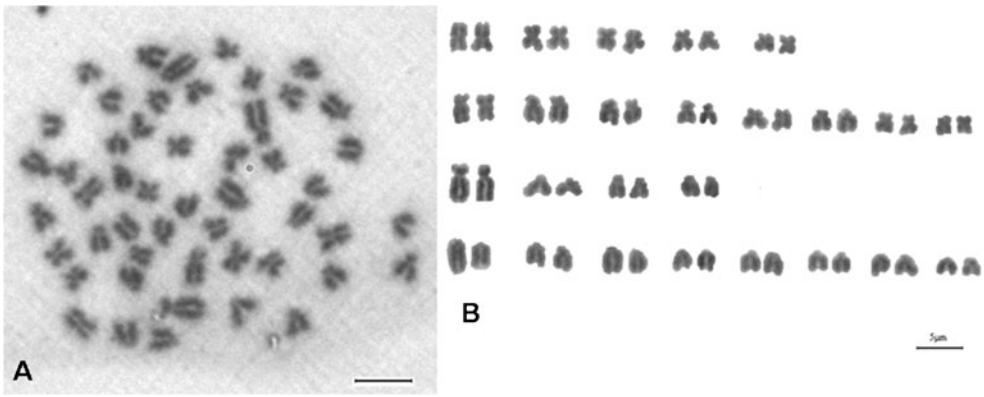

Figure 3. Karyotype analysis of CCM cells. *A*, metaphase chromosome numbers of CCM cells at passage 25. *B*, Karyotype of CCM cells indicates 25 pairs of chromosomes.

media supplemented with 10–15 % FBS can be used for growth and maintenance of this cell line.

The plating efficiencies (PE) of CCM cell line was determined at passage 25 with seeding concentration of 100, 500, and 1,000 cells in 25 cm^2 flasks (Freshney 2005). The plating efficiency was calculated to be 5.67± 1.53, 15.8±2.43 and 18.67±1.37, respectively with above cell densities. The PE data indicated that the CCM cell line performed better when seeded at a relatively higher density, i.e. 500 and 1,000 cells ml^{-1}. These results are in accordance with earlier reports (Swaminathan et al. 2010, 2011).

Flow cytometric analysis of CCM cells was carried out twice at intervals of 24 h to determine the relative proportions of CCM cells in different phases of the cell cycle following Ishaq Ahmed et al. (2009a). The results showed a distinct peak corresponding to the G_0–G_1 fraction and a relatively smaller peak corresponding to the G_2–M fraction of the cells at both intervals. The flow cytometric analysis also revealed a diploid cell population. The highest percentage of mitotic cells was observed at 48 h culture (19.38 %) with maximum in G_0–G_1 (66.12 %) and minimum cells in S-phase (14.5 %). The percentage of CCM cells in the S-phase was higher at 48 h than at 24 h of culture, as reported earlier (Ishaq Ahmad et al. 2009a, b).

Standard procedure was followed for preparing the chromosome spreads by the drop technique from monolayer cultures (Freshney 2005). Chromosomal counts of 100 metaphase plates at passage 25 of CCM cell line revealed that the number of chromosomes in the cells varied from 37 to 59. The majority of CCM cells (76 %) had a diploid chromosome number ($2N=50$), which is the modal chromosome number for this species (Patel et al. 2009). The abnormal chromosome number in a low percentage of CCM cells could be possibly due to loss of chromosomes or additions from nearby cells during karyotype preparation (Swaminathan et al. 2010). The metaphase spread with a normal diploid number (Fig. 3*A*, *B*) revealed a normal karyotype morphology, consisting of five pairs of metacentrics, eight pairs of submetacentrics, four pairs of subtelocentrics and eight pairs of telocentrics ($2n=10m+16$ sm$+8$ st$+16t$).

The origin of the CCM cell line was authenticated by partial amplification and sequencing of COI and 16s rRNA genes from the cells following Swaminathan et al. (2011). Briefly, DNA was isolated from 5×10^6 cells. The primers used in PCR amplification are given in Table. 1. The thermal cycling conditions included an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, annealing temperature of 50°C for 30 s, 72°C for 45 s and a final extension of 5 min at 72°C. The amplified PCR products were sequenced. DNA from whole blood cells of *C. catla* was used as positive control for PCR amplification and sequencing of the two mitochondrial genes. Amplification of the COI and 16S rRNA genes yielded PCR products of ~700 and ~600 bp, respectively from the cell line as well as *C. catla* blood (Fig. 4). The sequenced fragments of COI and 16s rRNA genes from CCM cells and catla blood showed a 100 % identity. The gene sequences also showed 99 % match to known *C. catla* mitochondrial DNA sequences in the GenBank. These results further confirmed that CCM cell line is truly derived from catla. The gene sequences from CCM cell line were submitted to NCBI GenBank (GenBank

Table 1. List of mtDNA primers

Sl. no.	Primers		Sequence (5′-3′)	No. of bases	Reference
1	COI	F	TCAACCAACCACAAAGACATTGGCAC	26	Ward et al. (2005)
		R	TAGACTTCTGGGTGGCCAAAGAATCA	26	
2	16S rDNA	L	CGCCTGTTTATCAAAAACAT	20	Palumbi et al. (1991)
		H	CCGGTCTGAACTCAGATCACGT	22	

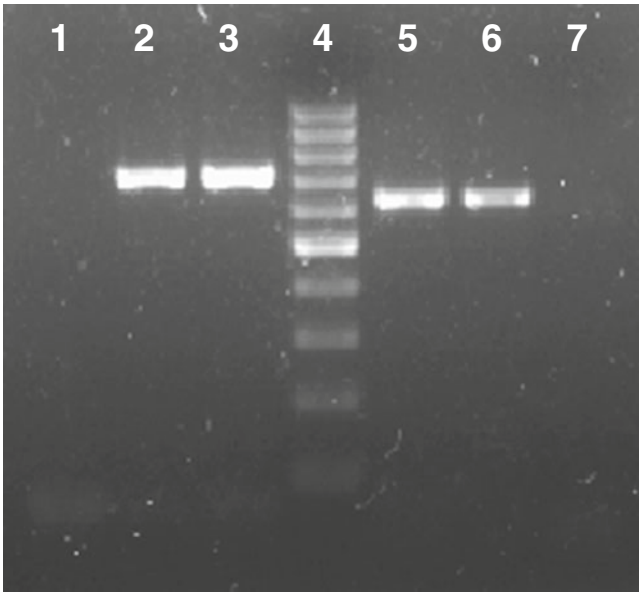

Figure 4 PCR amplification of ~700 and 600 bp sequences of the *C. catla* genome using universal oligonucleotide primers of the COI and 16S rRNA genes, respectively. Mitochondrial DNA amplification with COI primers: *lanes 1*, negative control; *2*, *C. catla* blood; *3*, CCM cells; *4*, 100 bp DNA ladder (Fermentas); mitochondrial DNA amplification with16S rRNA primers; *5*, CCM cells; *6*, *C. catla* blood; and *7*, negative control.

accession number: HM026494 and HM026495). The mitochondrial 16S rRNA, 12S rRNA and COI gene sequence alignment has been used as a reliable molecular method to accurately identify the origin of cell lines of many fish species such as catla (Ishaq Ahmad et al. 2009a), grouper (Ku et al. 2009), pearlspot (Swaminathan et al. 2010) and red-line torpedo (Swaminathan et al. 2011).

The phagocytic ability of the cultured cells was assessed by incubation with yeast cells and fluorescent latex beads. The yeast cell (*Saccharomyces cerevisiae*) suspension was prepared following Roy and Rai (2004). CCM cells grown on sterile coverslips were flooded with heat-killed yeast cell suspension for 60 min at 32°C. The coverslips were rinsed three times in PBS, fixed in methanol, and stained with Giemsa. The test was carried out in triplicates. The phagocytic capacity and the phagocytic index were determined following Jensch-Junior et al. (2006). At least 100 phagocytizing macrophages were counted without any predetermined sequence or scheme on each coverslip. The phagocytic capacity was calculated using the formula: number of cells showing phagocytosis/total number of macrophages counted ×100. The phagocytic index was determined by calculating the number of yeast cells inside macrophages divided by number of macrophages phagocytosing. CCM cells actively engulfed the yeast cells (Fig. 5*A*). A total of 69.6±5.18 CCM cells were found to have ingested yeast cells and the number of yeast cells per phagocytosing cell i.e. phagocytic index was 2.2± 0.23. In other experiment, fluorescent latex beads (1-μm diameter, Sigma-Aldrich) were suspended in DMEM and incubated with CCM cells for 30 min at 32°C in a tube (Ganassin et al. 2000). Thereafter, the cells were centrifuged at 200×*g* for 5 min and supernatant was discarded. The pellet was washed thrice with PBS and the cells were observed under fluorescent microscope. The cells also phagocytosed fluorescent latex beads (Fig. 5*B*). Though a majority of CCM cells ingested two to three beads but a few cells had ingested clump of beads. The ingested fluorescent beads were observed in different planes. Macrophage phagocytosis has been used as an immunologic parameter to evaluate the health/immune function of different fish species under different biotic and abiotic factors such as pollutants, nutrition, temperature, pathogens and genetic variation (Jensch-Junior et al. 2006). Therefore, the CCM cell line would be useful tool to study the influence of temperature on phagocytosis and determine if increased disease occurrence observed in this species during winters is related with reduced phagocytosis.

Nitric oxide production by CCM cells was detected following the method described by Wang et al. (1995). CCM cells (1×10^5 cells well^{-1}) in 96-well plate were incubated with DMEM containing different concentration (5–40 μg ml^{-1}) of lipopolysaccharide (LPS; Sigma-Aldrich). After 96 h, the culture supernatant was assayed for the presence of nitrite using a kit (cat. no. N165 kit; Amresco, Solon, OH). The nitrite concentration was determined by comparison with a standard sodium nitrite curve. The CCM cells showed a dose-dependent nitric oxide production in response to LPS. Maximum production of nitrite (5.8±0.46 μM) was observed

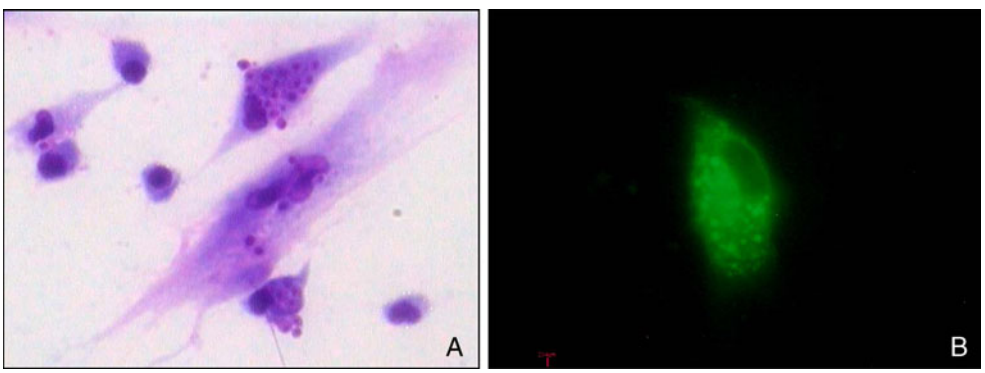

Figure 5. Phagocytic activity of CCM cells; *A*, CCM cells with ingested yeast cells (×1,000); *B*, a cell with engulfed fluorescent beads (×1,000) as seen under fluorescent microscope.

in cells incubated with DMEM containing LPS at a concentration of 10 μg ml^{-1}.

The detection of superoxide anion formation by the reduction of nitroblue tetrazolium (NBT) was performed following Wang et al. (1995). Briefly, macrophage monolayers in 96-well culture plates were washed twice with PBS. In one group of experiments, macrophages were incubated with DMEM containing fixed amount of LPS (10 μg ml^{-1}) and triggered with 100 μl of 1 mg ml^{-1} of NBT (Sigma-Aldrich) in culture medium containing differing amounts of PMA (12.5 to 100 ng ml^{-1}). In other experiment, macrophages were primed with DMEM containing different amounts of LPS (5 to 40 μg ml^{-1}) for 12 h. After removal of the old medium, the macrophage respiratory burst was detected by triggering the cells with 100 μl of 1 mg ml^{-1} NBT (Sigma-Aldrich) in culture medium containing 25 ng ml^{-1} PMA. The plates were incubated for 65 min at 32°C. The cells were fixed in absolute methanol, washed thrice with 70 % methanol and allowed to dry for several minutes. The reduced formazen within the macrophages was dissolved in 120 μl 2 M KOH and 140 μl DMSO and read in a spectrophotometer at 620 nm using KOH/DMSO as a blank. Results were expressed as means of at least three replicates±SD. In the present study, the long-term-cultured CCM cells produced ROS in response to PMA and LPS, as detected by NBT reduction assay. When CCM cells were primed with fixed amount of LPS, the maximum

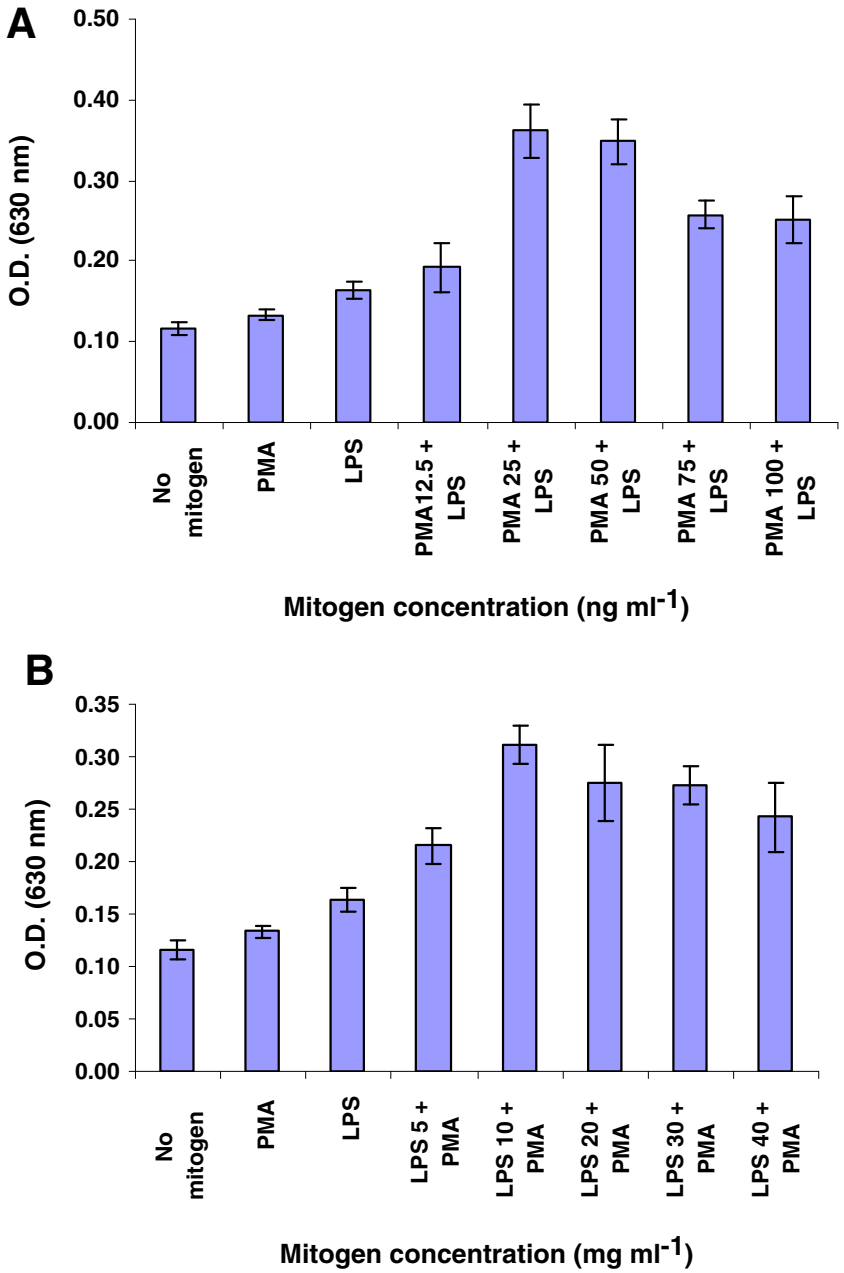

Figure 6. Respiratory burst activity of cultured CCM cells as detected by NBT reduction assay. Each bar represents mean O.D.±SE of three wells; *A* effect of priming with LPS for 24 h followed by triggering with different concentrations of PMA (12.5–100 ng ml^{-1}) on respiratory burst activity; *B* effect of priming with different concentrations of LPS for 24 h followed by triggering with PMA (25 ng ml^{-1}).

ROS production was observed in wells triggered with 25 ng ml^{-1} PMA (Fig. 6*A*). In other experiment, LPS at a concentration of 10 μg ml^{-1} induced maximum production of ROS (Fig. 6*B*). The results indicated that priming of the cells with LPS and triggering with PMA yielded much higher ROS production than the stimulation with earlier LPS or PMA alone, indicating an additive interaction between LPS and PMA. The ROS production was much lower in wells in which no mitogen was used. The respiratory burst of phagocytes has been intensively studied in mammals and has been demonstrated in many fish species including common carp (Weyts et al. 1997), goldfish (Wang et al. 1995) and rainbow trout (Ganassin and Bols 1998).

The lysozyme activity of the culture supernatant was measured using the turbitimetric assay (Sankaran and Gurnani 1972). Briefly, a solution of 20 mg of *Micrococcus lysodeikticus* (MP Biomedicals, Solon, OH) suspension was prepared in 100 ml of acetate buffer (0.02 M, pH 5.5). Hen egg white lysozyme (USB Corporation, Cleveland, OH) was used for preparing a standard curve. The culture supernatant of CCM cells collected for nitrite concentration determination was also used for determining lysozyme activity. For the test, 15 μl of culture supernatant was added to 150 μl of above suspension in a microplate and incubated at 32°C for 1 h. For the control, tissue culture medium DMEM was used in place of CCM culture supernatant. The lysozyme activity of the culture supernatant was determined by comparing decrease in O.D. to that of standard curve. Culture supernatant from CCM cells collected after 96 h of culture showed lysozyme activity of 2.67±0.61 μg ml^{-1}, as determined from the standard curve. The priming the CCM cells with LPS resulted in increase in lysozyme activity (Fig. 7). Maximum lysozyme activity was observed in culture supernatant collected from CCM cells primed with 10 μg ml^{-1} LPS. Spontaneous lysozyme synthesis and secretion is a characteristic feature of macrophages and of many established macrophage cell lines (Polansky et al. 1985; Ganassin and Bols 1998). The lysozyme concentration in culture supernatant of untreated wells falls within the normal range reported for catla serum (Saurabh and Sahoo 2008). Priming of CCM cells with LPS resulted in increase in lysozyme activity in culture supernatant. It has been reported that LPS stimulates fish lysozyme production in vitro (Paulsen et al. 2001) and in vivo (Paulsen et al. 2003).

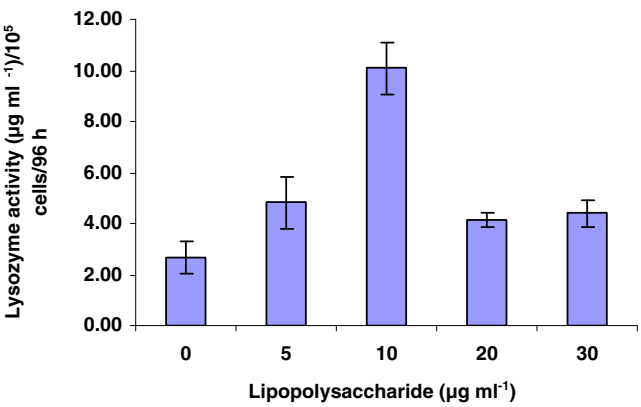

Figure 7. Lysozyme activity in culture supernatant of CCM cells after 96 h of priming with different amounts of lipopolysaccharide.

Cells were examined for the presence of Fc receptors, a marker reportedly present on phagocytes and NK cells. CCM cell smears were fixed in acetone methanol (1:1) and kept at 4°C. For the experiment, the slides were laid flat and incubated with pooled catla serum (n=5) for 30 min at room temperature. In control slides, PBS was used in place of catla serum. The slides were washed thrice with PBS and incubated with 1:1,000 dilution of mice anti-catla IgM antibody (raised in the laboratory) for 30 min. Following three washings, the slides were incubated with 1:100 dilution of anti-mouse IgG FITC conjugate (Sigma-Aldrich). The slides were again washed and mounted in buffered glycerol and observed under fluorescent microscope. Fc receptors were observed on CCM cells as marginal fluorescence. The control cells incubated only with anti-Catla IgM antibody did not show fluorescence indicating that CCM cells did not express surface IgM, a marker for B lymphocytes. FcR is a protein found on the surface of certain cells including natural killer cells, macrophages, neutrophils, and mast cells. The Fc receptors bind to the constant Fc portion of antibodies, mediate uptake of opsonised foreign material by the cells and can trigger cellular responses such as release of proinflammatory cytokines. The IgM receptors on macrophages have been demonstrated in red sea bream (Watanabe et al. 1997).

CCM cell smears were also stained for alpha-naphthyl acetate esterase activity using a commercial kit (cat. no. 91-A; Sigma-Aldrich). Briefly, the slides were fixed in citrate–acetone–methanol fixative for 30 s and washed thoroughly in deionised water. The slides were incubated in staining solution at 37°C for 30 min. Thereafter, the slides were washed in deionised water for 2 min. The slides were air dried and examined under a microscope. The cells were moderately positive for alpha-naphthyl acetate esterase enzyme. Esterase staining is regarded as most reliable cytochemical marker for mammalian macrophages (Kaplow 1981). Similarly, macrophages from fish species have also been found to be positive for esterase (Jorgensen et al. 1993; Wang et al. 1995).

The CCM cells grown on coverslips were briefly incubated with acridine orange to indicate the presence of lysosomes. CCM cells exhibited red-orange fluorescence in the cytoplasm. The particulate bodies showing the fluorescence were presumed to be lysosomes. These discrete bodies were observed following brief incubation (2 min) with acridine orange. The presence of lysosomes distinguished these cells from lymphocytes (Bayne 1986).

Neutral red (NR) uptake assay was carried out following Repetto et al. (2008). The assay is based on the ability of

viable cells to incorporate and bind NR, a weak cationic supravital dye that predominately accumulates intracellularly in lysosomes. The changes produced by toxic substances cause decreased uptake and binding of NR. This assay is one of the most commonly used cytotoxicity tests with many biomedical and environmental applications (Repetto and Sanz 1993) and inhibition of NR uptake in cultured fish cells is considered as a valuable tool for in vitro toxicity testing of a number of chemicals (Brandao et al. 1992). In the present experiment, CCM cells in 96-well plates were incubated with DMEM as well as in DMEM containing mercuric chloride (SRL, Mumbai, India) in increasing concentration (1.95, 3.91, 7.81, 15.63, 31.25, 62.5 and 125 μg ml^{-1}) and effect on NR uptake was determined using NR uptake assay. CCM cells were found to incorporate NR. The lysosomal integrity appeared to have been affected following incubation with DMEM containing mercuric chloride, evidenced by concentration-dependent decrease in uptake of NR by CCM cells. Nuclear condensation was observed in cells incubated with medium containing 62.5 and 125 μg mercuric chloride ml^{-1}. Therefore, CCM cell line can be used for in vitro screening of wide variety of chemicals for cytotoxicity and hence can be useful surrogate for fish in toxicity screening.

In conclusion, the CCM cells have morphological and functional characteristics of macrophages. The developed cell line will be useful in studying pathogen–macrophage interaction, the mechanisms of macrophage effector functions and in studying the role of macrophages in specific immune response. Moreover, CCM cell line can be potential source of fish-specific enzymes and cytokines.

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