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CHARACTERIZATION OF AN EQUINE MACROPHAGE CELL LINE: APPLICATION TO STUDIES OF EIAV INFECTION

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Abstract

EIAV is a monocyte/macrophage tropic virus. To date, even though EIAV has been under investigation for numerous years, very few details have been elucidated about EIAV/macrophage interactions. This is largely due to the absence of an equine macrophage cell line that would support viral replication. Herein we describe the spontaneous immortalization and generation of a clonal equine macrophage-like (EML) cell line with the functional and immunophenotype characteristics of differentiated equine monocyte derived macrophage(s) (eMDM(s)). These cells possess strong non-specific esterase (NSE) activity, are able to phagocytose fluorescent bioparticles, and produce nitrites in response to LPS. The EML-3C cell line expresses the EIAV receptor for cellular entry (ELR1) and supports replication of the virulent EIAV_{PV} biological clone. Thus, EML-3C cells provide a useful cell line possessing equine macrophage related properties for the growth and study of EIAV infection as well as of other equine macrophage tropic viruses.

Keywords

equine cells; Macrophage; lentivirus; equine infectious anemia virus; macrophage cell lines; equine viruses

INTRODUCTION

Equine Infectious Anaemia (EIA) was the first lentiviral disease to be recognized and its causal agent was the first *filterable* agent of a non-plant virus to be described. Numerous *in vivo* and

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in vitro studies have demonstrated that EIAV primary isolates are monocyte/macrophage restricted *in vivo* and *in vitro* (Harrold et al., 2000; Oaks et al., 1998; Payne et al., 1998). The capacity of lentiviruses to infect macrophages and other antigen-presenting cells (APC) plays a determinant role in the establishment, persistence, and pathogenesis of infection (Steinman et al., 2003). Progress in understanding EIAV/macrophage interactions, however, has been limited by the absence of permissive equine macrophage cell lines. Primary eMDM cultures have been used, but have been of relatively limited utility owing to their short-lived nature, to the laborious isolation procedures necessary to establish the cultures, and to the great variability revealed by these primary infections. To facilitate EIAV *in vitro* studies some authors have adapted primary EIAV strains to growth in equine dermal cells (ED) (Malmquist et al., 1973), fetal equine kidney cells (FEK) (Montelaro et al., 1982), and to the heterologous malignant canine thymus cell line (Cf2Th) (Bouillant et al., 1986). In addition, some EIAV strains have been shown to replicate in equine endothelial cells (Maury et al., 1998). However, EIAV field isolates do not grow in non-macrophage cell types without extensive adaptation. Thus, the availability of an EIAV permissive equine macrophage cell line to pursue *in vitro* EIAV/macrophage studies of cytopathicity and immunological functions is required to progress towards a more thorough understanding of EIAV infection and disease progression. Here we describe and characterize a novel equine macrophage-like cell line and demonstrate its ability to support replication of a virulent EIAV clone.

MATERIAL AND METHODS

Spontaneous immortalization and maintenance of equine macrophage-like cells (EML-3C)

Whole blood was obtained from a two-year-old male horse from the Portuguese autochthonous horse breed (the Garrano) immediately after slaughter. A volume of 500 mL of whole blood was collected in sterilized flasks containing 2mM ethylenediamine tetraacetic acid (EDTA). Blood collection occurred after brain insensibilization and during the bleeding procedure at an authorized slaughterhouse by Direcção Geral de Veterinária of Portugal and according to the European Union (EU) Animal Welfare legislation. Within two hours after blood collection, buffy coats were generated by a 45 min centrifugation at 1800 rpm at room temperature. Equine peripheral blood mononuclear cells (ePBMC) were obtained from the buffy coat after layering onto a Histopaque density (density=1.077gm/ml) gradient (Sigma-Aldrich Corporation) following the manufacturer's procedures. After removal from the density gradient, the ePBMC were incubated for 10 minutes at 37°C in a red cell lysis solution (10 mM Tris, 150 mM NH₄Cl, pH 7.4) and washed twice with Hank's solution (HBSS) (Gibco, Life Technology). Finally, the cells were resuspended at a density of $6-7.5 \times 10^6$ cells/ml in an enriched culture medium composed by DMEM Glutamax, high glucose (Gibco, Life Technology) culture medium supplemented with 10% of fetal bovine serum (Gibco, Life Technology), 10% of equine serum (HyClone Laboratories, Inc.), 1% of MEM non essential amino acids (Gibco, Life Technology) and 1% penicillin-streptomycin (Gibco, Life Technology). Cell suspensions (10ml) were incubated overnight in 10-cm diameter tissue culture dishes (TPP-Techno Plastic Products, Switzerland) at 37°C and 5% CO₂. After 24 hours of incubation the attached cells were washed a minimum of three times with HBSS to remove non-adherent cells, and fresh medium was added. At week 3 of incubation, adherent cells were harvested by treatment (5 minutes at 37°C and 5% CO₂) with trypsin-EDTA (Gibco, BRL), and about 1.5×10^6 cells were plated in T75cm² flasks (BD Falcon™). Cells were then successively transferred after trypsin treatment and plated into a new flask every week. After the fourth passage, a limiting dilution method was used to generate single cell clones from the parental cell population.

Maintenance, freezing and thawing of EML-3C cells

For maintenance and general growth, EML-3C cells were plated at $1-1.5 \times 10^4$ cells/cm² in fresh medium and moved into a new flask once a week. For passaging, EML-3C cells were

washed twice with HBSS, incubated with 1.5 ml of trypsin-EDTA solution for 2–3 minutes, and 10 to 15 ml of complete medium added immediately. To count cells a volume of 1mL of the cell suspension was taken from the cell cultures and centrifuged at 1100 rpm at 4°C for 7 minutes. To freeze down the EML-3C cells, two million cells were detached by trypsin-EDTA, centrifuged briefly and resuspended in 45% of fetal bovine serum 50% of equine serum, and 5% of DMSO. Cell suspensions were transferred to Cryotubes (NUNC) placed in isopropanol cryoboxes at –70°C for 16 hours and afterwards moved to liquid nitrogen containers. For thawing, frozen vials were immediately moved into a 37°C water bath and immediately placed into a T25cm² flask containing 10mL of pre-warmed enriched culture medium. After that, cells were allowed to adhere at 37°C and 5% CO₂ and medium replaced.

Cell lines: culture, viral stocks, and infections

RAW264.7 (TIB-71, ATCC) and NIH/3T3 (ATCC; Cat. No. CRL-1658) cells were grown in 10% FBS supplemented DMEM. Fetal equine kidney cells (FEK) were grown in MEM supplemented with 10% FBS as previously described (Montelaro et al., 1982). Equine monocytes and eMDMs were obtained from ePBMCs collected by jugular venipuncture. To obtain undifferentiated monocytes and eMDMs for culture and analyses, the same procedure and culturing conditions described above for the ePBMCs isolation and generation of eMDM/EML cell clones were followed with the following modifications. Briefly, monocytes were obtained from adherent ePBMCs after a overnight incubation at 37°C and 5% CO₂ in 10 cm dishes with 10mL of DMEM medium supplemented with 10% ES and 10% FBS. To remove non-adherent cells, cultures were washed four times with HBSS 18 hours post plating. Monocytes were differentiated into eMDMs by culturing for two to seven consecutive days in DMEM with 10% ES and 10% FBS without additional supplementation. For fluorescence microscopy analysis, non-specific esterase (NSE), and phagocytosis assays, approximately 2×10^5 EML-3C, RAW264.7, or NIH/3T3 cells were plated on coverslips and cultured in a DMEM enriched medium for 48 to 72 hours prior to the assays. For FACS analysis EML-3C cells were plated in a DMEM enriched medium, and the cells harvested by scraping after 3–4 consecutive days of culturing.

The virulent biological clone EIAV_{PV} has been described in detail previously (Lichtenstein et al., 1996). Briefly, EIAV_{PV} is a pathogenic biological clone derived from the EIAV primary Wyoming isolate.

For EIAV infections, EML-3C and FEK cells (10^4 cells per well) and eMDMs (2.5×10^5 cells per well) were seeded into 48 well plates 48 hours prior to infection. After two days, cell cultures were infected in triplicate with EIAV_{PV} at a MOI of 0.01. Tissue culture supernatants were screened for reverse transcriptase (RT) activity every three days (Raabe et al., 1998).

CFSE staining of EML-3C cells

Cellular division was examined by labeling EML-3C cells with 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), purchased from Molecular Probes (Leiden, The Netherlands). Briefly, 2×10^5 cells were plated in T25cm² flasks 48 hours prior to assay. Cells were labeled in a total volume of 7 mL with pre warmed PBS containing CFSE at a final concentration of 10μM by incubation for 15 min at 37°C. Afterwards, cells were washed twice with fresh medium and incubated at 37°C and 5% CO₂. After six days of culture, cells were detached by trypsinization, washed, and then analyzed by flow cytometry analysis. Rounds of cell division were determined by sequential halving (loss) of CFSE-fluorescence intensity with FlowJo 8.7.1 software (Ashland, OR).

Phagocytosis activity

To evaluate EML-3C cellular phagocytosis, *Escherichia coli* K12-Alexa 488 fluorescent bioparticles (Molecular Probes, Leiden, The Netherlands) were used according to the manufacturer's instructions. Two well-described murine cell lines, RAW264.7 and NIH/3T3, were used as control cell lines. The phagocytic RAW264.7 cells were used as the positive control while the fibroblastic NIH/3T3 cells were used as the negative control. Briefly, cells were grown directly on cover slips. Prior to the assay cells were washed, the culture medium replaced, and bioparticles added to the fresh medium. For control cell cultures, infection was carried out at 4° C. After 90 minutes of incubation at 37°C, the medium was removed and non-internalized fluorescent bioparticles quenched by adding trypan blue (1.2µg/ml). Immediately after, cells were washed in PBS, fixed in 3.7% formaldehyde (Sigma-Aldrich Corp.) and permeabilized with 0.18% Triton X-100 (Sigma-Aldrich Corp.). Cells were then washed twice in blocking buffer (PBS, 0.2% BSA and 2.5% equine adult serum), blocked, and stained with a Cy-3 conjugated anti-vimentin monoclonal antibody (V9 clone, Sigma-Aldrich Corp) for 30 minutes. After staining, cells were washed twice in PBS and mounted in 1:5 DAPI Vectashield (Vector Laboratories, CA, USA). Coverslips were examined under a Zeiss fluorescence microscope. A number of fields were examined, and the quantity of internalized bioparticles counted in parallel in both EML-3C and RAW264.7 cells.

Non-specific esterase (NSE) enzymatic activity

Coverslips were stained for NSE using a Pararosilin-HCl solution as the coupling agent for the naphthyl acetate substrate. The Pararosilin-HCl stock solution was made by dissolving 1g pararosilin base (Sigma-Aldrich Corp) in 20 ml distilled water to which 5ml concentrated HCL was added. This solution was gently heated, allowed to cool and filtered before use. A sodium nitrite/pararosilin-HCl solution (0.2ml) was made by adding sodium nitrite (40mg/ml) dropwise with vigorous mixing, at a 1:1 ratio, to the pararosilin-HCL solution, resulting in a corn-colored solution. Naphthyl acetate solution was prepared by mixing 62.5 ml naphthyl acetate (10 mg/ml acetone- Sigma), 1.8ml of 0.2 M phosphate buffer and 625 L distilled water. This solution was added to the sodium nitrite/pararosilin-HCl solution, vigorously mixed, and used immediately. NSE staining was performed for 10 min at room temperature without fixing the cells. Following staining, the coverslips were washed with PBS, the cells fixed with 4% paraformaldehyde for 30 minutes at room temperature, and counterstained with haematoxylin for 2 min followed by washing in running tap water for 10 min. Sodium fluoride (5mM- NaF) was included, where indicated, for the entire NSE incubation period, to inhibit macrophage esterase.

Nitrite production

The production of nitrites was estimated by the Greiss assay utilizing manufacturers instructions. Briefly, 2×10^5 cells of EML-3C and RAW 264.7 were plated in 60 mm dishes. The cells were then incubated at 37°C in a humidified 5% CO₂ incubator for 24 hours and stimulated with 1 µg/ml of LPS for 72 hours. At the end of the incubation, 100 µl of the culture medium were mixed with an equal volume of Greiss reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid) in triplicate. The absorbance at A₅₅₀ was measured, and the nitrite concentration was determined with a curve calibrated on sodium nitrite standards (0.5, 1, 1.5, 2, 2.5 and 3 nmol/assay).

FACS analysis

Monocytes, MDM, and EML-3C cells were immunophenotyped by flow cytometry. Briefly, after manual detachment, cell staining was performed in 96-well round-bottom plates (Greiner, Nürtingen, Germany) in staining buffer (PBS, 0.2% BSA, 0.1% NaN₃, 2.5% equine adult serum) with approximately 0.5×10^6 cells/well. A mouse antibody with the same isotype of

the test antibody was used as a negative control to define background staining. For intracellular staining, cells were fixed with 4% formaldehyde in PBS, washed, and then permeabilized with 0.2% saponin in PBS. A second staining step was performed using rabbit anti-mouse or goat anti-rabbit fluorochrome-conjugated antibodies. After staining, cells were washed three times with staining buffer and immediately analyzed in a FACScalibur, FACSaria or LSRII (Becton Dickinson, Mountain View, CA, USA). For each sample, 10,000–20,000 events were acquired using FSC/SSC characteristics and analyzed using CellQuest, FACSDiva (Becton Dickinson) and/or FlowJo.

Antibodies

The antibodies (Abs) used in this study were as follows: Cy3 conjugated mouse IgG1 anti-vimentin (V9 clone) from Sigma (Sigma-Aldrich Corp., Sintra, Portugal) for phagocytosis assays; FITC conjugated mouse IgG1 anti-human HLA-DR/DP/DQ/DM (clone Tu39) for equine MHC II staining; a mouse IgG1 anti-human CD86 (IT2.2 clone) from BD Pharmingen (San Diego, CA) for equine CD86 staining; anti-human CD68 (Ki-M6 clone) mouse monoclonal from Serotec (Denmark) for equine CD68 staining. The anti-equine CZ6 and CZ2.2 mouse monoclonals were a kind gift from Dr Antczack (Cornell, New York, USA). CZ6 was used for MHC class I staining, and CZ2.2 was used as a marker of equine macrophage. A rabbit polyclonal anti-Equine Lentivirus Receptor 1 (ELR-1) was previously described (Zhang et al., 2005). The anti-ELR-1 antibody was used for the detection of cell surface expression of the entry receptor for EIAV. As secondary antibodies, the following antibodies were used: goat FITC, Cy5 conjugated anti-mouse, and goat Cy5 conjugated anti-rabbit (Jackson ImmunoResearch Laboratories Inc, PA, USA).

RESULTS

Establishment of EML-3C cell line

The EML cell line, derived from isolated equine peripheral blood mononuclear cells (ePBMCs), was established by allowing a cellular fraction of ePBMCs to adhere and differentiate. The cells spontaneously acquired proliferation activity after 10–11 days in culture with a DMEM enriched medium containing 10% ES and 10% FBS. The cells were passaged via typical trypsinization procedures (Materials and Methods) and were carried with continual passage as a typical cell line (monocytes/macrophage are typically non-dividing cells). The cells grew stably at 37°C in the absence of exogenous cytokine treatment or viral infection. Moreover, freezing and thawing cycles of these cells under the described conditions did not interfere with cell viability assessed by trypan blue exclusion (>90%). Over numerous passages the cells maintained proliferation activity for more than three years and were consequently designated EML-3. At this point in the cellular history, the EML3 cell line was cloned by the limiting dilution method. The specific subclone produced from these standard techniques used for the current characterizations was termed EML-3C.

Morphology and cell division of EML-3C

Macrophages have a very distinctive cellular morphology. Vimentin staining and phase contrast micrographs revealed that the EML-3C cells (Figure 1A and B) were morphologically similar to previously described eMDM (Raabe et al., 1998). Early in the generation of the cell line, after passage four, the EML cells appeared as single cells. In general, these cells contained one large nucleus with one or two well-marked nucleoli, although binucleated cells continued to be observed (Figure 1B). In very confluent cultures ($\geq 90\%$) cells appeared with a fibroblastic-like morphology and formed typical multicellular aggregates and cellular clusters (data not shown). The individually derived EML parental and clonal cells lines such as the current EML-3C cells were similar with respect to the their evolution of morphological characteristics, as described.

Primary monocytic cells do not typically divide. Hence, characterization of the steady-state level of cellular proliferation or cellular division of the generated EML-3C cell line was obligatory. The expansion rate of EML-3C was consequently determined utilizing flow cytometry analyses of CFSE labeled cells. EML-3C cells were examined over a period of six days (Materials and Methods). Trypsinized cells were analyzed by flow cytometry. Dead cells were gated out using standard cytometry techniques. CFSE positive cells were compared to controls and proliferation measured (Figure 1C). FlowJo software algorithms were used to calculate specific cellular generations. The resultant data demonstrated seven generations of EML-3C in six days, and that 88.8% of the cells were proliferating with a 2.14 proliferation index (Figure 1C and D).

EML-3C functional properties

Macrophages possess certain distinctive functional characteristics including the ability to phagocytose foreign objects, non-specific esterase activity, and the production of nitrites in response to stimuli. The EML-3C cells were analyzed for their ability to accomplish these three properties.

Phagocytosis activity—Phagocytosis activity was measured utilizing *Escherichia coli* K12-Alexa 488 fluorescent bioparticles. Figure 2A reveals that EML-3C cells are able to phagocytose the bioparticles. The phagocytic activity of EML-3C cells was compared with the activity of an established positive control cell line, the murine phagocytic cell line, RAW264.7 and to the negative control cell line, the murine fibroblastic cell line, NIH/3T3. At 4°C both the RAW264.7 and EML-3C cells were not able to internalize the fluorescent bioparticles (Figure 2A panels 3 and 6). At 37°C the K12 Alexa 488 bioparticles were not detected in NIH/3T3 cells (Figure 2A-panel 1), but clearly detected in RAW264.7 and EML-3C cells (Figure 2A- panels 4 and 7). The RAW264.7 and EML3C cell lines differed in morphology and in the number of bioparticles phagocytosed per cell, which has been observed by others when comparing equine macrophage and RAW264.7 (Lunn et al.1995). The RAW264.7 cells possessed a rounded cellular morphology with a rim of cytoplasm that continually contained at least five bioparticles/cell (Figure 2A, fluorescence and phase contrast micrographs panels 4 and 5). By contrast, the EML-3C cell line had fewer bioparticles/cell even though they possess a larger portion of cytoplasm as compared to the RAW264.7 (Figure 2A- panels 4 and 5 versus 7 and 8). Although morphologically different from RAW264.7, the EML-3C cells have the capacity to phagocytose a large number of bioparticles (Figure 2A panels 7 and 8). The comparative analysis of microscopic fields of the two cell lines (Table 1) showed a phagocytosis activity rate of 53.8% for EML-3C and 60.9% for RAW264.7. In addition to these analyses, deconvolution microscopy was utilized to demonstrate that the bioparticles are located throughout different layers of the EML-3C cells as phagocytosed particles and not simply co-localized to the surface of the cells. Vimentin, a class III intermediate filament widely expressed in the phagosome was used as a cytoskeleton organization marker (Garin et al., 2001). The serial Z stacks presented in Figure 2B panels 2–4 demonstrate that the Alexa488 conjugated bioparticles are present in the cytoplasm of EML-3C cells in vimentin coated structures, (labeled in red) and not merely laying on the surface of the cells. These results show that the phagocytic activity of EML-3C is functionally equivalent to a classic reference phagocytic cell line.

Non-Specific Esterase activity—Due to the changes in morphology with increasing confluency, we measured NSE activity in EML-3C cells both at 50% confluency and 99% confluency (Figure 3A and B). This data demonstrates that EML-3C are clearly NSE positive cells. The distribution pattern of NSE staining differs accordingly with the cellular morphology and confluence of EML-3C cultures. In single cells NSE staining was found predominantly within the cytoplasm (Figure 3A, panel 1, arrows), however, in highly confluent cultures the

NSE activity was stronger and closer to the cellular membranes (Figure 3A, panel 2, arrow). Due to the “fibroblastic-like” morphology of highly confluent EML-3C cells, a NaF control with 99% confluent cells was included (Figure 3A, panel 3). NaF is an inhibitor of macrophage esterase (Andrew et al., 1990). EML-3C cells showed a strong NSE activity, and this activity was in turn inhibited in the presence of the NaF (10g/ml)(Figure 3A, panel 3).

Nitrite production—NO was measured as its stable oxidative metabolite, nitrite, by the classical Greiss Assay. EML-3C cells were shown to produce nitric oxide upon stimulation with LPS 1 µg/ml after 72 hours (Figure 3B). The levels secreted by these cells were similar to those produced by the murine macrophage cell line RAW264.7.

Results of NSE, phagocytic activity, and the nitrite production in response to LPS demonstrated that EML-3C cells retained major functional properties that are characteristic of primary macrophages.

EML-3C immunophenotype

Expression of macrophage-specific markers—Macrophages are characterized by selected features: the stellate morphology, expression of enzymes, namely non-specific esterase, and the non-specific uptake of particles. However none of these features are capable of definitively distinguishing a macrophage from other mesenchymal cells. Characterization of the expression of macrophage-specific cellular markers can provide additional discrimination of cell types. The CZ2.2 and the anti-CD68 Ki-M6 antibodies have been described as equine macrophage markers (Ibrahim and Steinbach, 2007; Lunn et al., 1998; Siedek et al., 2000). The Ki-M6 antibody is directed against an intracytoplasmic conserved domain of the CD68 molecule and recognizes equine CD68 (Siedek et al., 2000). The protein target of CZ2.2 antibody remains to be determined. In immunohistochemistry studies, CZ2.2 clone labeling of macrophages was shown to stain lymphoid tissues at germinal centers of follicles in lymph nodes (LN) and spleen, single large cells in bronchiolar epithelium, and parenchyma and single large cells in and under epidermis (Lunn et al., 1998). FACS analysis demonstrated that EML-3C cells are CZ2.2⁺ CD68⁺, and thus positive for two different cellular markers for equine macrophage (Figure 4A). The expression level of these markers was very similar to the levels presented by primary eMDMs.

Expression of macrophage differentiation markers—Equine macrophage differentiation markers have been reported and characterized previously (Lunn et al., 1998; Ibrahim and Steinbach, 2007; Kydd et al., 1994). To determine the EML-3C differentiation stage, we analyzed and compared the cellular immunophenotype with primary monocytes and eMDM. Cell surface expression of MHC-I and MHC-II as well as CD86 was analyzed using the anti-equine CZ6 (MHCI), the anti-human Tu39 (MHCII), and IT2.2 (CD86) monoclonal antibodies, respectively. CZ6 monoclonal antibody was raised against horse MHC-I and shown to specifically detect MHC-I (Kydd et al., 1994). The IT2.2 monoclonal antibody against human CD86 was shown to cross react with equine CD86 (Hammond et al., 1999). The Tu39 antibody against human MHC-II has been shown to recognize the equine MHC-II (Monos et al., 1989). Figure 3B shows a representative phenotypic characterization of equine monocytes, eMDMs, and EML-3C cells. FACS analysis of EML-3C cells revealed that they are MHC I and MHC II positive cells. CD86 is also expressed on EML-3C cells, although at very low levels. The surface expression levels of MHC I and II in EML-3C is decreased when compared to eMDMs, but increased when compared to those levels expressed in equine monocytes (Figure 4B). Taken together, this flow cytometry data confirms that EML-3C express cellular markers that are macrophage-like and that appear to be in a differentiated state.

EML-3C cells express the EIAV receptor

Our first approach to determine the permissiveness of EML-3C cells to EIAV infection was accomplished by FACS staining for the EIAV receptor required for cellular entry, the Equine Lentiviral Receptor 1 (ELR1). Resultant data demonstrated positive surface staining for the equine TNF-like receptor on the EML-3C cells at higher levels than those presented by eMDM (Figure 5A).

EML-3C cells can support the replication of EIAV_{PV}

The replication ability of the pathogenic biological clone EIAV_{PV} in EML-3C cells was compared to known permissive cell lines through parallel experimental infections in EML-3C, FEK cells, and eMDMs. Each cell type was infected at an MOI of 0.01 and screened for viral production over an 18-day period. Viral production was measured by the production of supernatant RT activity. Duplicate experiments measuring EIAV replication kinetics was performed individually in triplicate. Figure 5B demonstrates the respective replication profiles of EIAV_{PV} in EML-3C, eMDM, and FEK cells. The viral production observed in EML-3C cells was remarkably higher as compared to levels observed in the eMDMs and very similar to that presented by FEK cells. The eMDM viral replication peaked early and dropped off after 12 days, which happens to be the approximate life span of the primary cells and the point at which cell death typically occurs (data not shown). After a 12-day infection period the levels of viral production on EML-3C reached a plateau (approximately 5×10^4 cpm/10 μ l supernatant) and was slightly higher than the activity observed in FEK cells. The data represented in Figure 5B clearly demonstrates that EML-3C are able to sustain EIAV_{PV} replication at high levels over a time period of 18 days.

DISCUSSION

Macrophages are key players in inflammatory and immunological responses. Comparative immunology studies show that the equine model displays peculiar aspects of macrophage and dendritic cell biology (Mauel et al., 2006). During recent years a great effort has been made to generate equine immunological tools and to identify non-equine antibodies that would permit a comprehensive characterization of the equine immune system (Ibrahim and Steinbach, 2007; Kydd et al., 1994; Lunn et al., 1998; Marti et al., 2003). Nevertheless, the availability of *in vitro* cellular systems to pursue immunological studies is still scarce.

Here we report the spontaneous immortalization of an equine monocyte derived cell line from equine peripheral blood monocytic cells that retain the major functional properties and the immunophenotype of primary differentiated macrophages. The equine macrophage cell line developed in the present study was established in the absence of viral transformation, has been in continuous culture for more than three years, and has maintained macrophage-specific properties throughout this time, namely NSE and phagocytosis activity, nitrite oxide production, and the expression of two available equine macrophage-specific markers (CZ2.2, and Ki-M6). Spontaneous immortalization of monocyte derived cell lines has been previously reported with ovine monocytes (Olivier *et al.*, 2001) and in the generation of an equine macrophage (e-CAS) cell line (Werners et al., 2004). Monocytic mammalian cell lines have been established previously by viral infection of or by introduction of viral oncogenes into primary cells (SV40, V-myc and V-raf) (Blasi et al., 1989; Kreuzburg-Duffy and MacDonald, 1994; Nagata et al., 1983). Such cell lines can retain their original specialized functions while being adapted to prolonged life in culture, but most transformed cell lines obtained in this way characteristically show altered cellular phenotypes and functional properties when compared to their primary cell counterparts. Additionally, for studies principally analyzing viral infection, co-infection of transforming viruses or the presence of viral genes which might interact with the virus of study have potential complications for data interpretation. The EML-3C cell clone

was analyzed for the presence of equine herpesviruses as previously described (Diallo et al., 2006; Frampton et al., 2004) and found to be free of such transforming genes (data not shown).

Phagocytosis is a key immunological function of macrophages that previously could only be studied in primary equine macrophage. This ability to engulf materials is crucial for macrophage function as guardians and central effectors in innate immunity and host defense. The EML-3C cells demonstrated remarkable levels of phagocytosis with a phagocytosis rate not significantly different from the murine phagocytic cell line RAW264.7. However, it should be noted that phagocytosis is not restricted to cells of the monocyte/macrophage lineage. Other cell types such as the polymorpho-nuclear neutrophils are equally able to internalize materials or particles.

The EML-3C cells were also capable of nitrite production when stimulated by LPS. This function demonstrates that EML-3C cells maintained one of the most important functions of macrophages as key players of the modulation of inflammatory processes. Hence, this cell line can be a useful tool for the study of equine related inflammatory processes.

The EML-3C cells demonstrated NSE activity, another key function of macrophage. The NSE histochemical staining showed dissimilarities between EML-3C cells at different levels of confluency. In less confluent cultures macrophage like cells showed NSE activity mainly in the cytoplasm. In confluent cultures, the fibroblastic-like cells showed a stronger NSE activity when in close contact with other cellular membranes. The observations in this study suggest that NSE is a 'mobile' enzyme, whose subcellular localization changes according to macrophage activation status, thereby reconciling findings in earlier studies (Feng et al., 2002). Namely, NSE might be located within the cytoplasm when the macrophage is inactive, but it would move to the membrane to be secreted when the macrophage is activated. Yet, despite the morphological changes that occurs through culturing EML-3C cells until high levels of confluency, NSE expression is maintained.

In addition to positive assays of macrophage function, the EML-3C cells demonstrated detectable expression of specific equine macrophage cellular markers: the CZ2.2 and the intracytoplasmatic marker Ki-M6 (CD68). Siedek and colleagues (Siedek et al., 2000) have previously shown that the Ki-M6 and CZ2.2 are specific markers for equine macrophages. These particular markers are not present in polymorphonuclear leukocytes. While the anti-human Ki-M6 clone was shown to target equine CD68, the anti-equine CZ2.2 target molecule remains to be characterized. The distribution pattern of these two markers differed in immunocytochemistry assays suggesting that they recognize macrophages at distinct differentiation stages (Siedek et al., 2000). The expression of macrophage specific markers further confirms that EML-3C cells are of the monocyte-macrophage lineage. The monocyte-derived cells can also be differentiated and characterized in subsets of cells with pro-inflammatory or antigen presenting cell properties. For that degree of characterization, a complex system of cell markers has been identified and is used in studies of human and murine cell types, although studies in the equine model have shown dissimilarities among these markers and species. Namely, MHC II expressions in humans and murine models are activation markers, while in equines MHC II was detected in resting lymphocytes (Crepaldi et al., 1986; Lunn et al., 1993). Additionally, within the horse model the absence of a comprehensive panel of equine-specific reagents for the characterization of monocyte subsets hinders better characterization of EML-3C clone. EML-3C cells were shown to be MHC-I⁺MHC-II⁺ and to express very low levels of CD86. MHC I and MHC II cell surface expression levels in EML-3C cells were lower as compared to the levels expressed by eMDM, but higher to those found in monocytes. The reported levels of expressions of MHC I, MHC II, and CD86 markers in primary monocytes and eMDM are highly variable among equine studies. These differences can be assigned to horse breed, age, and gender of the animals, in addition to the dissimilarities

of isolation and culture procedures used by different investigators. In this study the levels of MHC II presented by eMDMs and EML-3C were very similar to those reported for dendritic cells isolated by treatments with reqIL4 and huGMSF (Rivera et al, 2005) or by reqIL4 and eqGMSF (Mauel et al, 2006). The increased expression of this marker can be explained by the presence of 10% equine serum in our medium formulation. The cytokines and chemokines present in the equine serum can induce differentiation of macrophages. Similar to what was reported for the human macrophage cell line, KG-1, it would be of interest to further study the influence of specific cytokines and stimulation molecules on the differentiation of these cells into dendritic-like cells (Teobald et al., 2008).

Proliferation of EML-3C induces morphological changes during the culturing process. Macrophage like morphology with large cytoplasmic regions and a stellate morphology can be seen in single cells in less confluent cultures (Figure 1B and 3A). In more confluent culture EML-3C cells become fibroblastic-like with characteristic dendrite like projections (figure 2A). Another characteristic of EML-3C confluent cell cultures is the formation of multicellular aggregates similar to those reported by Langerhans cells (data not shown).

Collectively NSE staining, phagocytic activity, nitrite production in response to LPS, and the expression of macrophage specific and differentiation markers indicate that the EML-3C is a differentiated macrophage-like cell line that can be used as a valuable tool for the study of equine macrophage functions in immunity and infection.

A major goal of this study, however, was the generation of a suitable cell line that would enable the development of a model system for the *in vitro* study of interactions between EIAV and macrophage to dissect lentiviruses persistence and cytopathicity mechanisms. EML-3C cells express the EIAV cellular receptor, ELR1, at higher levels than those observed in primary macrophages. EML-3C cells were also capable of sustaining continuous viral replication of the pathogenic virus clone, EIAV_{pv}, at high levels for long periods. EML-3C cells hence now offer a new system for reproducible and reliable studies of the interactions of EIAV and macrophage *in vitro*, in contrast to the variability observed in EIAV infections of primary equine macrophages. Although not demonstrated in the work presented here, the availability of such an equine macrophage cell line can also be useful for the propagation of EIAV primary isolates and in turn possibly the analysis of macrophage/EIAV interactions and their cell killing properties.

The data presented here confirms that the EML-3C cell line is an equine macrophage cell line that spontaneously arose without the complications of co-infecting oncoviruses or exogenous introduction of viral transforming genes. This cell line has been demonstrated to possess functional properties of macrophage, to possess the cellular receptor of and be infectable by an equine macrophage-tropic lentivirus. Taken together these data indicate that the described cell line will be of use for multiple disciplines that include but are not limited to studies of inflammation, numerous equine microbial infections, and macrophage phagocytosis.

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Figure 1. EML cells possess macrophage-like morphology but divide like a cell line

Phase contrast micrographs and fluorescent microscopy of **(A)** equine primary monocyte derived macrophages after seven consecutive days of culture; and **(B)** newly established EML-3C cell line. Immunofluorescence staining of both cell types represents cellular vimentin (red) and nuclei (blue). After passage four, EML cells became more homogeneous morphologically, and some cells appeared to be binucleated; in less dense cultures they show extended filopodia and large cytoplasmic extensions. (Scale bars: micrographs = 12µm; fluorescent microscopy = 6µm). **(C)** Histogram showing loss of CFSE fluorescence through cellular divisions of the EML-3C cell line. The number of cell divisions over a six-day CFSE staining period was determined by FlowJo Software. The cellular divisions are indicated on

the top of histograms and were calculated by sequential halving of CFSE fluorescence. **(1D)** Contour plot of CFSE data analyses. Cell generations were also calculated by FlowJo. For specifics of CFSE assay see Materials and Methods.

Figure 2. EML-3C cells phagocytose E. Coli K12 Alexa 488 bioparticles

(A) Fluorescent microscopy and phase contrast of representative fields taken from phagocytosis assays performed in cultures of NIH/3T3 (panels 1–2), RAW264.7 (panels 3–5), and EML-3C cells (panels 6–8). Phase contrast cells were imaged at 37°C (panels 2, 5, and 8). Imaging of phagocytosis immunofluorescence was performed at 4°C (panels 3 and 6) and 37°C (panels 1, 4 and 7). Scale bar = 25µm. Fluorescent bioparticles are imaged as white particles in the micrographs. (B) Phase contrast micrographs of EML-3C cells (B, panel 1) and deconvoluted microscopy of EML-3C cells displaying progressive Z stacks showing internalization of fluorescent bioparticles (panels 2–4). Merged immunofluorescence micrographs of Alexa 488 bioparticles (green) with an anti-vimentin-Cy3 antibody (red) and DAPI staining of nuclei (blue) (B, second, third and fourth panel). Scale bar = 12.2µm.

A**Non Specific Esterase**

50%confluency

99% confluency

+Sodium Fluoride (+NaF)

B**Nitrite production** **Figure 3. EML-3C cells display macrophage-like functional enzymatic characteristics**

Non-specific esterase enzymatic activity (**A**) of EML-3C cells in 50% confluent (first panel) and $\geq 90\%$ confluent (second panel) cultures. Control EML-3C culture (third panel) was assayed in the presence of sodium fluoride (NaF), a specific inhibitor of macrophage non-specific esterase enzymatic activity (see Materials and Methods for details). Note the different NSE staining patterns in EML-3C cultures at different confluency levels (first and second panel). Nitrite production (**B**) in response to LPS ($1\mu\text{g/ml}$, 72 hours of incubation) was determined by the Greiss assay in both EML-3C and RAW264.7 cells.

A. Macrophage specific markers

B. Differentiation markers

Figure 4. FACS analysis of EML-3C cells

Immunophenotyping of EML-3C cells, eMDMs, and equine monocytes with macrophage-specific markers (A) and differentiation markers (B). (A) Macrophage-specific CZ2.2 surface expression and CD68 (Ki-M6 clone) intracytoplasmic staining was analyzed in eMDM and EML-3C cells. (B) MHC I (CZ6 clone, a kind gift of Dr Antzack), MHC II (Tu39 clone) and CD86 (IT2.2 clone) surface expression of EML-3C cells, eMDMs and equine monocytes was measured as described in Materials and Methods. White histograms represent isotype controls.

Figure 5. EML-3C cells are susceptible to EIAV infection

(A) EML-3C cells and eMDMs were stained for the cellular receptor of EIAV, ELR-1, and analyzed by FACS. Dotted lines represent isotype controls. (B) Replication kinetics of EIAV_{PV} was measured in different cell types: EML-3C cells, FEK and eMDM. Cells were all infected at a MOI of 0.01 and supernatant samples collected for reverse transcriptase activity analysis over an 18 day period.

Table 1

Comparative analysis of the proportion of cells with intracytoplasmic fluorescent bioparticles in Raw 264.7 and the newly established EML-3C cell line*

Cell line	N° of fields	Total N° of cells counted	N° of cells with fluorescent bioparticles	%
Raw 264.7	8	323	198	60.9%
EML-3C	35	325	175	53.8%

* For details see Materials and Methods. The significantly smaller number of cells counted in the same number of fields of the two cell cultures reflects the difference in size between the equine and the murine cells. See text for differences in number of particles per cell.