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Postprint: In Vitro Induction, Culture, and Identification of Bovine Peripheral Blood Dendritic Cells

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Abstract

Dendritic cells were induced from peripheral blood mononuclear cells in vitro using granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), establishing a foundation and providing a cellular model for dendritic cell immunotherapy in the treatment of bovine mastitis. Bovine peripheral blood mononuclear cells were isolated using lymphocyte separation medium and cultured in a 6-well plate for 2 h. The supernatant containing abundant T cells and B cells was then discarded, with the adherent cells being predominantly monocytes. After washing five times with phosphate-buffered saline, 2 mL of culture medium containing GM-CSF and IL-4 was added for a 3-day induction. Subsequently, 1.4 mL of medium was carefully aspirated and discarded from the top of the culture, followed by the addition of 1.8 mL of fresh medium containing GM-CSF and IL-4 to continue the induction for an additional 3 days. Cell morphology was observed daily under a microscope. On day 7, the expression of cell surface antigens CD11c, CD14, Major Histocompatibility Complex class II (MHC II), CD40, CD80, and CD86 was detected by flow cytometry. The results showed that: 1) On day 2, some cells developed spikes accompanied by pseudopodia formation. On day 3, spikes and pseudopodia on the cell surface became increasingly abundant. On days 4 and 5, cells bearing spikes and pseudopodia began to aggregate and fuse. By day 6, monocytes were essentially differentiated into dendritic cells, with the cell surface exhibiting numerous clearly visible spikes and pseudopodia. 2) Flow cytometry detection revealed that CD14-, CD11c-, and MHC II-positive cells accounted for 6.8%, 65.0%, and 75.9% of the induced cells, respectively, while CD80- and CD86-positive cells accounted for 2.0% and 1.2% of the induced cells, respectively. In summary, bovine dendritic cells of certain purity can be obtained through in vitro induction of bovine peripheral blood monocytes.

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Full Text

Bovine Peripheral Blood Monocyte Derived Dendritic Cell Culture and Identification in Vitro

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Abstract: This study aimed to induce dendritic cells from bovine peripheral blood monocytes using granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), thereby establishing a foundation and providing a cellular model for treating bovine mastitis via dendritic cell immunotherapy.

Bovine peripheral blood mononuclear cells were isolated using lymphocyte separation medium and cultured in 6-well plates for 2 hours. The supernatant containing large numbers of T and B cells was discarded, leaving adherent cells that were predominantly monocytes. After washing five times with phosphate-buffered saline (PBS), 2 mL of culture medium containing GM-CSF and IL-4 was added for 3 days of induction. Subsequently, 1.4 mL of medium was carefully aspirated from the top of each well and replaced with 1.8 mL of fresh medium containing GM-CSF and IL-4 for an additional 3 days of induction. Cell morphology was observed daily under a microscope. On day 7, flow cytometry was performed to detect expression of cell surface antigens CD11c, CD14, major histocompatibility complex II (MHC II), CD40, CD80, and CD86. The results showed: (1) On day 2, some cells began to develop spikes accompanied by pseudopodia growth. By day 3, spikes and pseudopodia became increasingly abundant. On days 4-5, cells with spikes and pseudopodia started to aggregate and fuse. By day 6, monocytes were essentially fully induced into dendritic cells with numerous clearly visible spikes and pseudopodia on their surfaces. (2) Flow cytometry analysis revealed that CD14-, CD11c-, and MHC II-positive cells accounted for 6.8%, 65.0%, and 75.9% of the induced cells, respectively, while CD80- and CD86-positive cells represented 2.0% and 1.2% of the induced population. In conclusion, bovine peripheral blood monocytes can be induced in vitro to obtain bovine dendritic cells of considerable purity.

Keywords: cellular immunotherapy; monocyte; dendritic cell; bovine mastitis

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Research on dendritic cell phenotype, function, and cellular immunotherapy has become a hot topic in recent years [1]. Cellular immunotherapy primarily involves collecting blood from diseased animals, isolating peripheral blood mononuclear cells in vitro, directionally inducing them into dendritic cells under specific culture conditions, co-incubating the induced cells with pathogens or antigens, and finally injecting them back into the animal via intravenous administration [2]. Bovine mastitis is one of the most important diseases in dairy cows,

directly causing decreased milk production and even culling, which results in serious economic losses to the livestock industry [3]. Therefore, using immature dendritic cells to treat bovine mastitis holds significant importance. However, the challenge of obtaining sufficient purity of immature dendritic cells in vitro has hindered the development of cell immunotherapy for mastitis. Moreover, the number of immature dendritic cells in animal peripheral blood is extremely low, comprising only 1% of peripheral blood mononuclear cells, making direct sorting of immature dendritic cells from peripheral blood unfeasible. Most precursor cells in animals are monocytes, which can directionally differentiate into target cells under certain conditions. Therefore, this study obtained monocytes in vitro and induced them with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) cytokines to obtain immature bovine dendritic cells. This approach also helps elucidate the relationship between nutrition and immunity in dairy cows.

Numerous antigen-presenting cells exist in humans and animals, such as monocytes, macrophages, and dendritic cells. Among these, dendritic cells are the most potent antigen-presenting cells in the body. Dendritic cells undergo two stages during differentiation: immature and mature [4]. Werling et al. [5] isolated bovine dendritic cells from peripheral blood in vitro and used them to capture and absorb bovine respiratory syncytial virus, demonstrating that bovine dendritic cells could process this virus. Schreiner et al. [6] co-cultured dendritic cells, T cells, and Staphylococcus aureus regulatory peptides, finding that these peptides could enhance dendritic cell and T cell function and modulate naive T cell activity. Guermonprez et al. [7] co-cultured dendritic cells with malaria parasites, inducing T cell activation, particularly CD8 cytotoxic T cells, which could inhibit parasite proliferation. This study aimed to induce peripheral blood monocytes into immature dendritic cells in vitro, observe morphological changes, identify surface molecule expression, and provide material for co-culturing immature dendritic cells with bovine mastitis pathogens, thereby establishing a cellular model for treating bovine mastitis via cell immunotherapy.

1 Materials and Methods

1.1 Materials

Blood samples were collected from the jugular veins of three Holstein cows into sterile heparin sodium anticoagulant tubes, placed on ice, and immediately transported to the laboratory. RPMI-1640 complete culture medium and fetal bovine serum (Australia) were purchased from Gibco; penicillin (100 U/mL) and streptomycin (100 μg/mL) from Sigma; GM-CSF and IL-4 from Kingfisher; lymphocyte separation medium from Cedarlane; 2-mercaptoethanol (2-ME) from Amresco. CD11c, CD14, MHC II, CD40, CD80, CD86, and isotype control IgG1, IgM, and IgG2a (all mouse monoclonal antibodies, 1:50) were purchased from the University of Washington. PerCP-Rat Anti-Mouse IgG1 (1:4) was from BD; PE-Goat Anti-Mouse IgM and FITC-Goat Anti-Mouse IgG2a (1:50) were from Southern Biotech.

1.2 Isolation of Bovine Peripheral Blood Monocytes

Twenty milliliters of blood was collected from dairy cows into heparin sodium anticoagulant tubes and diluted 1:1 with PBS containing 100 U/mL penicillin and 100 µg/mL streptomycin, yielding a total volume of 40 mL. Lymphocyte separation medium was added to the bottom of a 50 mL centrifuge tube, and the diluted bovine blood was slowly layered on top. The sample was centrifuged at 2000 rpm for 30 minutes. After centrifugation, cells separated into distinct layers: serum/diluent, lymphocytes, separation medium, and erythrocytes/granulocytes. The lymphocyte layer was transferred to a new 50 mL tube, PBS was added to bring the total volume to 40 mL, and the sample was centrifuged at 2000 rpm for 10 minutes before discarding the supernatant. The cell pellet was resuspended in 800 µL of erythrocyte lysis buffer, then additional lysis buffer was added to reach 20 mL total volume and incubated at room temperature for 5 minutes. After centrifugation at 2000 rpm for 10 minutes, the supernatant was discarded. The pellet was resuspended in 800 µL PBS, then PBS was added to 40 mL total volume and centrifuged at 2000 rpm for 10 minutes; the supernatant was discarded. The cells were resuspended in 1 mL PBS, and the contents of four tubes (4 mL total) were transferred to a 15 mL centrifuge tube and kept on ice. Cells were diluted 80-fold for counting.

1.3 Differentiation Induction of Bovine Peripheral Blood Dendritic Cells

Based on cell counting, 10^7 cells were seeded into each well of a 6-well plate with 3 mL of culture medium containing final concentrations of 50 ng/mL GM-CSF, 50 ng/mL IL-4, 0.5 mol/L 2-ME, 100 U/mL penicillin, and 100 µg/mL streptomycin, then incubated at 37°C in a 5% CO_2 incubator for 3 days. Subsequently, an appropriate volume of medium was aspirated from the top of each well and replaced with fresh medium containing the same components. Cultures were maintained for 7 days, with cells harvested on day 7 for flow cytometric analysis. Cell morphology was observed daily under a microscope throughout the 6-day culture period.

1.4 Phenotypic Identification of Bovine Dendritic Cells

A suspension of 10^6 induced cells was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 2000 rpm for 3 minutes; the supernatant was discarded. Cells were resuspended in 100 µL PBS, and 50 µL of primary antibody was added for a total volume of 150 µL, then incubated at 4°C for 30 minutes. The cells were then centrifuged at 4°C, 2000 rpm for 3 minutes; the supernatant was discarded, and this washing step was repeated three times. Corresponding secondary antibodies (PerCP-Rat Anti-Mouse IgG1, PE-Goat Anti-Mouse IgM, and FITC-Goat Anti-Mouse IgG2a) were added and incubated at 4°C in the dark for 30 minutes. After centrifugation at 4°C, 2000 rpm for 3 minutes, the supernatant was discarded, and the washing step was repeated three times. Dendritic cell phenotypes were detected using a FACS Aria flow cytometer.

Isotype control primary antibodies were added to each sample as controls to eliminate non-specific fluorescence differences.

2 Results

2.1 Morphological Identification of Bovine Peripheral Blood Dendritic Cells

As shown in Figure 1 [Figure 1: see original paper], on day 1 (2 hours after seeding), peripheral blood mononuclear cells began to adhere, though attachment was not firm. Numerous suspended cells remained in the supernatant, mostly T and B cells. After adding GM-CSF and IL-4 cytokines, monocytes began to differentiate. On day 2, some cells developed surface spikes accompanied by pseudopodia growth. By day 3, spikes and pseudopodia became increasingly abundant. On days 4-5, cells with spikes and pseudopodia began to aggregate and fuse. By day 6, the cells exhibited typical dendritic cell morphology under microscopy, with more prominent surface spikes and extensions projecting outward. Some cells increased in size and displayed numerous dendrites of varying lengths and sizes, with some projections being relatively straight while others were curved and stacked to form larger protrusions. Meanwhile, a small number of cells began to senesce and detach.

2.2 Phenotypic Identification of Bovine Dendritic Cells

As shown in Figures 2 [Figure 2: see original paper] and 3 [Figure 3: see original paper], after 6 days of induction with GM-CSF and IL-4 cytokines, CD14-positive cells accounted for 6.8% of the induced population, while CD11c-positive cells represented 65.0%, and MHC II-positive cells comprised 75.9%. These results indicated that most adherent monocytes had been successfully induced into dendritic cells. However, dendritic cells exist in two states in vivo. Typically, dendritic cells in the bloodstream are in a mature state, monitoring for foreign invaders and immediately initiating immune responses upon detection of foreign antigens, at which point immature dendritic cells become mature. The surface markers used to identify mature dendritic cells are CD80 and CD86. As shown in Figure 4 [Figure 4: see original paper], CD80- and CD86-positive cells represented only 2.0% and 1.2% of the induced population, respectively, indicating that the induced dendritic cells were in an immature state. At this stage, they could efficiently uptake, process, and present antigens while maintaining strong migratory capacity. In contrast, mature dendritic cells can effectively activate naive T cells and are responsible for initiating, regulating, and maintaining immune responses.

3 Discussion

3.1 Culture and Morphological Analysis of Bovine Dendritic Cells

Dendritic cells were first discovered by Canadian scientist Steinman in 1973 and are currently known as the most potent antigen-presenting cells, named for their numerous dendritic or pseudopodia-like projections [8]. They possess unique functions in inducing primary immune responses, with immature dendritic cells exhibiting strong capacity for antigen uptake, processing, and handling, while mature dendritic cells show reduced antigen uptake and processing [9]. Their role in cellular immunity and their unique ability to activate naive T cell responses have made them an important research target for disease therapy. However, research on dendritic cells in livestock has been limited, particularly regarding their use in treating bovine mastitis. Bovine mastitis has long been a major obstacle to dairy industry development. Current treatments typically involve large amounts of antibiotics, which lead to antibiotic resistance in pathogens and, more importantly, antibiotic residues in milk that pose health risks to humans. Guermonprez et al. [7] demonstrated that dendritic cells could activate T cell immune responses during malaria infection, effectively inhibiting parasite growth. Schreiner et al. [6] showed that Staphylococcus aureus phenol-soluble modulin peptides could modulate dendritic cell function and activate regulatory T cells in vitro. Lei et al. [10] co-cultured dendritic cells with Mycobacterium avium subspecies paratuberculosis, finding that infected bovine dendritic cells had impaired function and activity, suggesting that paratuberculosis infection limits dendritic cell phenotype and function. Cruz-Adalia et al. [11] studied mouse dendritic cells capturing antigens and presenting them to T cells, ultimately showing that T cells could kill bacteria and provide immune protection to mice. Dendritic cells originate from hematopoietic stem cells through two main pathways: myeloid stem cells differentiate into dendritic cells under stimulation by GM-CSF and IL-4, forming myeloid dendritic cells that share common progenitors with monocytes and granulocytes, including Langerhans cells, epidermal dendritic cells, and monocyte-derived dendritic cells. The second pathway originates from lymphoid stem cells, forming lymphoid dendritic cells [12] that share common progenitors with T cells and natural killer cells. In vivo, dendritic cells constitute less than 1% of peripheral blood mononuclear cells but possess abundant antigen-presenting molecules MHC I and MHC II and co-stimulatory factors CD40, CD40L, CD80, and CD86, making them powerful professional antigen-presenting cells. However, myeloid and lymphoid dendritic cells differ in morphology and function. Our results showed that by day 6, the induced dendritic cells exhibited typical dendritic morphology under microscopy, with prominent surface spikes indicating abundant antigen-capturing receptors on the cell membrane.

3.2 Identification of Bovine Dendritic Cells

Adding GM-CSF and IL-4 cytokines to monocytes represents the optimal approach for obtaining dendritic cells in vitro. However, dendritic cells derived

from bone marrow and peripheral blood exhibit inconsistent phenotypes. Borkowski et al. [13] and Galy et al. [14] identified two distinct lineages of dendritic cells in humans and mice: myeloid and lymphoid. Our results showed that dendritic cells induced from peripheral blood monocytes displayed a $CD11c^{+}/CD14^{-}$ phenotype. Miyazawa et al. [15] studied bovine peripheral blood dendritic cell phenotypes and identified them as $CD11c^{+}/CD172A^{+}$, indicating myeloid dendritic cells. Bovine dendritic cells from the thymus exhibited a $CD1^{+}/CD172A^{+}$ phenotype, further suggesting that myeloid dendritic cells lack CD1 molecules. Mouse peripheral blood dendritic cells have also been reported to express CD11c [16], and Renjifo et al. [17] detected CD11c expression on bovine peripheral blood dendritic cells. These results demonstrate that $CD11c^{+}$ can serve as a phenotypic marker for peripheral blood dendritic cells. Functionally competent dendritic cells must be capable of activating naive T cells and initiating primary immune responses. Dendritic cells play crucial roles in mucosal immune systems, as intestinal mucosa and mammary tissue are frequently attacked by foreign pathogens [18]. When the mammary gland is infected by bacteria, resident lymphocytes such as dendritic cells and natural killer cells capture antigens and immediately initiate immune responses to kill pathogens. Meanwhile, mammary epithelial cells suffer lethal damage from bacteria, ultimately leading to decreased milk production. Therefore, integrating nutrition and immunity has profound implications for livestock development, particularly regarding recent findings on dendritic cell capture, processing, and presentation of foreign antigens to T cells in lymph nodes for killing effects. Our results demonstrated that dendritic cells induced from peripheral blood monocytes in vitro exhibited a $CD11c^{+}/CD14^{-}/CD80^{-}/CD86^{-}$ phenotype, characteristic of typical immature dendritic cells that can serve as cellular material for immunotherapy to treat bovine mastitis.

Conclusion: 1. After 6 days of in vitro induction of peripheral blood monocytes into dendritic cells, the cell surfaces contained numerous clearly visible spikes, and cells extended outward with dendritic pseudopodia. 2. Flow cytometric analysis of induced cell phenotypes revealed that CD14-, CD11c-, and MHC II-positive cells accounted for 6.8%, 65.0%, and 75.9% of the induced population, respectively, while CD80- and CD86-positive cells represented 2.0% and 1.2%, indicating that monocytes had been induced into immature dendritic cells. 3. Bovine dendritic cells of considerable purity can be obtained by in vitro induction of bovine peripheral blood monocytes.

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