# Comparison of the Requirement of Porcine Peripheral Blood Monocytes and Intestinal Lamina Propria Macrophages as Accessory Cells in Primary Allogeneic Mixed Leukocyte Responses and Oxidative Mitogenesis

Dominic Mukama KAMBARAGE*, Paul BLAND, and Chris STOKES

*Department of Veterinary Medicine, Langford House, Langford, Bristol. BS18 7DU. UK*

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ABSTRACT. Differences and/or similarities of the accessory cell activity of circulating peripheral blood monocytes (PBM) and intestinal lamina propria macrophages (LPM) in the pig have never been evaluated. Therefore this study was designed to compare the accessory cell activity of these cells in the induction of primary allogeneic mixed leukocyte responses (MLR) and polyclonal mitogenesis. Splenic adherent cells (SPAC) were used as control accessory cells. It was observed that PBM, LPM and SPAC induced significant T cell proliferation in response to allo-antigens. Significantly higher responses were elicited by SPAC, followed by PBM whereas, LPM induced low responses when cultures were established a T cell: accessory cell ratio of 1:1. PBM was the only accessory cell which induced significant responses at a cell ratio of 10:1. Whereas LPM and SPAC were able to induce significant oxidative mitogenesis, PBM were poor stimulators of this response. The observed difference between PBM, SPAC and LPM is attributed partly to differences in number of major histocompatibility complex (MHC) class II positive cells and the intensity of expression of the glycoproteins.—KEY WORDS: accessory cell, macrophage, MHC class II antigen, mixed leukocyte response, monocyte and oxidative mitogenesis.

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The presence of non-lymphoid cells in the intestinal lamina propria in animals is well documented [7, 13, 18, 20, 24, 25]. These cells are thought to be responsible for the induction or suppression of certain immune responses in the gastro-intestinal tracts of animals. For instance, murine [16] and human [11] intestinal macrophages have been shown respectively to suppress and induce T cell proliferation in response to allo-antigens *in vitro*. This species difference has never been fully studied, but may be due to differential secretion of suppressive factors as shown in some studies [3, 16].

Peripheral blood monocytes constitute the main adherent cell population in the blood. However, their role as antigen presenting cells (APC) has been shown to vary with species. For instance, whereas human peripheral blood monocytes are poor stimulators of T cell responses [3], bovine cells have been shown to be effective APC [5]. Because circulating monocytes are recruited into normal as well as inflammatory sites [14, 15, 19, 20], they are able to modulate responses induced by local APC. Therefore, it was of interest to evaluate similarities or differences in the accessory cell activity of porcine peripheral blood monocytes (PBM) and intestinal lamina propria macrophages (LPM) in order to assess the probable effect on mucosal immune responses when the former are recruited into the gastro-intestinal tract of the pig. In this study splenic adherent cells (SPAC) were used as control APC and systems used to assess the effectiveness of the accessory cells were mixed leukocyte responses (MLR) and oxidative mitogenesis.

* PRESENT ADDRESS: KAMBARAGE, D. M., Department of Veterinary Medicine and Public Health, Faculty of Veterinary Medicine, Sokoine University of Agriculture, P. O. Box 3021, Morogoro, Tanzania.

## MATERIALS AND METHODS

*Animals used*: Large White pigs of 6–8 months of age from minimal-disease and enzootic pneumonia-free herds were used in this study.

*Media and antibodies*: Tissue culture medium used was RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate, 20 mM Hepes (Flow Laboratories, Irvine, Scotland), 10 $\mu$M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, U.S.A.), penicillin / streptomycin preparation used at 100 units and 100 $\mu$g per m*l* respectively and 40 $\mu$g per m*l* gentamicin. Tissue culture medium but with only 5% FCS was used for enzyme digestion and washing of intestinal cells. Monoclonal antibody, MSA3 (mouse anti-pig SLA II, (MHC class II) DR epitopes) was kindly provided as a hybridoma cell line by Dr. J. Lunney (United States Department of Agriculture, Beltsville, Maryland).

*Preparation of cells*:

*Peripheral blood monocyts (PBM)*: Blood, aseptically collected from jugular veins, was incubated at 37°C for 2 hr and the plasma was layered on Ficoll-pague (Flow Laboratories) and centrifuged at 800 g for 40 min at room temperature. Cells at the interface were collected, washed three times in Hanks balanced salt solution (HBSS) for 5 min at 400 g. They were then incubated on Petri dishes [4]. Non-adherent cells were removed by three times repeats of swirling the Petri dishes with warmed HBSS and decanting. Adherent cells were eluted by incubating the cells in tissue culture medium supplemented with 10% ethylene diamine-tetra acetic acid (EDTA). Eluted cells were washed three times in HBSS (400 g for 5 min) and are referred to as PBM.

*Lamina propria macrophages (LPM)*: Aseptically collected jejunal portion of the small intestine, devoid of Peyer's patches, was cut into small piece (0.5 × 0.5 cm) after the serosal muscle layer was peeled off. Tissues were stirred six times in HBSS supplemented with 1 mM EDTA on a magnetic plate each for 30 min. Tissues were then stirred in tissue culture medium containing 5% FCS and 100 units per m*l* of collagenase type V (Sigma) and hyaluronidase type A-S (Sigma) for 90 min. Cell suspension was discarded and the procedure was repeated twice. The cell suspensions from the last two stages were pooled and passed through a bolting cloth (Plastock Holdings, Birkenhead, UK) and washed (300 g for 8 min at 5°C) three times in tissue culture medium with 5% FCS (washing medium). Lamina propria cells were then re-suspended in 3 m*l* of 20% Nycodens (Nycomed AS, Oslo, Norway, density 1.15 g/m*l*), layered on 5 m*l* of 40% Nycodens and centrifuged at 650 g for 20 min at room temperature. The cells which were collected at the interface were washed three times in washing medium. Cell viability was determined by trypan blue exclusion.

*Splenic adherent cells (SPAC)*: Pieces of spleens were meshed through wire sieve into cold HBSS and the cell suspension was passed through a bolting cloth. The cells were washed three times in HBSS and red blood cells were lysed by hypotonic shock. The remaining cells were then passed through nylon wool columns [9]. SPAC eluted by teasing the columns in warm tissue culture medium were used as control accessory cells in proliferative assays.

*T cells*: Pieces of mesenteric lymph nodes aseptically excised were passed twice through nylon wool columns [9] and the eluted non-adherent cells are referred to as T cells. They were used as responding cells in proliferative assays.

*Proliferative assays*: Periodate-treated [2] and untreated T cells ($2 \times 10^5$ cells/200 $\mu l$/well (in tissue culture medium) were cultured either alone or with PBM, SPAC or LPM at a T cell: APC ratio of 1:1 for 72 hr. This experiment was repeated four times. In some cultures (three experiments), allogeneic T cells were cultured either alone or with APC for 120 hr at 37°C with 5% $CO_2$. Cells were cultured in six replicates in 96-well plates (Nunclon, Nunc Inter Med, Roskilde, Denmark). Four hr before termination of cultures, cells were pulsed with 1.0 $\mu$Bq of [$^3$H] thymidine (Amersham International PLC, Buckinghamshire, UK) in 20 $\mu l$ of tissue culture medium. Cells were then harvested onto glass discs (Skatron, AS, Lier, Norway) using a multiharvester (Skatron AS) and radioactivity was measured by a liquid scintillation counter (LKB Wallac Rack Beta, Wallac Oy, Turku, Finland). Results are presented as counts per min with standard error of means (CPM+SEM) and the data was analyzed using multiple regression analysis of variance. Regression coefficient ratios were used to analyze statistical significant difference between the various treatments.

*Evaluation of expression of MHC class II antigens*: In order to establish reasons for the differences in the accessory cell effect of the cells, LPM, PBM and SPAC were assessed for the number of major histocompatibility complex (MHC) class II positive cells and the intensity of expression of the molecules. Briefly, the cells were incubated with either MSA3 used at a final dilution of 1:10 or tissue culture medium (control cells) for 45 min at 4°C. The cells were then washed three times in ice-cold HBSS supplemented with sodium azide (0.1%) at 4°C and then incubated with goat anti-mouse FITC conjugate (Sigma) used at a final dilution of 1:3,000 for 45 min. Cells were washed three times and immediately analyzed with an Epics CS Flow Cytometer (Epics Division, Coulter Corporation, Hialeah, U.S.A.). Positive staining was determined by using "Overtons Cumulative Subtraction" method which is a one-parameter histogram subtraction routine for test histograms with weak immunofluorescence.

## RESULTS

*Periodate mitogenesis*: The data of four experiments carried out to assess the accessory cell activity of PBM, SPAC and LPM in oxidative mitogenesis are summarised in Table 1. The results show that proliferative values of cultures containing untreated T cells and the various accessory cells were not statistically different from those of control cultures (T cells alone) ($p>0.001$). In three experiments with the exception of number 2, the responses evident in cultures containing sodium periodate-treated T cells alone were comparable to those of other controls.

Table 1. Accessory cell activity of LPM, PBM and SPAC in oxidative mitogenesis

| | Untreated T cells with | | | | Treated T cells with | | | |
|---|---|---|---|---|---|---|---|---|
| | No APC | SPAC | PBM | LPM | No APC | SPAC | PBM | LPM |
| Exp. 1 | 195±24 | 515±75 | 301±45 | 274±34 | 177±23 | 9103±940 | 980±104 | 3416±441 |
| Exp. 2 | 225±54 | 415±75 | 200±39 | 198±32 | 12047±1028 | 86825±9922 | 10127±510 | 48513±5132 |
| Exp. 3 | 461±70 | 934±103 | 529±70 | 571±76 | 602±132 | 5440±688 | 847±121 | 2127±182 |
| Exp. 4 | 217±62 | 289±40 | 400±52 | 327±48 | 568±93 | 9725±510 | 1067±196 | 4459±574 |
| Mean | 282±61 | 538±140 | 358±70 | 343±81 | 3349±2901 | 27773±19706 | 3255±22910 | 14629±11305 |

Data showing T cell proliferations in response to sodium periodate treatment following culturing untreated and treated T cells either alone or with accessory cells for 72 hr. Data are presented as counts per minute±standard error of means.

Table 2. Comparison of the accessory cell activity of LPM and PBM SPAC in the induction of allogeneic MLR

| | T cells with | | | | T cells with | | |
|---|---|---|---|---|---|---|---|
| | T cell: APC ratio of 1:1 | | | | T cell: APC ratio of 10:1 | | |
| | No APC | SPAC | PBM | LPM | SPAC | PBM | LPM |
| Exp. 1 | 123±27 | 29148±2701 | 15741±1806 | 7449±1158 | 330±75 | 16486±2001 | 440±40 |
| Exp. 2 | 362±73 | 26758±3366 | 17969±2786 | 4079±765 | 450±77 | 5997±783 | 480±125 |
| Exp. 3 | 150±15 | 29874±4888 | 9719±1522 | 7358±1578 | 465±98 | 4376±995 | 629±129 |
| Mean | 212±77 | 28593±959 | 14476±2510 | 6295±1129 | 415±44 | 8953±3867 | 516±59 |

Data showing the effectiveness of LPM, PBM and SPAC in the induction of primary allogeneic MLR after culturing the cells for 120 hr. Data are presented as counts per minute±standard error of means.

Significantly higher responses were induced only in cultures containing sodium periodate-treated T cells and either SPAC or LPM than in all controls ($p<0.001$). It was further observed that in all the experiments, SPAC induced significantly higher responses than those elicited by LPM ($p<0.001$). However, in contrast to the effectiveness of both SPAC and LPM, PBM were not capable of inducing significant responses of sodium periodate treated T cells.

*Allogeneic mixed leukocyte responses (MLR):* The data on the ability of LPM, SPAC and PBM to stimulate proliferation of allogeneic T cells show that the three accessory cells were capable of stimulating significantly higher T cell proliferation ($p<0.001$) in response to allo-antigens than those observed in control cultures (T cells cultured alone) (Table 2). However, differences in proliferative levels induced by the three accessory cells were observed at T cell:APC ratios of both 1:1 and 10:1. At a cell ratio of 1:1, SPAC induced significantly higher responses than PBM and LPM ($p<0.001$) and, the least effective accessory cell was LPM.

In contrast to the effectiveness of all the three APC in eliciting T cell proliferation in response to allo-antigens at a T cell:APC ratio of 1:1, it was only PBM which induced significantly higher responses at a cell ratio of 10:1 ($p<0.001$). Both SPAC and LPM did not induce significant ($p>0.001$) T cell proliferation at the T cell:APC ratio of 10:1 compared to the responses seen in control cultures.

*MHC class II antigen expression*: Flow cytometry results of the evaluation of MHC class II glycoprotein expression show that 49% of PBM, 40% of SPAC and 24% of LPM were MHC class II positive (Fig. 1). Comparison of the intensity of expression of MHC class II antigens showed that SPAC expressed the molecules slightly more intensely than PBM (the mean peak channel being 705±119 and 563±172 for SPAC and PBM respectively). However, the intensity of expression of the glycoproteins was more in LPM than the other two cell types.

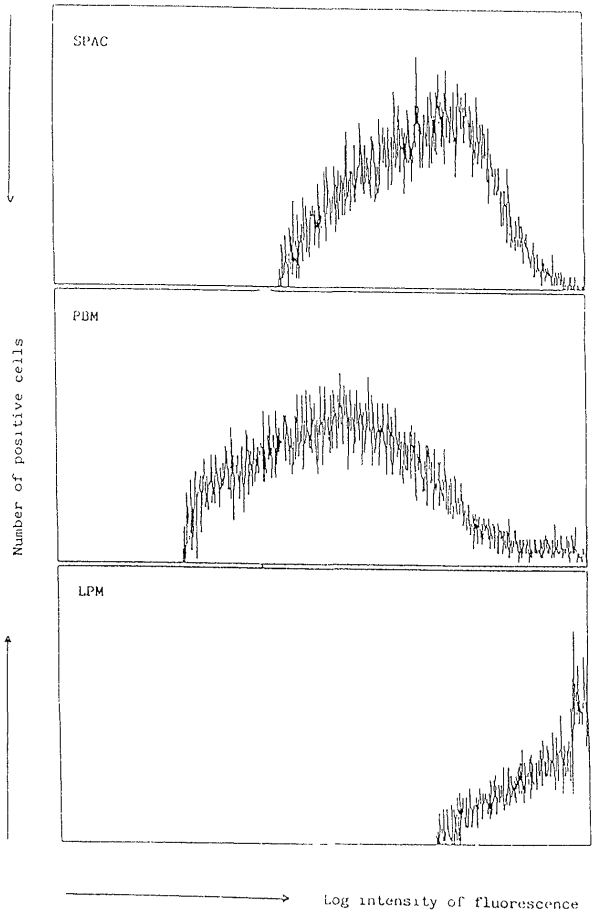


Fig. 1. Flow cytometry results showing positive staining for MHC class II antigens in SPACs, PBMs and LPMs as evaluated by indirect immunofluorescence. Y-axis represents frequencies (number of positive cells), whereas X-axis is the log intensity of green fluorescence.

## DISCUSSION

These results indicate that LPM, PBM and SPAC were capable of inducing significant allogeneic MLR. This ability of porcine LPM concurs with the observation made with human intestinal [11] and foetal placental [21] macrophages, but in contrast to murine intestinal cells which were shown to be suppressive [16]. The effectiveness of porcine PBM compares with that of bovine monocytes [5].

Differences in the accessory cell effect were observed between the three APC; SPAC were more effective than both PBM and LPM at T cell: APC ratio of 1:1 but at a higher ratio it was only PBM which were effective. Flow cytometry evaluation of MHC expression by the three APC showed differences in the number of MHC class II-positive cells and the intensity of expression of the glycoproteins. It was observed that although only few LPM were positive, they expressed the molecules more strongly than both SPAC and PBM. Slightly less intensity was evident on PBM which had more MHC class II positive cells than SPAC and LPM. Thus, it is possible that a combination of the difference in number of MHC class II positive cells and intensity of expression of the glycoproteins were responsible for the observed differences in the induction of T cell proliferation in MLR responses. For instance, despite LPM expressing the molecules more intensely than other cells, the weak responses induced by the former may partly be due to the fewer number of MHC class II positive cells than those in other APC types. However, adhesion molecules and other co-stimulatory factors may also be partly responsible for the observed differences in responses induced by the various accessory cells.

Both LPM and SPAC induced significant oxidative mitogenesis, although the latter were more effective than the former. PBM were poor stimulators of periodate mitogenesis, a T cell response which is both accessory cell- [1, 2] and MHC class II- [17] dependent. The poor accessory cell effect of porcine peripheral blood monocytes in oxidative mitogenesis has also been reported in man [22, 23].

Although it has been reported in one study that allogeneic MLR can occur without the involvement of interleukin-1 [13], it is believed that all T cell responses require a number of costimulatory factors [8]. In this study it was observed that PBM were able to stimulate primary allogeneic MLR and had more MHC class II positive cells than either SPAC and LPM. In addition, the intensity of expression of the MHC class II antigens was comparable to that of SPAC, which was the most effective accessory cells in oxidative mitogenesis. Thus, these results indicate that factors other than MHC class II glycoproteins were responsible for the ineffectiveness of PBM in oxidative mitogenesis. This poor accessory cell effect of PBM in periodate mitogenesis is probably due to lack of specific adhesion molecules which enable contact between the interacting cells. For instance, it has been reported that the poor antigen presenting activity of resting B cells was partly due to the relative inability to interact physically with T cells and this was overcome by neuraminidase treatment [10]. Other costimulatory factors needed specifically in this type of T cell response may also be lacking or deficient. This however requires further studies.

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