EVALUATION OF THE ABILITY OF
MYCOBACTERIUM CELL WALL FRACTION
(MCW) IMMUNOSTIMULANT TO ALTER BLOOD
LEUKOCYTE POPULATIONS IN NEWBORN
CALVES

(EXPERIMENT #47 FOR VETREPHARM RESEARCH, INC.)

DECEMBER 08, 1999

1. TITLE- Evaluation of the ability of Mycobacterium Cell Wall Fraction (MCW) immunostimulant to alter blood leukocyte populations in newborn calves

2. INTRODUCTION

2.1 Background

The newborn ruminant is highly susceptible to infectious disease and over 70% of deaths, during the first year of life, occur during the neonatal period (less than 28 days of age) [1]. This high disease susceptibility may be due, in part, to a functionally immature immune system. Studies of immune responses in newborn calves have identified a number of functional deficiencies. These deficiencies have included an age-related increase in blood T-lymphocyte populations [2], sub-optimal neutrophil function [3], decreased complement activity [4], poor induction of delayed hypersensitivity reactions [5], weak lymphocyte proliferative responses [6,7], and low levels of interleukin (IL)-2 production [8]. These decreases in immune function were most pronounced during the first two weeks after birth. Many of these changes may reflect normal development processes within the immune system. However, the presence of a cortisolemia for approximately 10-14 days after birth may also limit the functional capacity of leukocytes in the newborn calf [8,9].

Activation of the immune system in the newborn calf, particularly non-specific immune defences, could significantly reduce the risk of infection during this period. Mycobacterial components have been used in a wide variety of ways to activate various immune functions [10]. Mycobacterial cell wall extract fraction (MCW), of Mycobacterium phlei, has been shown to activate both specific and non-specific immune

responses. Thus, MCW has been used as an adjuvant for a variety of vaccines [11,12]. Furthermore, MCW has been shown to activate macrophages [13] and induce cytokine secretion [15]. These observations suggest that the administration of MCW may be an effective strategy to activate the immune system and enhance disease resistance in the newborn calf. In December 1995, MCW was licensed by the USDA for use in 1-4 day-old calves to treat *E. coli* infections, caused by strain K99. MCW is marketed by Vetrepharm Research, Inc. (Athens, GA) under the trade name IMMUNOBOOST.

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2.2 Study Objective

Field studies have revealed that the intravenous (IV) administration of MCW to newborn calves, less than 24 hours of age, induces statistically significant weight gain in comparison with non-treated calves. It is speculated that the weight gain may be induced by MCW's ability to activate a non-specific immunological response. Activation of the immune system may assist newborn calves to overcome disease during the first 10 days of life. Due to the efficiency of an activated non-specific immune response, less energy may be required to fight infectious disease agents and this conserved energy may be converted to an increased body weight. Thus, the objective of the present study was to evaluate the ability of MCW to alter either the phenotype or function of blood leukocytes in newborn calves, following a single injection of MCW at less than 24 hours of age. This analysis of blood leukocyte phenotype and function was used to determine if the immune system had been activated

3. REFERENCES

3.1 Production Outline for Mycobacterium Cell Wall Fraction Immunostimulant. Product Code 9300.02

4. PERSONS AND RESEARCH LABORATORIES INVOLVED IN THE STUDY

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4.2 Laboratories

This study was completed at the Veterinary Infectious Disease Organization (VIDO) in Saskatoon, Saskatchewan. All calves were housed in individual pens within the Animal Care facility at VIDO. The serum cortisol analyses was done by the Endocrine Laboratory, Prairie Diagnostic Services, Western College of Veterinary Medicine (WCVM) in Saskatoon, Saskatchewan, Canada. Clinical haematology was completed in the Clinical Diagnostic Laboratory, Prairie Diagnostic Services, WCVM. Post-mortem examinations were conducted at Prairie Diagnostic Services, WCVM, and histological evaluation of tissues was completed at VIDO. All immunological assays, including leukocyte function and phenotype analyses, were performed in the Immunology Laboratory at VIDO.

5. DATES OF START AND END OF THE STUDY

Clinical studies with calves began on September 08, 1999 and were completed on October 06, 1999. Data collection, analysis and collation were completed on November 20, 1999.

6. SUMMARY

The present investigation examined changes in the phenotype and function of blood leukocytes isolated from newborn calves. These parameters were used to evaluate the capacity of MCW to activate the immune system of newborn calves. Within 24 hours after birth, calves were given either a single subcutaneous injection of saline (Control; n = 5) or a single injection of 250 µg MCW. MCW was injected either intravenously (MCW-IV; n =5) or subcutaneously (MCW-SC; n = 5). Four days after MCW treatment, the MCW-SC group had significantly ($p < 0.05$) higher numbers of MHC Class II$^+$ CD4$^+$ T-cells/ ml of blood than Control calves. **This observation indicated that a single subcutaneous injection of MCW (250 µg) increased the frequency of activated lymphocytes in the blood of newborn calves.** Other comparisons between MCW-SC and Control groups revealed several differences on day 4 post-treatment that were consistent with this conclusion. These differences included a higher mean value for IFNγ production, a higher mean value for MHC Class II$^+$ monocytes, and a higher mean value for CD25$^+$ CD4 T-lymphocytes. These changes were not significantly different from the Control group but, collectively, these observations support the conclusion that a single subcutaneous injection of MCW increased the level of immune activation in newborn calves. There were no significant differences in the clinical parameters monitored (body temperature, body weight, hydration, behaviour, milk consumption, fecal consistency, and serum cortisol levels) that may explain these differences in immune activation.

7. MATERIALS AND METHODS

7.1 Description of Test Animals

Newborn, clinically healthy, male, colostrum-deprived Holstein calves were purchased by VIDO from four commercial dairy farms, within a 25 mile radius of Saskatoon, Saskatchewan. Newborn calves were collected from the farm of origin within 8 hours after birth. On arrival at VIDO, serum was collected from each calf and a zinc sulphate turbidity test was performed twice, at 3-hour intervals, to confirm that transfer of maternal antibody had not occurred. Each calf was identified with an All-flex ear tag, marked with a unique identification number. This identification number was attached to all blood and serum samples. Calves were housed in individual pens, within an open shelter, that prevented direct contact with other animals and were bedded with straw. Each pen was cleaned daily and disinfected between calves. Calves were fed twice daily with fresh milk obtained from the Dept. of Animal and Poultry Science Dairy facility, University of Saskatchewan (Saskatoon, Canada). No other medications or vaccinations, except the test product, were administered throughout the study period. Calves that did not survive the four-day study period were eliminated from the study.

7.2 Description of Immunostimulant

Mycobacterium Cell Wall Fraction Immunostimulant (Serial Number 98064A; Expiry Date- 10. 23. 00) was produced according to the current production outline standards filed with the USDA as product code 9300.02. The trade name of this product is IMMUNOBOOST®. IMMUNOBOOST contains 250 µg of MCW/ml, 30 µg/ml gentacin, and 20 µl/ml Squalene oil. These components are emulsified in phosphate

buffered saline (PBS) solution containing an emulsifier. This product was supplied

directly from Vetrepharm Research, Inc., Athens, GA.

7.3 Experimental Design

Fifteen (15) calves were randomly divided into three (3) groups: **Group I**- Five (5) calves

received a single intravenous (IV) injection of MCW (MCW-IV); **Group II**- Five (5)

calves received a single subcutaneous (SC) injection of MCW (MCW-SC); and **Group**

III- Five (5) calves received a single, one (1) ml SC injection of physiological saline

solution. The dose for MCW was 250 µg of active material in an injection volume of one

(1) ml. MCW and saline were administered once to calves at less than 24 hours of age.

The designated injection site was the left side of the neck. All blood and serum samples

were collected from the right jugular vein to ensure that any reactions at the site of

injection were specific to MCW.

7.4 Clinical Observations

A clinical veterinarian examined the calves twice daily and recorded body

temperature, behaviour, milk consumption, hydration, and fecal consistency. Behaviour,

milk consumption, and fecal consistency were scored on a scale of 0 (normal) to 3 (severe

symptoms) and dehydration was scored on a scale of 0 (normal) to 2 (severe dehydration).

Calves were weighed each morning, prior to feeding. The injection site was shaved and

observed daily for any signs of swelling, induration, or pain.

7.5 Blood and Serum Collection

Blood and serum collection was performed between 8-9:00 AM, following the morning feeding. Whole blood was collected in sterile Vacutainers containing EDTA. One tube was collected for clinical haematology and a second tube was collected for flow cytometry and the IFNγ production assay. Serum samples were collected in sterile, siliconized, blood separation Vacutainers. Blood and serum samples were collected immediately prior to treatment with MCW or saline and then daily for 4 days after treatment. Serum was separated within 4 hours after collection and duplicate samples were stored at -70 $^{\circ}$C.

7.6 Clinical Haematology

Total white blood cell (WBC) counts and differential counts were performed by medical laboratory technicians in the Clinical Pathology Laboratory, Prairie Diagnostic Services, Western College of Veterinary Medicine (WCVM), Saskatoon, Saskatchewan. Total WBC counts were performed with a Cell-Dyn 3500 R Analyzer (Abbott Laboratories) and Sheath Reagent (WIC/HGB Lyse Diluent). Differential counts of monocytes, neutrophils, eosinophils, and lymphocytes were performed on Wright's stained blood smears that were stained on the automated Hematek Slide Stainer. One hundred (100) leukocytes are microscopically differentiated by a technologist and if there was variation from accepted 'normal' values then a second technologist counts another 100 leukocytes on a second slide.

7.7 Serum Cortisol

Serum cortisol analysis was performed in the Endocrine Laboratory, Prairie Diagnostic

Services, Western College of Veterinary Medicine (WCVM), Saskatoon, Saskatchewan.

Serum samples were stored at -70 ^{o}C and were analyzed using a flourescence polarization

immunoassay (TDx System, Abbott Laboratories, Irving, TX). Crossreactions in this

assay were 4.7% and 4.5% for 11-deoxycortisol and corticosterone, respectively, and <

0.1% for progesterone and testosterone. Sensitivity was 12.4 nmol/L. The range of the

standard curve was 10-1200 nmol/L. All samples were determined in one assay in which

the intracoefficient of variations were 5.4% (32.0 nmol/L), 3.2% (110.3 nmol/L) and

3.2% (407.5 nmol/L).

7.8 Flow Cytometry

Whole blood was lysed using an ammonium chloride solution. This procedure generated

a leukocyte population that included monocytes, lymphocytes, and polymorphonuclear

leukocytes (PMNs). This population is referred to as peripheral blood leukocytes (PBL).

Single cell suspensions were prepared from prescapular and mediastinal lymph nodes

(LNs) by mincing the tissue in phosphate buffered saline containing 0.1% EDTA and then

filtering the cell suspension through a 20 μm nylon mesh. PBL and LN cells were labelled

with the following monoclonal antibodies (mAbs) that are designated by clone number

and antigen specificity in brackets: Pig45A (immunoglobulin of the M-isotype-IgM);

MM1A (CD3); IL-A11 or CACT138B (CD4); CACT80C (CD8); BAQ4A (WC1 antigen

expressed on γδ T-cell receptor [TCR] T-lymphocytes); DH59B (monocytes and

granulocytes); TH14B (MHC Class II molecules); and CACT116A- (CD25). Labelling of

leukocytes with a single mAb was detected using FITC-conjugated goat-anti-mouse Ig

and dual-labelling of leukocytes with two mAbs was detected using FITC- and PE-

conjugated, isotype-specific goat anti-mouse Ig. Flow cytometric analyses were

performed using a FACScan flow cytometer and the CellQuest program. PMNs were

excluded from the flow cytometric analysis by using electronic gates based on forward

angle light scatter and right angle light scatter. This gating of data collection ensured that

DH59B$^+$ cells were monocytes and facilitated the calculation of cell number/ml blood for

individual mononuclear leukocyte populations. Blood was collected for flow cytometric

analysis of PBL prior to treatment of newborn calves and daily for four days after

treatment. Lymph nodes were collected five days after the treatment of newborn calves.

7.9 Assay for Interferon-gamma (IFNγ) Secretion

The production of IFNγ was assayed with the PBL population isolated for flow cytometric

analyses. PBL were cultured in 96-well plates with 2×10^5 cells/well in a final volume of

200 µl of serum-free medium (AIM-V; GIBCO/BRL) supplemented with 2% fetal bovine

serum and 2×10^{-5} M 2-mercaptoethanol. Triplicate cultures of PBL were incubated for

48 hours in the presence or absence of 10 µg/ml of Concanavalin A (Con A). Cell-free

culture supernatants were collected and stored at $-70\,^{\circ}$C. IFNγ production was assayed

with PBL isolated prior to treatment (Day 0) and daily for 4 days after treatment. The

level of IFNγ in culture supernatants was determined using a capture ELISA. The ELISA

plates were coated with the IFNγ-specific mAb 2-2-1 prior to adding 2-fold dilutions of

the culture supernatant. Captured IFNγ was detected with polyclonal rabbit anti-bovine

IFNγ. Known concentrations of recombinant bovine IFNγ were also added to the ELISA

plate to establish a standard curve for absorbance. This standard curve was used to

convert absorbance with culture supernatants to pg/ml of IFNγ.

7.10 Post-Mortem Examination and Histology

Calves were euthanized on the fifth day of the study. A complete post-mortem

examination was performed to identify any gross lesions of infectious disease. The

injection site was excised, examined for macroscopic evidence of pathology and

preserved in 10% phosphate buffered formalin for histopathological examination.

Sections of the prescapular and mediastinal lymph nodes were collected in ice-cold

phosphate buffered saline and used to isolate cells for flow cytometric analysis of

lymphocyte phenotype. Sections of the same lymph nodes were also fixed in 10%

phosphate buffered formalin for histopathological examination. Tissues samples of liver,

spleen, lung, and small intestine (jejunum and ileum) were also collected in 10%

phosphate buffered formalin. Carcasses were incinerated by Prairie Diagnostic Services

(WCVM) after the post-mortem examination was completed.

7.11 Data Analysis

Statistical analysis was performed using GraphPad Prism 2.01 software (Graphpad

Software, Inc. San Diego, CA). Differences among groups were determined using a one-

way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test if the

ANOVA revealed a significant difference ($p < 0.05$) among the groups. The analysis of

leukocyte activation (CD25 and MHC Class II molecule) was based on the assumption

that the Control group (saline) represented the normal state of activation in newborn

calves and that we could detect perturbations of this state by MCW treatment. However,

clinical records and haematology revealed that some calves displayed signs of co-

incidental infection. An infectious agent may also activate the immune system and alter

the level of leukocyte activation. Therefore, the analysis of leukocyte activation, which

was most pronounced on Day 4 post-treatment, was restricted to calves that had a total

clinical score less than one (1). The application of this criteria resulted in the exclusion of

one calf from each of the three experimental groups. This exclusion is clearly indicated in

all Figures by either presenting data for individual animals or stating that n = 4.

8. RESULTS

8.1 Clinical Haematology

The objective of this study was to determine if MCW treatment altered blood leukocyte populations. The first step in evaluating this question was to determine if, following MCW treatment, there were changes in the number of leukocytes present in blood. White blood cells includes monocytes, polymorphonuclear cells (neutrophils, eosinophils, and basophils), and lymphocytes. Each leukocyte subpopulation was quantified by first counting the total number of leukocytes/ml of blood and then determining what percentage each subpopulation contributed to the total leukocyte count. There were no significant differences among the three groups of calves when total white blood cell counts were compared either before or after treatment (Figure 1a). All three groups of calves displayed similar changes in blood leukocytes during the four-day post-treatment period. Briefly, there was a gradual increase in the total number of lymphocytes (Figure 1b) and a gradual decline in the number of mature neutrophils (Figure 1c). These changes were consistent with a decreasing level of serum cortisol since elevated serum cortisol is known to induce a neutrophilia and a lymphopaenia. All three groups of calves also displayed a gradual increase in the total number of monocytes (Figure 1d). The presence of immature neutrophils in blood is often an indicator of infection. Immature neutrophils were observed in some calves from all three groups but there was not a significant difference among the groups when comparing mean values throughout the study period (Figure 2a). The MCW-SC group appeared to have the highest level of neutrophils on days 1 and 2 post-treatment. However, a comparison of individual animal values for day 1

post-treatment revealed that this apparent elevation was due to a single animal (Figure

2b). Thus, clinical haematology did not reveal any significant differences in blood

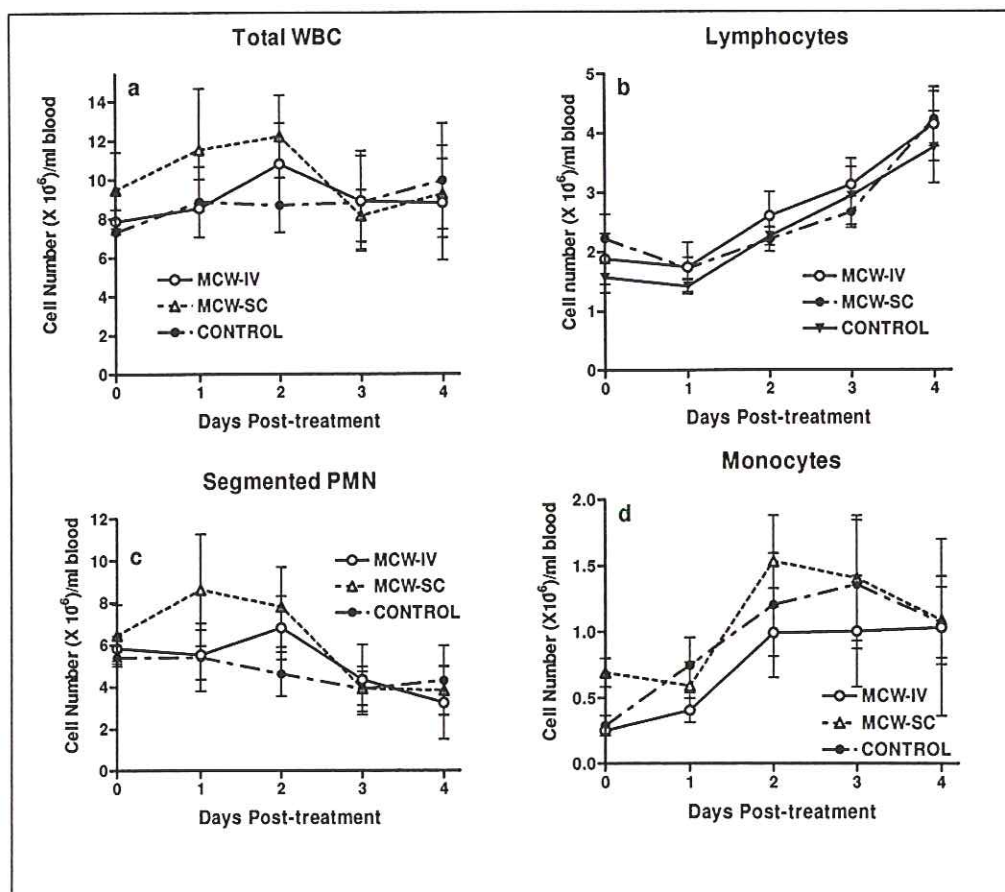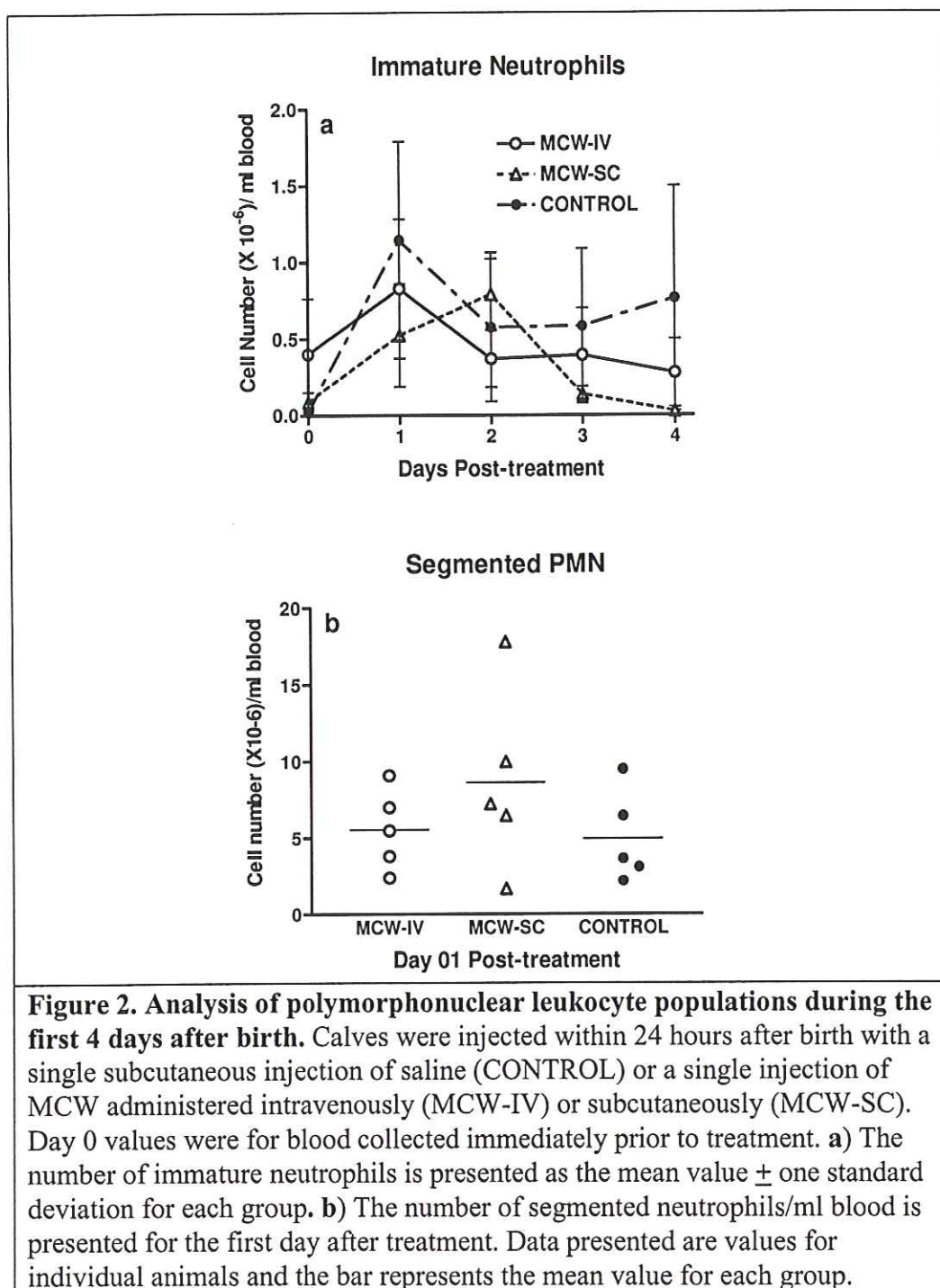leukocyte populations following IV or SC injection of MCW.

Figure 1. Analysis of blood leukocyte populations during the first four days after birth. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Day 0 values were for blood collected immediately prior to treatment. **a)** The total number of blood leukocytes (Total WBC) was counted and the number of **b)** lymphocytes, **c)** neutrophils (Segmented PMN), and **d)** monocytes were calculated by multiplying the 'Total WBC' by percentages obtained from blood smears. Data presented are the mean value $\pm$ one standard deviation for each group.

Immature Neutrophils

Segmented PMN

Figure 2. Analysis of polymorphonuclear leukocyte populations during the first 4 days after birth. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Day 0 values were for blood collected immediately prior to treatment. **a)** The number of immature neutrophils is presented as the mean value ± one standard deviation for each group. **b)** The number of segmented neutrophils/ml blood is presented for the first day after treatment. Data presented are values for individual animals and the bar represents the mean value for each group.

8.2 Flow Cytometry

The use of flow cytometry and specific mAbs permits a much more detailed analysis of

monocyte and lymphocyte subpopulations in blood. Lysis of whole blood was used to

obtain leukocytes for the flow cytometric analyses. This method of cell isolation was selected since it allowed us to relate flow cytometric data to the clinical haematology data and directly calculate the number of cells/ml of blood for specific lymphocyte subpopulations.

The flow cytometric analysis of the major mononuclear leukocyte subpopulations revealed no significant differences in cell number/ml of blood for the three groups of calves. All groups displayed similar increases in T- and B-lymphocytes (Figure 3a and 3b) and similar increases in the number of monocytes (Figure 3c). Further analysis of T-lymphocyte subpopulations revealed that all groups displayed a substantial increase in the number of CD4$^+$ T-lymphocytes/ml of blood (Figure 4a). In contrast, the number of CD8$^+$ (Figure 4b) and $\gamma\delta$TCR$^+$ (Figure 4c) T-lymphocytes remained relatively constant within each experimental group. Statistical analyses did not reveal any significant differences among the groups when T-lymphocyte subpopulation numbers/ml of blood were compared.

Lymphocyte activation is closely linked to the expression of several different surface molecules and the frequency of cells expressing these activation molecules provides an indication of immune activation. Flow cytometry was used to quantify the number of CD4$^+$ T-lymphocytes/ml of blood that expressed two different activation molecules. The first surface molecule analysed was the alpha chain of the interleukin-2 (IL-2) receptor (CD25). Expression of CD25 is an early event during T-lymphocyte activation and indicates that a T-lymphocyte can respond to the important growth signal, interleukin-2. Newborn calves have very few CD25$^+$CD4$^+$ T-lymphocytes present in blood (Figure 5a) but the number of CD25$^+$ T-lymphocytes/ml of blood increased in all

calves on days 3 and 4 after birth. On day 4 post-treatment, the highest number of

$CD25^+CD4^+$ T-lymphocytes/ml of blood was observed in MCW-SC treated calves but

this difference was not significant when compared with the the Control group. Similarly,

all newborn calves had very few MHC Class II^+ $CD4^+$ T-lymphocytes in blood but the

number of these cells/ml of blood increased in all calves on days 3 and 4 after birth

(Figure 5b). Again, the MCW-SC group displayed the highest number of MHC Class II^+

$CD4^+$ T-lymphocytes/ml of blood but the difference was not significant when compared
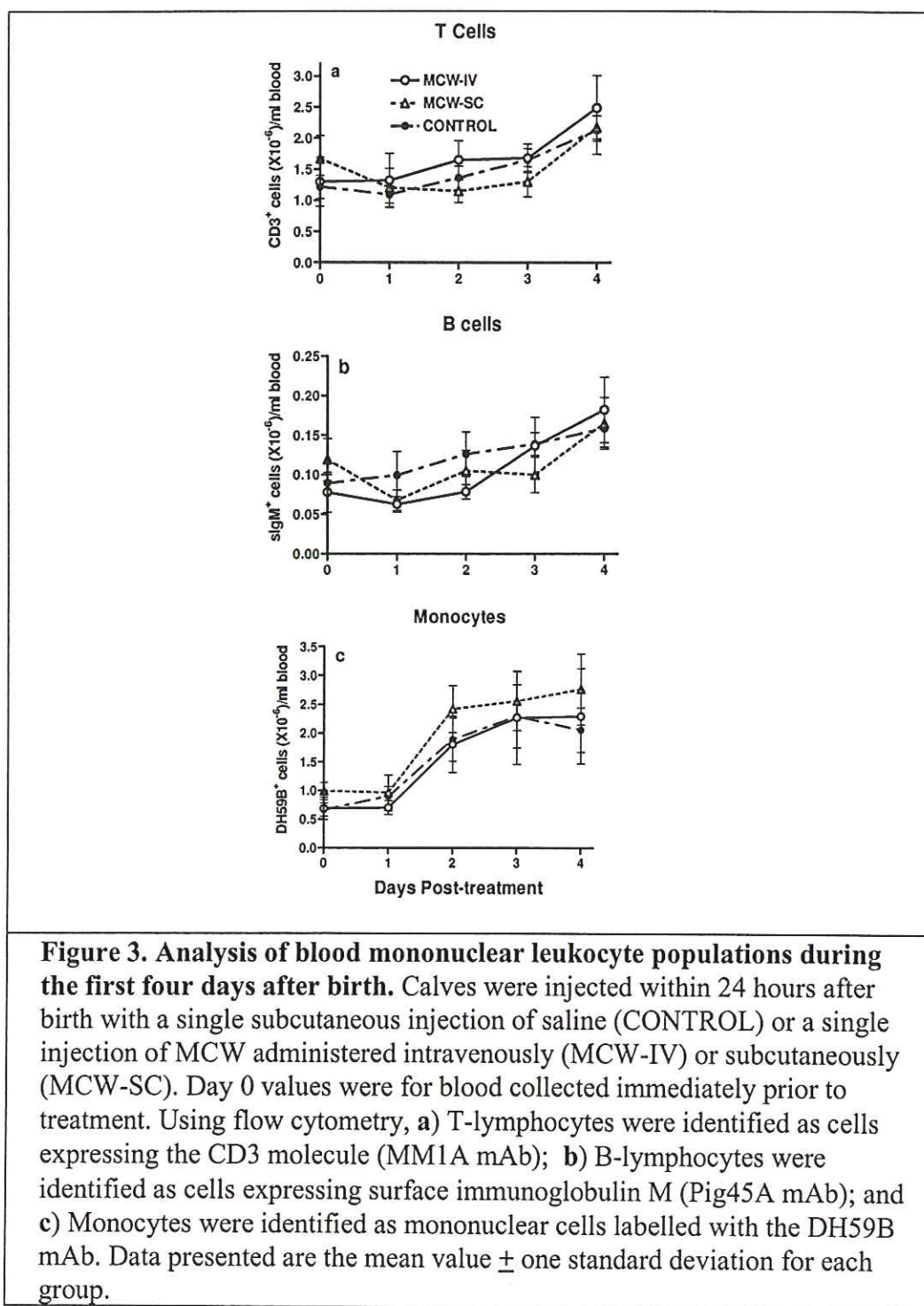
to Control calves.

A review of clinical observations (Section 8.4) indicated that some calves in each

experimental group had clinical score indicative of an infection. This observation raised

the concern that the state of the immune system may have been altered in these calves.

Therefore, the analysis of $CD4^+$ T-lymphocyte activation on day 4 post-treatment was

restricted to 'clinically normal' calves. A 'clinically normal' calf was defined as a calf with

a clinical score less than one (1). The application of this criteria eliminated one (1) calf

from each of the three experimental groups. Restricting the analysis to 'clinically normal'

calves confirmed that MCW-SC treated calves had a higher number of $CD25^+CD4^+$ T-

lymphocytes/ml of blood than the Control calves (Figure 6a). **Furthermore, there was a**

significantly ($p < 0.05$) greater number of MHC Class II^+ $CD4^+$ T-lymphocytes in the

blood of MCW-SC treated calves when compared to CONTROL calves (Figure 6b).

These observations provided direct evidence that MCW activated the immune system of

newborn calves.

The expression of MHC Class II is essential for monocytes to function as antigen

presenting cells. Therefore, the functional capacity of the immune system in newborn

calves was further investigated by quantifying the number of MHC Class II$^+$ monocytes /ml of blood. Calculations of total monocyte number (DH59B$^+$ cells)/ml of blood, based on flow cytometry and clinical haematology, indicated that there was not a significant difference among the three experimental groups (Figure 7a). However, by day 4 post-treatment, the MCW-SC group had a substantially greater number of MHC Class II$^+$ monocytes/ml of blood than the Control group. This difference was not significant, even when the analysis was restricted to 'clinically normal' calves, since there was considerable variation within each group (Figure 7c). Thus, the data on MHC Class II$^+$ monocytes was not definitive but is consistent with the conclusion that MCW can activate the immune system of newborn calves.

The level of immune activation in newborn calves was further characterized by analyzing the phenotype of cells isolated from lymph nodes. The prescapular lymph node was selected since this lymph node drains the neck region, which was the site of MCW injection. The mediastinal lymph node of the lung was selected as a second site that was independent of the MCW injection site. These phenotypic analyses did not reveal any significant differences among the treatment groups when the percentage of T- and B-lymphocytes were compared (Figure 8). Further analysis of specific T-lymphocyte subpopulations, including CD4, CD8, and $\gamma\delta$ TCR cells, did not reveal any significant differences among the treatment groups (Figure 9). Finally, an analysis of CD25 and MHC Class II expression on CD4$^+$ T-lymphocytes confirmed that these cells were also present in lymphoid tissue but did not reveal significant differences among the treatment groups (Figure 10). These analyses were restricted to the percentage of cells expressing a specific phenotypic marker and could not be used to determine the absolute frequency of

a specific cell subpopulation in a lymph node. This may have limited the usefulness of

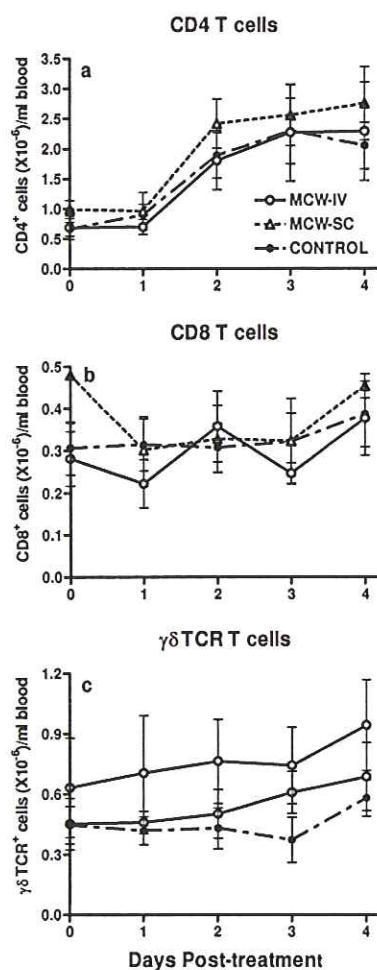these analyses for evaluating immune activation on newborn calves.

Figure 3. Analysis of blood mononuclear leukocyte populations during the first four days after birth. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Day 0 values were for blood collected immediately prior to treatment. Using flow cytometry, **a**) T-lymphocytes were identified as cells expressing the CD3 molecule (MM1A mAb); **b**) B-lymphocytes were identified as cells expressing surface immunoglobulin M (Pig45A mAb); and **c**) Monocytes were identified as mononuclear cells labelled with the DH59B mAb. Data presented are the mean value ± one standard deviation for each group.

Figure 4. Analysis of T-lymphocyte sub-populations during the first four days after birth. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Day 0 values were for blood collected immediately prior to treatment. **a)** CD4$^+$ T-lymphocytes were identified as cells labelled with IL-A11 mAb. **b)** CD8$^+$ T-lymphocytes were identified as cells labelled with CACT80C mAb. **c)** $\gamma\delta$ TCR$^+$ T-lymphocytes were identified as cells labelling with the BAQ4A mAb. Data presented are the mean value $\pm$ one standard deviation for each group.
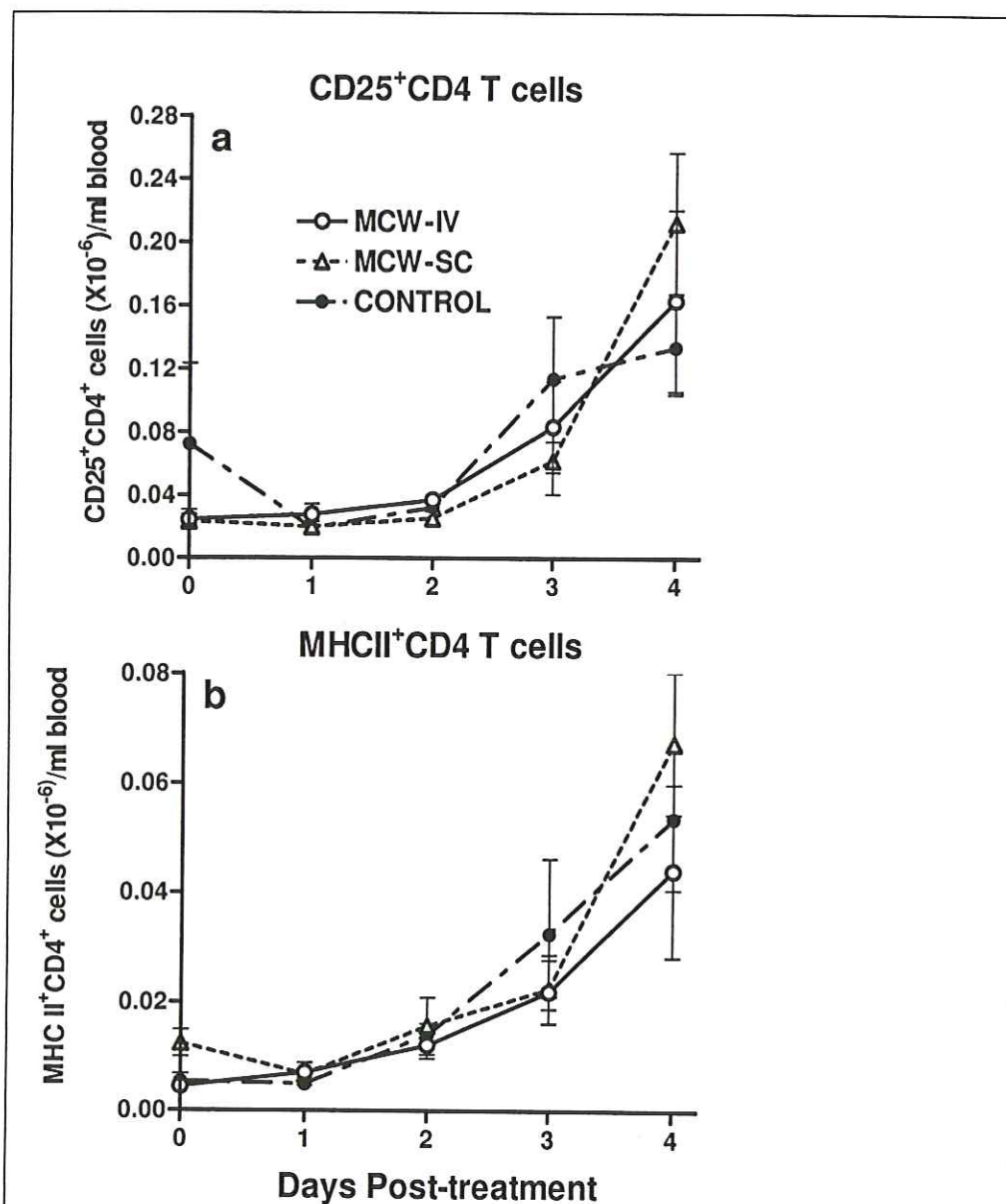
Figure 5. Analysis of CD4⁺ T-lymphocyte phenotype during the first four days after birth. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Day 0 values were for blood collected immediately prior to treatment. **a**) CD25⁺CD4⁺ T-lymphocytes were identified as cells that co-labelled with CACT116A (CD25) and IL-A11 (CD4) mAbs. **b**) MHC Class II⁺CD4⁺ T-lymphocytes were identified as cells that co-labelled with TH14B (MHC Class II) and IL-A11 (CD4) mAbs. Data presented are the mean value $\pm$ one standard deviation for each group (n = 5).

CD25⁺CD4 T cells
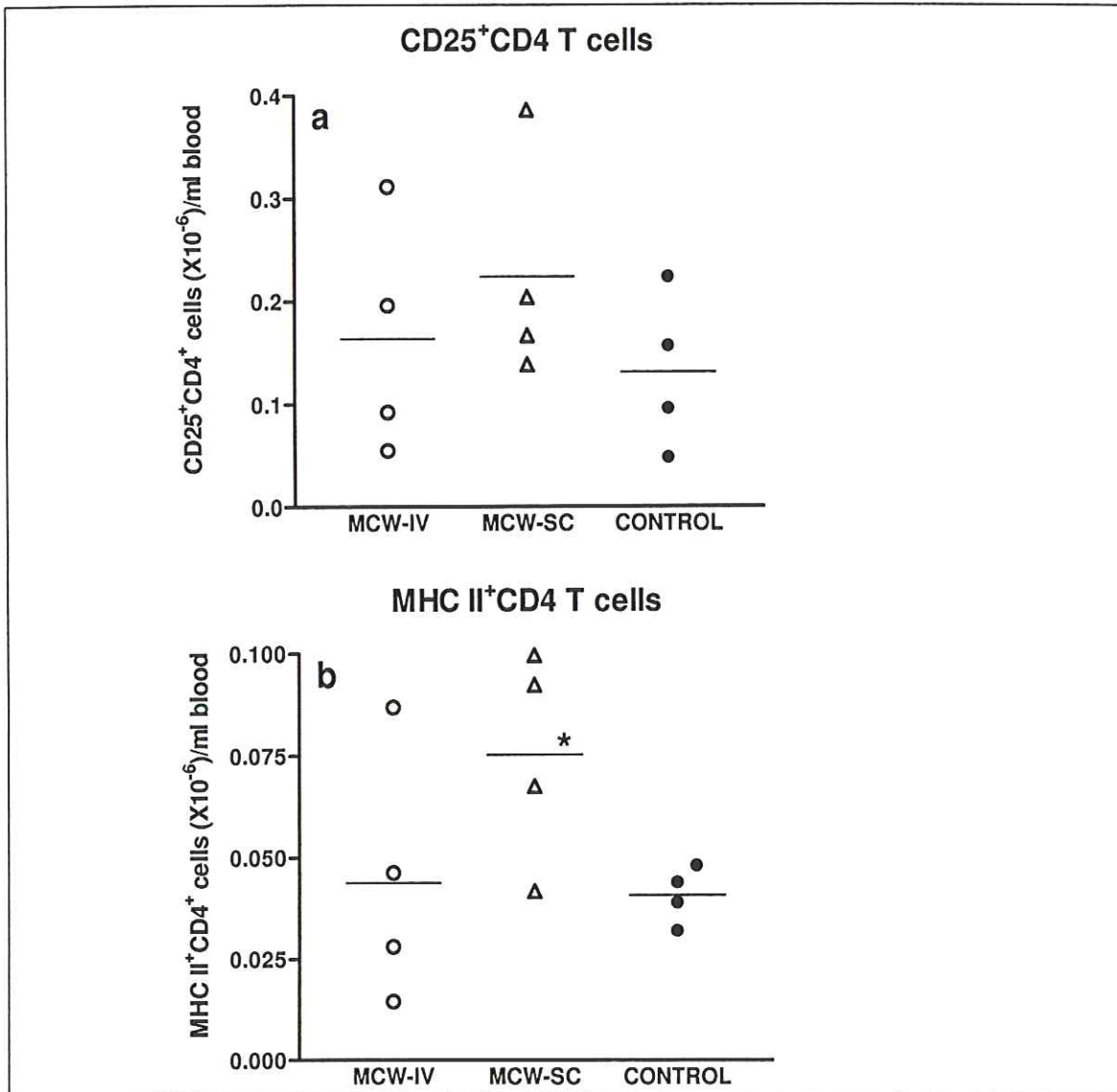

MHC II⁺CD4 T cells

Figure 6. Analysis of CD4⁺ T-lymphocyte phenotype on day four after birth. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Day 0 values were for blood collected immediately prior to treatment. **a**) CD25⁺CD4⁺ T-lymphocytes were identified as cells that co-labelled with CACT116A (CD25) and IL-A11 (CD4) mAbs. **b**) MHC Class II⁺CD4⁺ T-lymphocytes were identified as cells that co-labelled with TH14B (MHC Class II) and IL-A11 (CD4) mAbs. Data presented are values for individual animals and bars represents mean values for each group (n = 4). Clinically normal (clinical score less than one) calves only were included in these analyses. * MCW-SC group was significantly (p < 0.05) different from Control group.
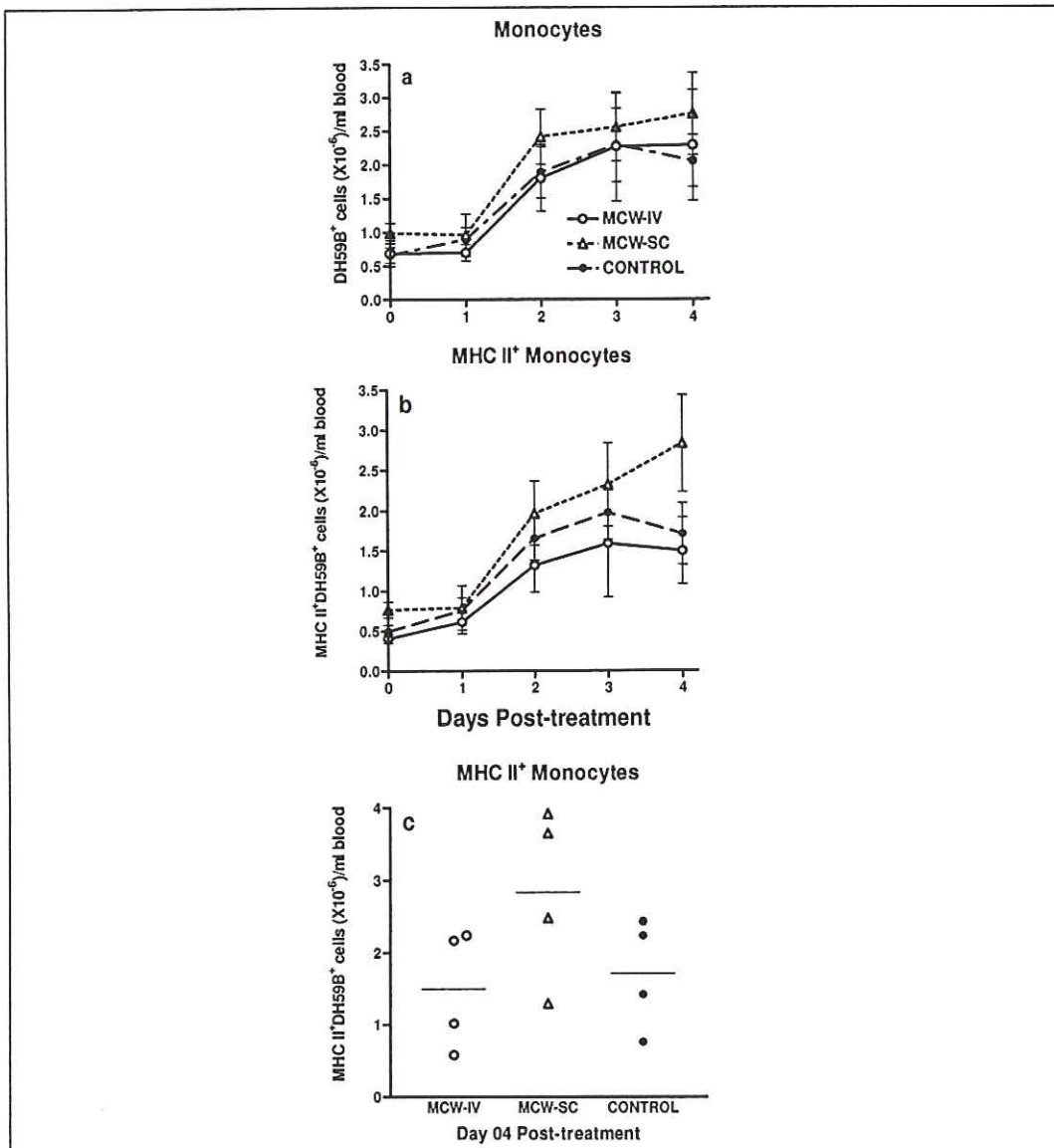
Figure 7. Phenotypic analyses of blood monocytes during the first four days after birth. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Day 0 values were for blood collected immediately prior to treatment. **a)** Blood monocytes were identified as cells that labelled with DH59B mAb. **b)** MHC Class II$^+$ monocytes were identified as cells that co-labelled with TH14B (MHC Class II) and DH59B mAb. **a and b)** Data presented are the mean value ± one standard deviation for each group (n = 5). **c)** Data presented are values for individual animals on day 4 post-treatment and bars represents mean values for each group (n = 4). Calves with clinical scores less than one were included in these analyses.
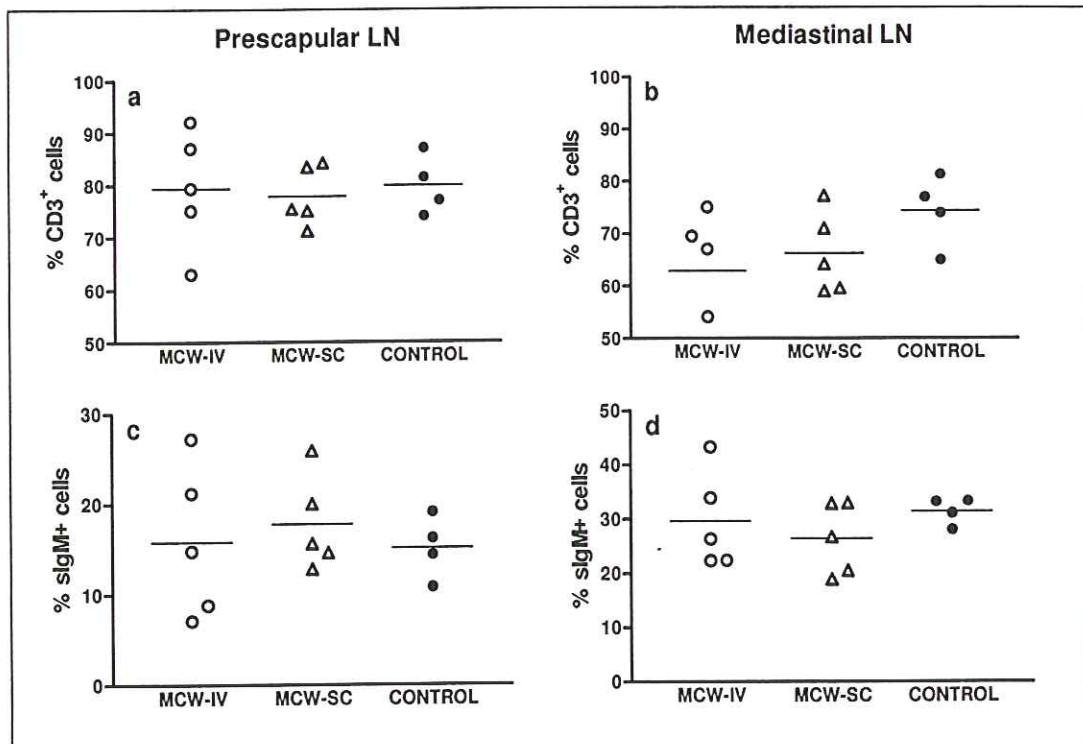
Figure 8. Analysis of T- and B-lymphocyte composition of prescapular and mediastinal lymph nodes. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Tissues were collected for flow cytometric analyses on day 5 post-treatment. T-lymphocytes in the prescapular (**a**) and the mediastinal lymph nodes (**b**) were identified as cells that labelled with MM1A (CD3) mAb. B-lymphocytes in the prescapular (**c**) and the mediastinal lymph nodes (**d**) were identified as cells that labelled with PIG45A (sIgM) mAb. Data presented are values for individual animals and bars represents mean values for each group.
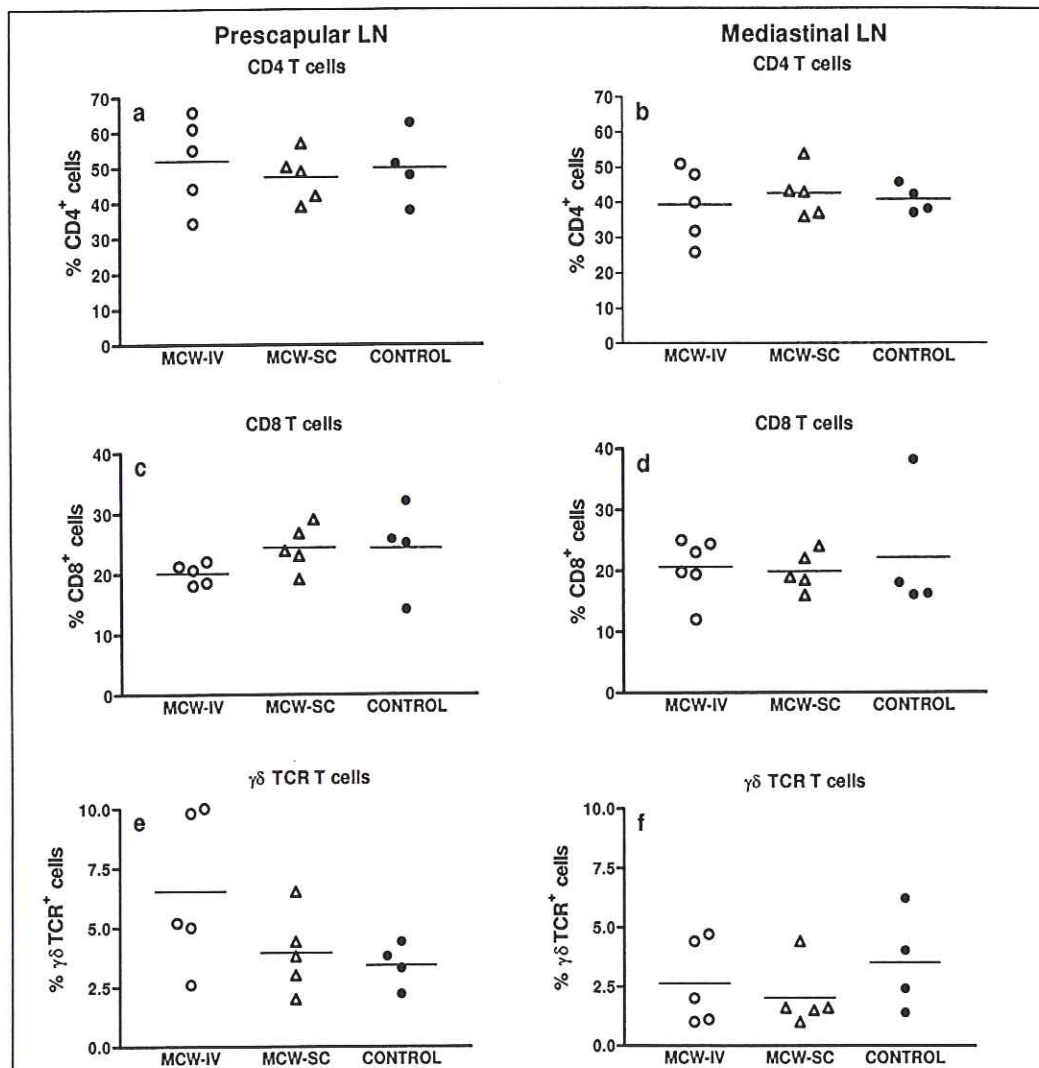
Figure 9. Analysis of T-lymphocyte subpopulations in prescapular and mediastinal lymph nodes. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Tissues were collected for flow cytometric analyses on day 5 post-treatment. $CD4^+$ T-lymphocytes in prescapular (**a**) and mediastinal lymph nodes (**b**) were identified as cells labelled with IL-A11 mAb. $CD8^+$ T-lymphocytes in the prescapular (**c**) and mediastinal lymph nodes (**d**) were identified as cells labelled with CACT80C mAb. $\gamma\delta$ TCR^+ T-lymphocytes in prescapular (**e**) and mediastinal lymph nodes (f) were identified as cells labelling with the BAQ4A mAb. Data presented are values for individual animals and bars represents mean values for each group.
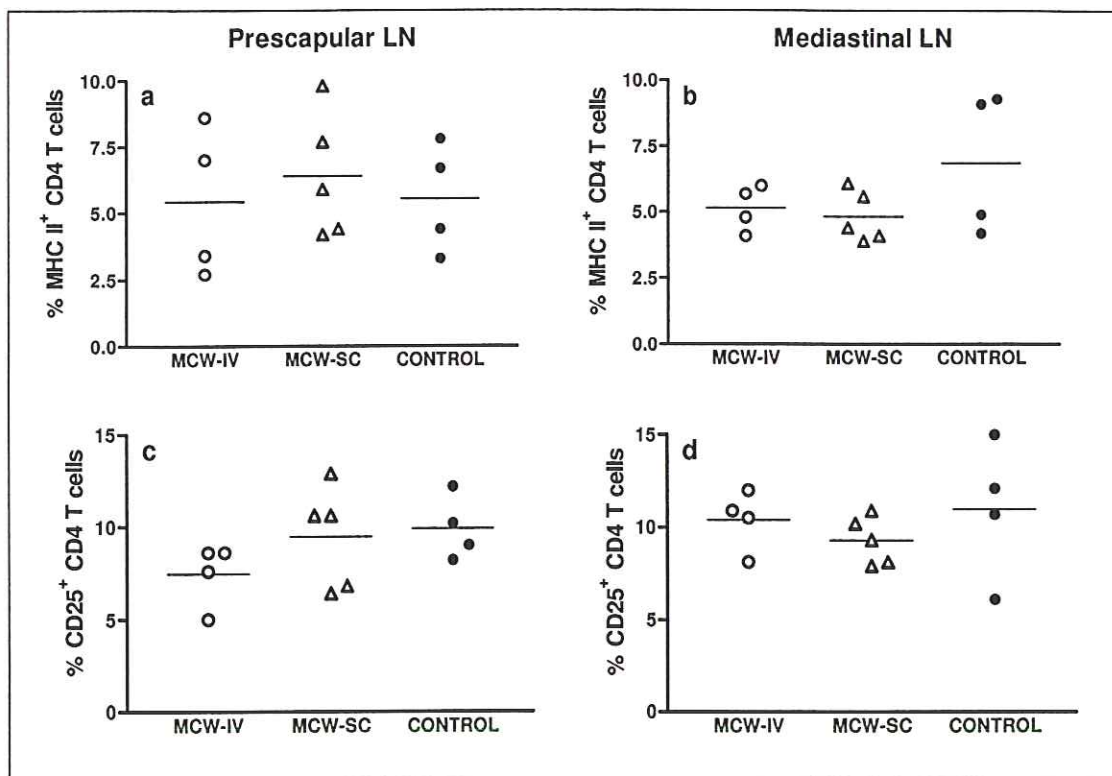
Figure 10. Analysis of the CD4⁺ T-lymphocyte population in prescapular and mediastinal lymph nodes. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Tissues were collected for flow cytometric analyses on day 5 post-treatment. MHC Class II⁺CD4⁺ T-lymphocytes in prescapular (**a**) and mediastinal (**b**) lymph nodes were identified as cells that co-labelled with TH14B (MHC Class II) and IL-A11 (CD4) mAbs. CD25⁺CD4⁺ T-lymphocytes in prescapular (**c**) and mediastinal (**d**) lymph nodes were identified as cells that co-labelled with CACT116A (CD25) and IL-A11 (CD4) mAbs. Data presented are values for individual animals and bars represents mean values for each group.

8.3 IFNγ Secretion

IFNγ is a cytokine that plays a central role in regulating the immune system and activating effector cells. For example, IFNγ is critical for activating immune defences against many intracellular pathogens, such as viruses and some bacteria. Therefore, measuring IFNγ production is another way to measure the capacity of the immune system to respond to

infectious agents. An *in vitro* stimulation assay, using Concanavalin A, was used to measure the capacity of blood leukocytes to secrete IFNγ.

The present assay revealed that leukocytes, isolated from the blood of newborn calves, can produce very little IFNγ but the capacity of these cells to produce IFNγ increased on days 3 and 4 after birth (Figure 11a). It is notable that the most pronounced increase in IFNγ production was observed with blood leukocytes from MCW-SC treated calves (Figure 11a). An analysis of 'clinically normal' calves on day 4 post-treatment, revealed that all calves treated with MCW-SC had a level of IFNγ production that exceeded 3000 pg/ml (Figure 11b). In contrast, only half the control calves produced a similar level of IFNγ (Figure 6b). Due to the broad range of IFNγ production by blood leukocytes from Control calves, IFNγ production was not significantly different when MCW treated and Control calves were compared.

The high level of IFNγ secretion by leukocytes isolated from MCW-SC calves was consistent with an increased frequency of activated CD4$^+$ T-lymphocytes in the blood of these calves. Since CD4$^+$ T-lymphocytes are a major source of IFNγ in cattle we investigated further if there was a possible correlation between the frequency of activated T-lymphocytes and IFNγ secretion. First, the number of CD4$^+$ T-lymphocytes cultured in each assay was calculated (Figure 12a). These calculations revealed that there were significantly fewer CD4$^+$ T-lymphocytes present on Day 2 and 3 of the assay than on day 4. However, the number of CD4$^+$ T-lymphocytes added to cultures on day 0 and 3 were not significantly different from cell number on day 4. Therefore, fewer CD4$^+$ T-lymphocytes/well could not explain the low level IFNγ secretion at birth and 3 days later.

In contrast, the number of CD25$^+$ (Figure 12b) and MHC Class II$^+$ (Figure 12c) CD4$^+$ T-lymphocytes/well appeared to follow closely the level of IFNγ production (Figure 11a). This apparent correlation was analyzed by doing a linear regression analysis of MCW treated calves on day 4 post-treatment. Regression analysis of the number of CD4$^+$ (Figure 13a), MHC Class II$^+$CD4$^+$ (Figure 13b), and CD25$^+$CD4$^+$ T-lymphocytes (Figure 13c) did not reveal a significant correlation between any of the specific T-lymphocyte subpopulation analyzed and IFNγ production. Therefore, we concluded that measuring IFNγ production and analysing activation molecules on T-lymphocytes provided independent measures of immune system activation in newborn calves.
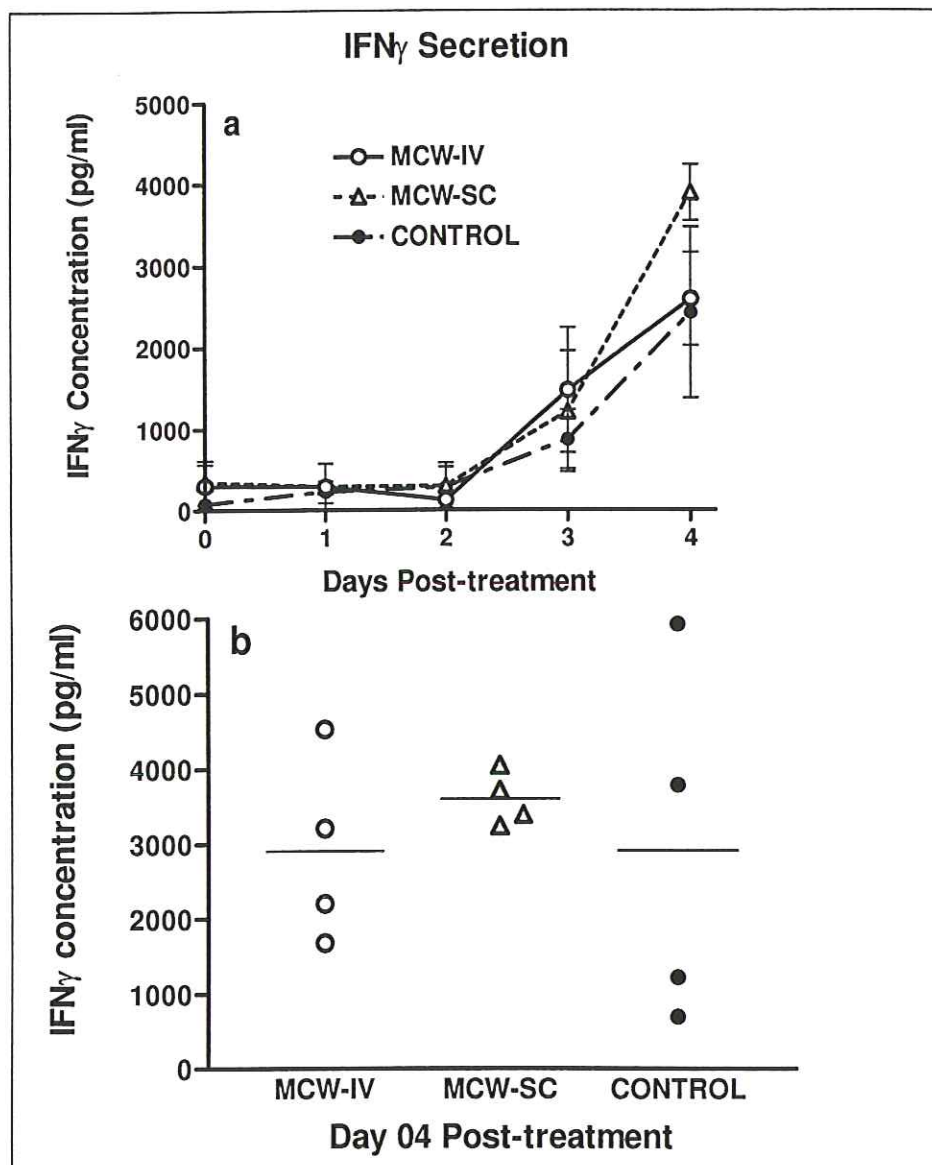
Figure 11. IFNγ secretion by blood leukocytes stimulated with Concanavalin A (Con A). Blood leukocytes were obtained by lysing whole blood and 2×10^5 cells/well were cultured in 200 μl of medium. Cells were cultured in triplicate, in the presence and absence of Con A. Culture supernatants were collected after a 48 hour incubation and the concentration of IFNγ was assayed with a capture ELISA. IFNγ was not detected when cells were cultured in the absence of Con A. **a)** Data presented are the mean value ± one standard deviation for each group (n = 5). **b)** Data presented are values for individual animals on day 4 post-treatment and bars represents mean values for each group (n = 4). Calves with clinical scores less than one were included in these analyses.
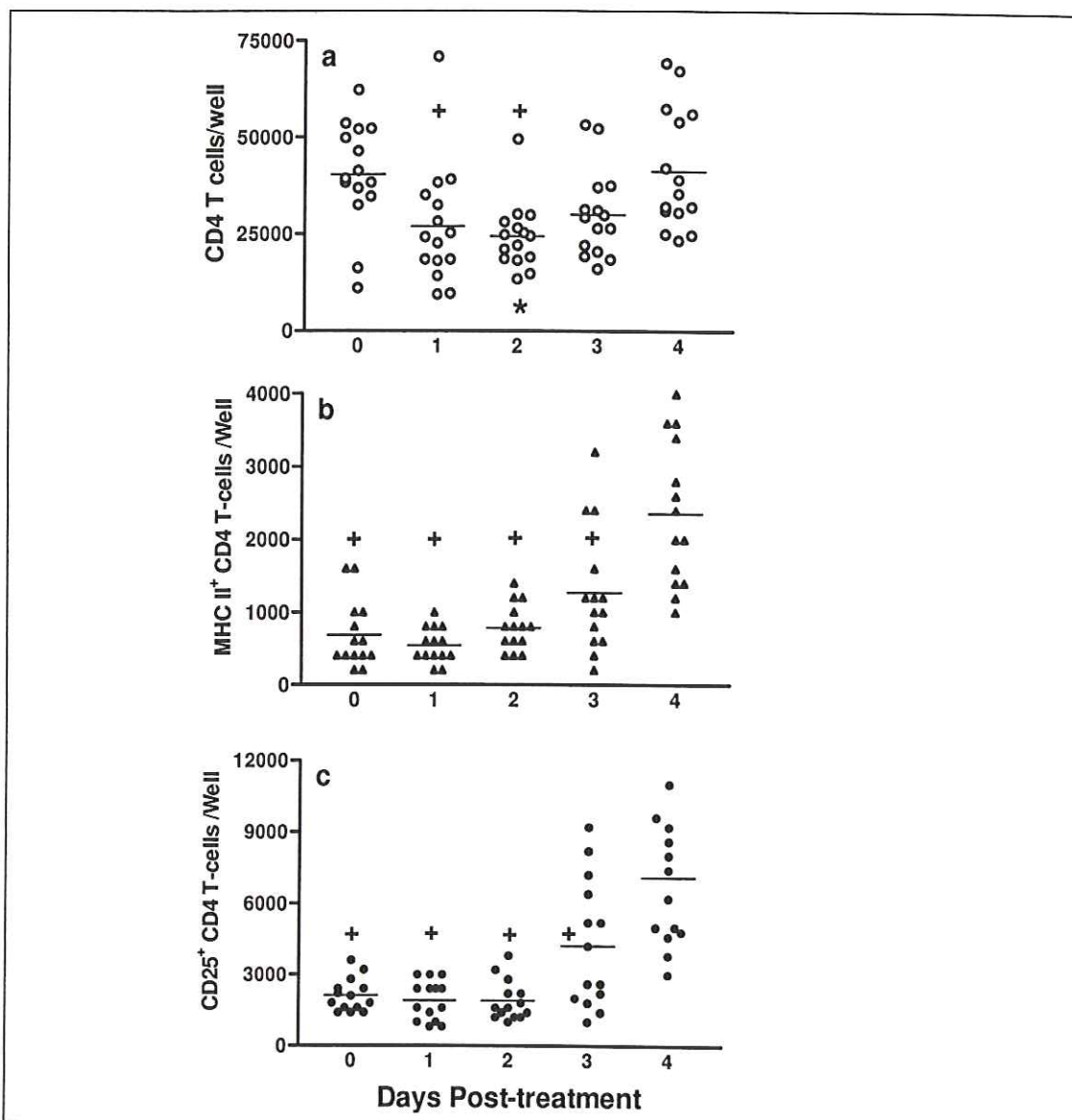
Figure 12. The number of CD4+ T-lymphocytes added to cultures when assaying IFNγ production. Cell number/well was calculated by multiplying the percentage of cells, determined by flow cytometric analyses to have a specific phenotype, by the number of cells added to each well (2 X 10^5 cells). **a)** CD4+ T-lymphocytes were identified as cells that labelled with the IL-A11 mAb. **b)** MHC Class II+CD4+ T-lymphocytes were identified as cells that co-labelled with TH14B (MHC Class II) and IL-A11 (CD4) mAb. **c)** CD25+CD4+ T-lymphocytes were identified as cells that co-labelled with CACT116A (CD25) and IL-A11 (CD4) mAb. Data presented are values for individual animals and bars represents mean values for each day. Data represents values for calves from all three experimental groups (n = 15). * p < 0.01 for values that are significantly different from Day 0.   + p < 0.01 for values that are significantly different from Day 4.
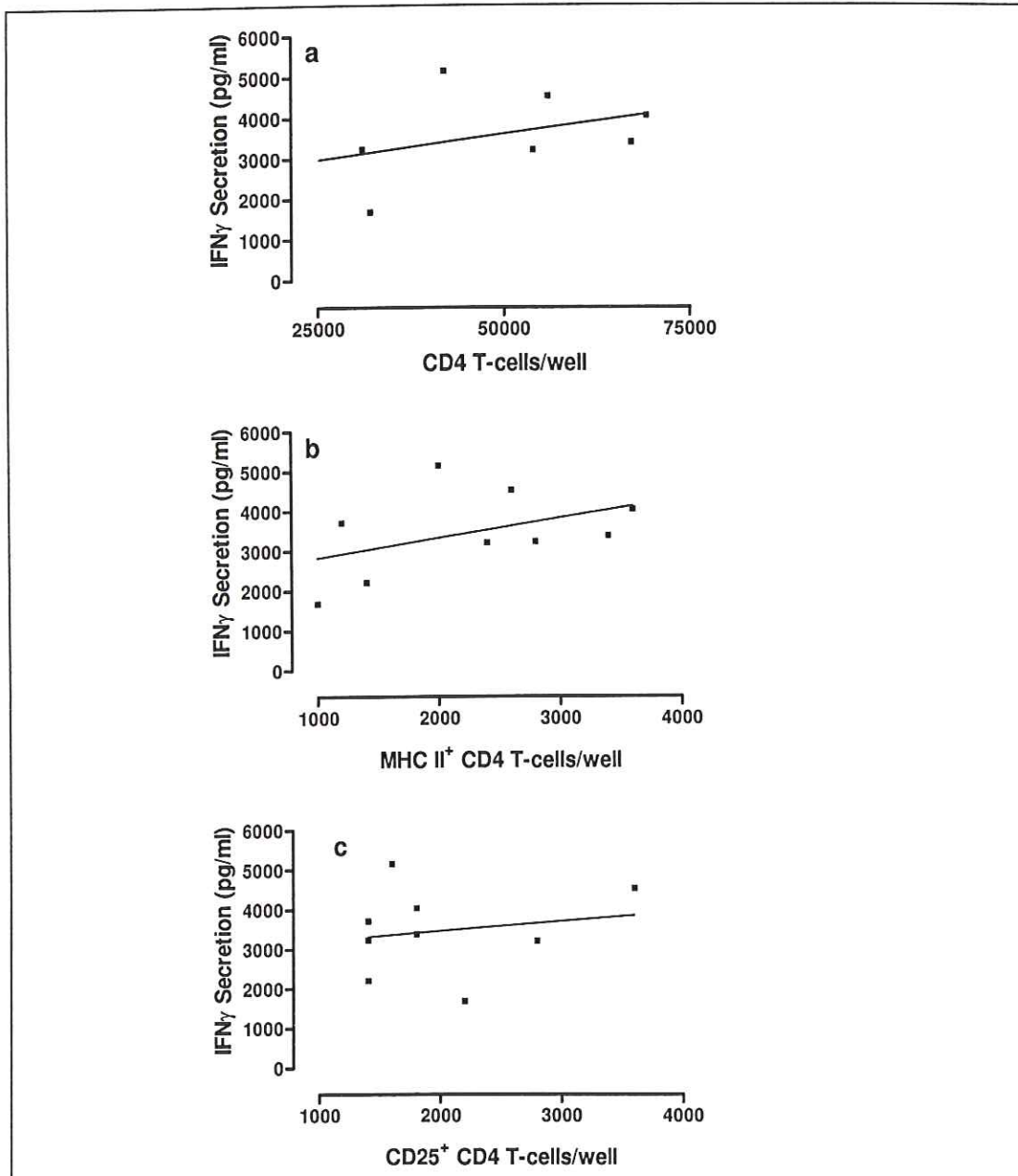
Figure 13. Analysis of a possible correlation between CD4$^+$ T-lymphocyte number/well and IFNγ secretion in culture. Data used for the linear regression analyses were values from calves injected 4 days prior with MCW, either intravenously or subcutaneously. The Control group (saline injection) was not included since half this group had very low IFNγ production on Day 04 post-treatment (see Figure 10b). **a**) Correlation between total CD4$^+$ T-lymphocyte number/well and IFNγ secretion. **b**) Correlation between MHC Class II$^+$ CD4$^+$ T-lymphocyte number/well and IFNγ secretion. **c**) Correlation between CD25$^+$ CD4$^+$ T-lymphocyte number/well and IFNγ secretion. None of the regression lines were significantly different from zero.

8.4 Serum Cortisol

Corticosteroids have diverse effects on cell metabolism and the immune system. For example, corticosteroids can induce lymphocyte death (i.e. thymic involution), alter leukocyte traffic (neutrophilia and lymphopaenia), inhibit phagocytosis by macrophages, and inhibit the secretion of cytokines and other mediators of inflammation. Therefore, 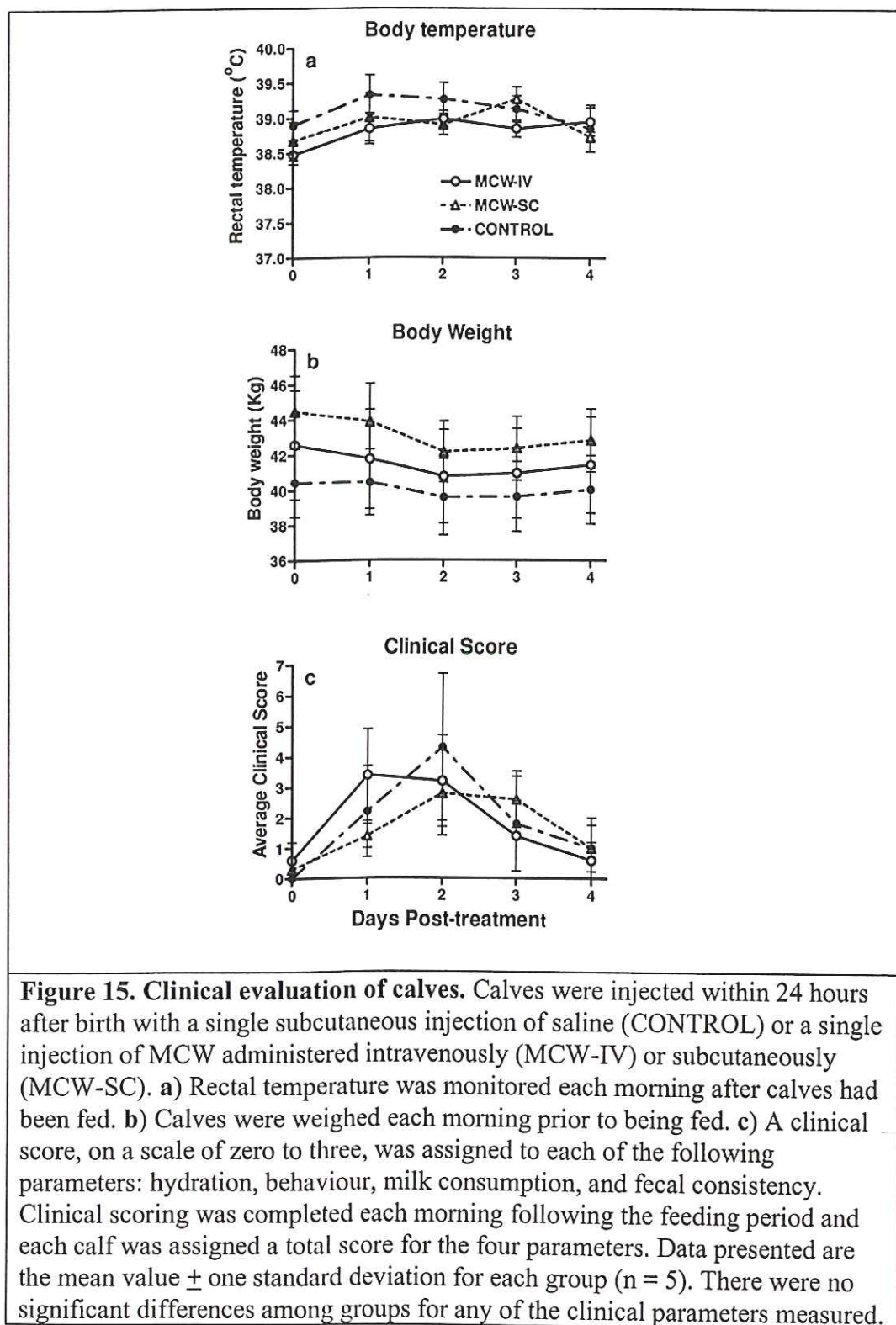any evaluation of immune activation in newborn calves should consider serum cortisol levels. Serum cortisol levels were monitored daily throughout the study. As reported previously, there was a high cortisolemia in newborn calves but the level of serum cortisol began to decline on days 3 and 4 after birth (Figure 14). Calves from all groups had similar serum cortisol levels at the beginning and the end of the study (Figure 14a). However, calves in the Control group displayed a different pattern of cortisolemia throughout the study when compared with the MCW treated calves. Three of the five Control calves displayed an increased level of serum cortisol 24 h after birth but, at the same time, seven of the ten MCW treated calves displayed a decreased level of serum cortisol (Figure 14b). These differences in serum cortisol were not significant when MCW treated calves were compared with Control calves.

In conclusion, fetal cortisol provides the signal for parturition in cattle. A high cortisolemia is present in the newborn calf (Figure 14) and may explain the low level of immune system activation that is observed during the first 3 days after birth (Figures 5 and 11). The increased number of activated T-lymphocytes (MHC Class II$_+$CD4$^+$ cells in the MCW-SC group,) on day 4 post-treatment, suggests that the subcutaneous injection of MCW may be able to overcome some of the immune suppression induced by endogenous cortisol.

Figure 14. Changes in serum cortisol levels during the first 4 days after birth. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). Day 0 values were for serum collected immediately prior to treatment and all serum samples were collected each morning after the calves were fed. Total serum cortisol was determined using a competitive binding assay. Data presented are the mean value ± one standard deviation for each group (n = 5). Throughout the experiment, there were no significant differences in serum cortisol levels among the three treatment groups.

8.5 Clinical examination

All calves were examined twice daily to evaluate hydration, behaviour, milk consumption, fecal consistency, and body temperature. The calves were also weighed once daily. There were no significant differences among the groups when these parameters were compared (Figure 15). It was evident throughout this study that some calves displayed clinical signs of an active infection. These signs included the presence of immature neutrophils (Figure 2a), elevated body temperatures that were less than one (1) °C (Figure 11a), and mild depression that was associated with either reduced milk intake or some diarrhea (Figure 11c). These clinical observations were used as the basis for defining a 'clinically normal' state for calves that were used to analyse in detail the state of immune activation.

Figure 15. Clinical evaluation of calves. Calves were injected within 24 hours after birth with a single subcutaneous injection of saline (CONTROL) or a single injection of MCW administered intravenously (MCW-IV) or subcutaneously (MCW-SC). **a)** Rectal temperature was monitored each morning after calves had been fed. **b)** Calves were weighed each morning prior to being fed. **c)** A clinical score, on a scale of zero to three, was assigned to each of the following parameters: hydration, behaviour, milk consumption, and fecal consistency. Clinical scoring was completed each morning following the feeding period and each calf was assigned a total score for the four parameters. Data presented are the mean value $\pm$ one standard deviation for each group (n = 5). There were no significant differences among groups for any of the clinical parameters measured.

8.6 Pathology

The calves were killed on day 5 after birth and a complete post-mortem exam was performed on each calf. The injection sites were also examined for both gross and histological changes. Only one calf displayed gross lesions that indicated an active infection. A diagnosis of bacterial pneumonia was confirmed by bacterial culture. This calf (99-087) was also one of the calves removed from the data analyses on the basis of clinical observations. None of the calves displayed gross lesions at the site of MCW injection. Histopathology revealed that one calf (99-092) displayed a mild, perivascular infiltration of mononuclear cells at the site where MCW was injected subcutaneously. The examination of prescapular and mediastinal lymph nodes did not reveal any consistent histological changes.

9. DISCUSSION

Previous investigations in cattle and other species have clearly demonstrated that MCW can activate the immune system. MCW activation of the immune system in cattle has been demonstrated primarily through the use of MCW as a vaccine adjuvant in calves and mature cattle. The present investigation analyzed the capacity of MCW to activate the immune system in newborn calves. Activation of the bovine immune system in this age group is faced with a number of potential limitations. These limitations include an immune system that may be immature and the presence of a cortisolemia, which can inhibit a variety of immune functions. Thus, the state of the immune system may be changing rapidly during the first days after birth.

The present investigation, through a detailed analysis of blood leukocyte phenotype and function, confirmed that the state of immune activation changes dramatically during the first four days after birth. The newborn calf was characterized by a virtual absence of activated CD4$^+$ T-lymphocytes in blood (Figure5), a low number of MHC Class II$^+$ monocytes in blood (Figure 7), and mitogen-activated blood leukocytes were unable to produce IFNγ (Figure 11). Control (saline injected) calves displayed some increases for all these parameters on day 4 after birth but, for each parameter, there was marked variability within the group. These observations raised questions regarding possible immunomodulatory effects by environmental factors, such as a coincidental infection. It was critical that the analysis of MCW immunostimulatory activity be done in comparison to the 'normal' development of the newborn immune system and that the effects of MCW treatment be clearly separated from other environmental factors. Therefore, the results of daily clinical examinations were used to define Control and

MCW-treated calves that were 'clinically normal' and these calves were used for further analyses. These analyses revealed that a single subcutaneous injection of MCW can significantly increase the number of activated (MHC Class II^{+}) CD4^{+} T-lymphocytes in newborn calves. This provided direct evidence that MCW can function as an immune stimulant in newborn calves.

Several procedures were instituted during the present investigation to minimise the possibility that factors, other than the experimental treatment (saline versus MCW), would influence immune function in the newborn calves. Calves were colostrum-deprived to minimise the possibility that variable transmission of maternal antibody, complement, and other colostral components would alter the state of the immune system. Furthermore, calves were housed in individual pens to minimise contact between animals and to reduce the risk of disease transmission. However, calves were purchased from multiple herds and it was not possible to standardise environmental exposure prior to delivery at VIDO's Animal Care Facility. Clinical haematology (Figure 2) and clinical examinations (Figure 15c) indicated that some calves were exposed to infectious agents. Calves with clinical scores exceeding one (1) were subsequently removed from the analysis of data for day 4 post-treatment.

There were no significant differences among the experimental groups during the first three (3) days post-treatment. Previous investigators have identified the early post-natal period as a time of extremely low immune responsiveness. This has been attributed primarily to the cortisolemia that is associated with parturition. The present investigation examined the early post-partum period in much greater detail and confirmed that at birth there is a cortisolemia (Figure 14). This cortisolemia is associated with a paucity of

lymphocytes in blood (Figure 1 and 3) and few of the T-lymphocytes express surface molecules, such as CD25 and MHC Class I, that are associated with cell activation (Figure 5). Furthermore, these lymphocytes are unable to secrete IFNγ when activated with the mitogen, Concanavalin A (Figure 11a). These observations confirmed that the immune system of newborn calves was functionally deficient. Thus, it was of considerable interest to determine if the injection of MCW, a known immune stimulant, could alter the state of the immune system in newborn calves.

A single subcutaneous injection of MCW had a significant effect on the development of the immune system in newborn calves. MCW treatment significantly increased the number of MHC Class II$^+$ CD4$^+$ T-lymphocytes/ml of blood (Figure 6). This observation confirmed that treatment with MCW could enhance the state of immune activation in newborn calves. Several other observations were consistent with an increased level of immune activation on day 4 following the subcutaneous injection of MCW. These differences included increased mean values for the number of MHC Class II$^+$ monocytes (Figure 7) and for IFNγ production (Figure 11). The expression of MHC Class II on monocytes is essential for the presentation of antigen and the induction of specific immunity. Furthermore, IFNγ plays a critical role in immune defences against intracellular pathogens, such as viruses and some bacteria. Therefore, these observations indicate that MCW activation of the immune system in newborn calves could result in an increased capacity to fight infectious disease.

10. CONCLUSIONS

1. There are very few activated leukocytes present in the blood of newborn calves.

2. A single subcutaneous injection of MCW significantly increases the frequency of activated T-lymphocytes in the blood of newborn calves.

3. MCW can function as an immune stimulant in newborn calves

Research Director   **Date**

December 08, 1999

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