



Short Communication

Changes in some pro-and anti-inflammatory cytokines produced by bovine peripheral blood mononuclear cells following foot and mouth disease vaccination

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ABSTRACT

Interleukin (IL)-17 is exclusively produced by CD4 helper T-cells upon activation. It most often acts as a pro-inflammatory cytokine, which stimulates the release of pro-inflammatory cytokines IL-6, IL-8, TNF- α , and granulocyte-macrophage colony-stimulating factor (GM-CSF). In this study, we studied the in-vitro IL-17 response to specific antigens and a variety of mitogens and compared the IL-17 response to IL-2, IL-4, IL-5, IL-6, IL-10, and IFN- γ responses. We used a foot and mouth disease (FMD) vaccine as specific antigens and mitogens (phytohemagglutinin [PHA], pokeweed mitogen [PWM], and concanavalin A [Con A]) to stimulate peripheral blood mononuclear cells (PBMCs) of vaccinated calves. Cell culture supernatant was harvested and analyzed for cytokines, using commercially available bovine ELISA kits. The mitogens induced a significant increase in IL-17 production. IL-17 was produced at high levels in response to the T cell-stimulated mitogens, PHA, and Con A, and at low levels in response to PWM mitogens. In contrast, level of the produced IL-17 cytokines in response to the FMDV antigens was lower as compared to those produced by mitogens. The FMDV antigens and mitogens significantly increased IL-17 production. There was not a correlation between IL-17 production and type-1 cytokine, IFN- γ , and IL-2, while there was a correlation between type-2 cytokine, IL-4, and IL-5 at either cytokine level produced by PBMCs stimulated by FMDV antigens. Moreover, there was an interaction between IL-17 and IL-6, that is, as IL-6 cytokine level elevated or diminished, IL-17 cytokine level increased or decreased, as well.

Keywords: Interleukin-17, Vaccine, Foot and mouth disease, Peripheral blood mononuclear cells

Modifications dans certaines cytokines pro- et anti-inflammatoires libérées par les cellules mononucléées du sang périphérique des bovins après une vaccination contre la fièvre aphteuse

Résumé: L'interleukine (IL)-17 est exclusivement produite par les cellules T auxiliaires CD4 après activation. L'IL-17 agit le plus souvent comme une cytokine pro-inflammatoire capable de stimuler la libération d'autres cytokines pro-inflammatoires (IL-6, IL-8, TNF- α) ainsi que les facteurs de stimulation des colonies de granulocytes-macrophage (GM-CSF). Dans cette étude, nous avons étudié la réponse *in vitro* IL-17 vis-à-vis d'antigènes spécifiques et d'une série de substances mitogènes. La réponse IL-17 a été ensuite comparée à celles d'autres cytokines, en l'occurrence l'IL-2, IL-4, IL-5, IL-6, IL-10, et l'IFN- γ . Dans ce but, les antigènes spécifiques du vaccin de la fièvre aphteuse (foot and mouth disease vaccine, FMDV) et des substances mitogènes comme la phytohémagglutinine (PHA), l'agent mitogène de la phytolaque (pokeweed mitogen, PWM) ainsi que la concanavaline A (Con A) ont été utilisés dans le but de stimuler les cellules mononucléées du sang périphérique (PBMC) des veaux vaccinés. Le surnageant des cultures cellulaires a été prélevé et son contenu en cytokines analysé en utilisant des kits ELISA bovins commerciaux. Selon nos résultats, les agents mitogènes induisent une hausse significative de la libération d'IL-17. Des taux élevés de cette cytokine sont effectivement produits à la suite d'une stimulation des cellules T par la PHA et la Con A alors que la réponse au mitogène PWM semble moins importante.

En revanche, le taux d'IL-17 généré en réponse des antigènes spécifiques du FMDV est bien inférieur comparé à ceux induits par les mitogènes. Les antigènes FMDV et les substances mitogènes testées augmentent donc de façon significative la production IL-17. Il n'y a cependant pas de corrélation entre la libération d'IL-17 et la concentration des cytokines de type 1 « IFN- γ , et IL-2 », contrairement au cas des cytokines de type 2 « IL-4 et IL-5 » dont la production par les PBMC est stimulée par les antigènes FMDV. De plus, un lien direct a été établi entre les taux des cytokines IL-17 et IL-6 libérés lors des différentes stimulations.

Mots clés: Interleukine-17, Vaccin, Fièvre aphteuse, Cellules mononucléées du sang périphériques

INTRODUCTION

T helper (Th) cells play an important role in regulating immune responses through coordinating the function of other immune cell types. Effector Th cell subsets are characterized by their differential cytokine production profiles and immune regulatory functions. The Th1 and Th2 performance has prevailed in immunology (Dong and Flavell, 2000; Korn et al., 2009). Despite the fact that most recent studies describe IL-17 as a T cell-secreted cytokine, much of the interleukin (IL)-17 released during an inflammatory response is produced by innate immune cells (Cua and Tato, 2010), and it appears that IL-17-producing Th cells can function with Th1 cells to mediate protective immunity against pathogens (Mills, 2008). Furthermore, these cytokines play key regulatory roles in host defense and inflammatory diseases (Jin and Dong, 2013). The induction or function of Th17 cells is regulated by cytokines secreted by the other major subtypes of T cells including interferon-gamma (IFN- γ), IL-4, IL-10 and at high concentrations, transforming growth factor beta 1 (TGF- β) (Korn et al., 2009). The main function of IL-17-secreting T cells is to mediate inflammation through stimulating production of inflammatory cytokines such as TNF- α , IL-1 β , and IL-6, and inflammatory chemokines that promote the recruitment of neutrophils and macrophages (Cua and Tato, 2010; Espir et al., 2014). IL-17 and other pro-inflammatory cytokines have a mutual role as a strong synergy with IL-1 β in inducing IL-6 production by synovial fibroblasts (Chaubaud et al., 1999; Mills, 2008) and TNF- α and IL-1 β cytokine production in macrophages stimulated with IL-17 (Jovanovic et al., 1998; Mills, 2008). Moreover, the in-vitro addition of recombinant IL-17 to synovial fibroblasts induced

secretion of the pro-inflammatory cytokines of IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and prostaglandin (PG) E2 (Fossiez et al., 1996). Few studies have investigated the interaction response of IL-17, IL-6, IL-2, and IFN- γ as pro-inflammatory and inflammatory cytokines against IL-4, IL-5, and IL-10 as anti-inflammatory cytokines to viral antigen stimulation in the bovine peripheral blood cells (Mingala et al., 2009). Thus, in this study, we investigated pro- and anti-inflammatory cytokine production in response to specific antigens and general mitogens that stimulate specific cytokine profiles; inactivated foot and mouth disease vaccine (FMDV) stimulates Th2 cells, pokeweed (PWM) stimulates T cells, particularly Th cells and B cells (Miller et al., 1991), phytohemagglutinin (PHA) has similar effects as PWM, but is less potent in stimulation of B cells, and concanavalin A (Con A) is reported to stimulate T cells, particularly cytotoxic T cells (Simon et al., 1986), suppressor inducer T cells (Morimoto et al., 1985), and "virgin" T cells (Miller et al., 1991). We studied expression of pro-inflammatory cytokines such as IL-17, IL-6, IL-2, and IFN- γ , and anti-inflammatory cytokines including IL-4, IL-5, and IL-10 at cytokine level in response to antigen and mitogen stimulation of peripheral blood mononuclear cell (PBMC), and compared IL-17 to other well-characterized cytokines such as IFN- γ , IL-2, IL-4, IL-5, IL-6, and IL-10.

MATERIALS AND METHODS

Subjects. Fifteen Holstein calves, aged 2.5-3 months, were used in this study. All the calves were in good health and were divided into two groups of vaccinated calves (ten calves) as the treatment group and unvaccinated calves (five calves) as the control group.

The treatment group was vaccinated with tetravalent FMDV, and the control group received phosphate-buffered saline (PBS) alone. This vaccine is specifically produced in Iran by Razi Vaccine and Serum Research Institute, which contains O pan, O2010, A05IR, and Asia1 strains. The treatment group received 5.0 ml of tetravalent-FMD vaccine in the first vaccination and 2.0 ml of the same vaccine in the second vaccination (booster) one month after the first vaccination. Further, all the animals were monitored on a daily basis for temperature and clinical signs after vaccination. Blood samples were collected one week after booster dose for proliferation and cytokine assay.

Media and reagents. All the materials, reagents, and media cultures were obtained from Sigma Chemical Co., St. Louis, Mo. USA. The cell culture medium consisted of RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 10 mM of HEPES buffer, 2 mM of l-glutamine, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. PBMCs were isolated from whole blood through centrifugation by Ficoll-Hypaque solution (Histopaque-1077; Sigma Chemical Co., St. Louis, Mo 63103, USA).

Cell culture and antigen stimulation. In general, 10-15 ml of blood samples were taken from the jugular vein of calves and were collected in sterile heparin tubes, and PBMCs were isolated from whole blood as follows. The whole blood was mixed with an equal volume of PBS and overlaid onto 15 ml Histopaque (Sigma). The gradients were centrifuged for 30 min, at 20-25 °C at 1300 x g. Cells at the interface were aspirated and washed in PBS by centrifugation for 10 min at 670 x g. If after washing, red blood cell (RBC) contamination was evident, the cells were incubated with 0.83% (w/v) ammonium chloride buffer for 5 min before three further washes as described above. PBMCs were isolated and viability was estimated via trypan blue dye exclusion. The viability of PBMCs was 94%; freshly isolated PBMCs were cultured at 1.1×10^6 cells/ml in RPMI 1640 containing non-essential amino acids, l-glutamine, 2 g/l of sodium bicarbonate, 10 µM of HEPES, and 100 µg/ml of gentamycin,

supplemented with 10% fetal calve serum (FCS) in 96-well U-bottomed microtitre plates (Nunc, Napierville, US). PBMCs were stimulated with optimal concentrations of FMDV and mitogens, which resulted in maximal proliferative response. FMDV at 1:100 dilution contained 5 µg/ml of Con A, 2.5 µg/ml of PHA, and 2.5 µg/ml of PWM (Sigma, St Louis, MO 63103, US). Thereafter, 180 µl of cell suspension was added to each well of a 96-well tissue culture plate in five replicates. Next, 20 µl of the diluted FMDV, mitogens as described above, and medium (for control) were added to the wells, yielding a final concentration of 2×10^5 cells/well. Finally, PBMC cultures were incubated at 37 °C in 5% CO₂ for up to four days (96 h).

Proliferation assay. FMDV and mitogen-induced proliferations were determined for the treatment and control groups at 24, 48, 72, and 96 hours using MTT assay. Accordingly, 10 µl of MTT (5 µg/ml in phosphate buffer saline [Roche Co., France]) solution was added to the wells during the last six hours of cultivation. At the end of the cultivation, the plates were centrifuged at 400 x g for 10 min and the culture supernatant was accumulated. Afterwards, 100 µl of dimethyl sulfoxide (DMSO) was added to each well, after which the plates were vigorously shaken to ensure that all crystals were completely dissolved. The amount of MTT formazan produced during the incubation was quantified by an ELISA reader (Bio-Tek ELx800, US) at test wavelength of 550 nm and a reference wavelength of 630 nm. The results were based on optical density at the wavelength of 550 nm (OD550) and were expressed as a stimulation index (SI), which was calculated as follows and the culture supernatants were collected and stored at -70°C for cytokine assay by ELISA (Kondo et al., 1996; Daenicke et al., 2011).

$$SI = \frac{(\text{mean OD550 of stimulated PBMCs}) - (\text{mean OD550 of blank})}{\text{mean OD550 of unstimulated PBMCs}}$$

Cytokine assays. Cell culture supernatants were harvested and analyzed for cytokines using bovine IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, and IFN-γ commercially available ELISA kits (all USCN Life Science Inc.,

China). The limits of detection (LOD) for the individual assays were as follow: IL-2, 12.9 pg/ml; IL-4, 6.2 pg/ml; IL-5, 6.2 pg/ml; IL-10, 12.4 pg/ml; IL-17, 14.2 pg/ml; IL-6, 12.6 pg/ml; and IFN- γ , 12.8 pg/ml.

Statistical analysis. Due to variability of the cytokine response over time, for each individual subject and each antigen treatment the mean cytokine response was calculated over the three time points of PBMC culture to represent the overall cytokine response to the antigen. Mean values for each treatment are graphically represented in the presented figures. To analyze the data, Mann-Whitney U-test and Spearman's rank test were performed using SPSS, version 18.

RESULTS AND DISCUSSION

Antigen and mitogen-induced proliferation of PBMCs. The stimulation index (SI) determined for each antigen and mitogen at 24, 48, 72, 96, and 120 h were used as a measure of PBMC proliferation in response to the FMDV (as a specific antigen) and PHA, Con A, and PWM mitogens. For each of the specific antigens and mitogens, the mean SI increased over time (Figure 1-a). The highest proliferative response was observed at 96 h after stimulation, and the order of preferential stimulation was PWM>PHA>Con A>FMDV>cell control (Figure 1-b).

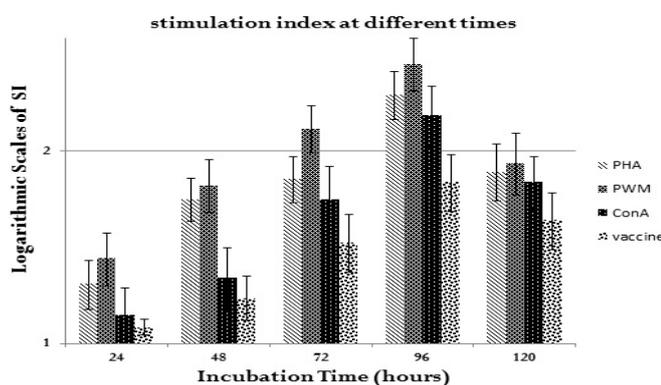


Figure 1a. Logarithmic scales of stimulation index (SI) of peripheral blood monocyte cells in response to antigen and mitogens; Con A (5 μ g/ml); PHA and PWM (2.5 μ g/ml); FMDV (1:100) at 24, 48, 72, 96, and 120 h after culture in RPMI 1640 at 37°C in 5% CO₂. The x-axis represents incubation time and the y-axis represents the stimulation index, based on log₁₀.

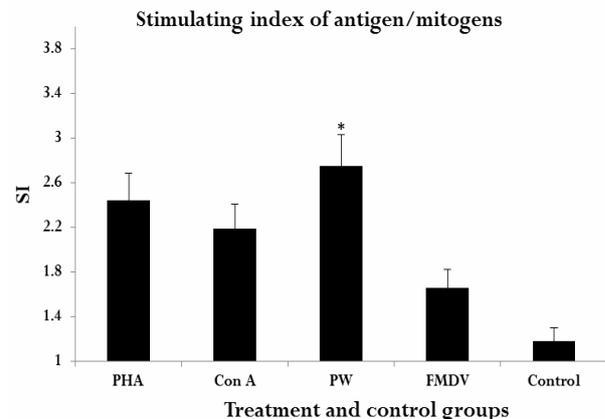


Figure 1b. Stimulation index (SI) of peripheral blood monocyte cells in response to antigens and mitogens; concanavalin A (5 μ g/ml); phytohemagglutinin and PW (2.5 μ g/ml); foot and mouth disease vaccine (1:100) at 96 h (or 4 days) after culture in RPMI 1640 at 37°C in 5% CO₂.

Antigen and mitogen stimulation of pro-inflammatory cytokines. IL-17: In contrast to mitogens, antigen stimulation produced low levels of IL-17 cytokines. Median IL-17 levels were 1087.06 pg/ml for FMDV antigen, 1422.96 pg/ml for PHA, 1367.91 pg/ml for Con A, 1217.08 pg/ml for PWM, and 301.54 pg/ml for cell control. The highest and lowest levels of the secreted IL-17 cytokines were observed in response to PHA and Con A mitogens, respectively, the order of IL-17 cytokine secretion in the stimulated PBMCs was PHA>Con A>PWM>FMDV>cell control (Figure 2).

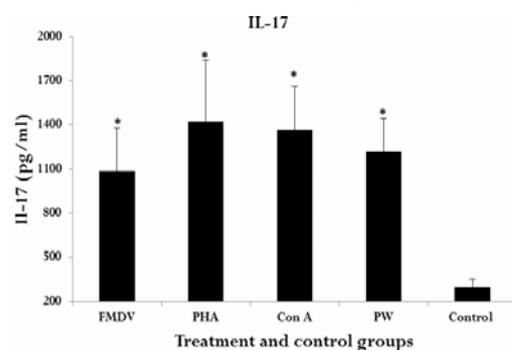


Figure 2. Interleukin-17 production of the stimulated peripheral blood monocyte cells in response to antigens and mitogens at 96 h (or 4 days) after culture in RPMI 1640 at 37°C in 5% CO₂.

IL-2: According to the results presented in Figure 3, IL-2 production in response to FMD antigens and mitogens showed a wide range of values, as PWM

mitogen was more effective in stimulating the PBMC cells in IL-2 synthesis compared to other mitogens and antigens. The median values of IL-2 production due to the antigen and mitogen stimulatory effects were 123.41 pg/ml for FMDV antigen, 188.23 pg/ml for PHA, 212.7 pg/ml for Con A, 252.37 pg/ml for PW, and 23.18 pg/ml for cell control. Moreover, the data were in the following order: PWM>Con A>PHA>FMDV>cell control (Figure 3).

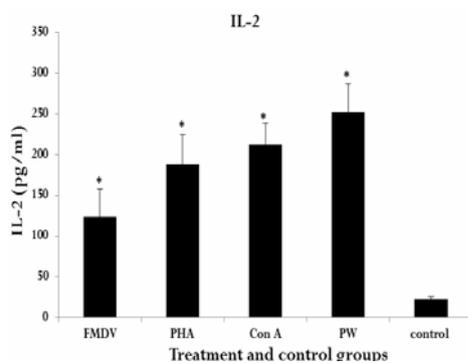


Figure 3. Interleukin-2 production of the stimulated peripheral blood monocyte cells in response to antigens and mitogens at 96 h (or 4 days) after culture in RPMI 1640 at 37 °C in 5% CO₂.

IL-6: The results of IL-6 production are shown in Figure 4, demonstrating a wide range of values. The median values of IL-6 production due to the antigen and mitogen stimulatory effects were 75.34 pg/ml for FMDV antigen, 138.38 pg/ml for PHA, 72.5 pg/ml for Con A, 116.49 pg/ml for PW, and 28.2 pg/ml for cell control. The order of preferential stimulation was PHA>PWM>FMDV>Con A>cell control (Figure 4).

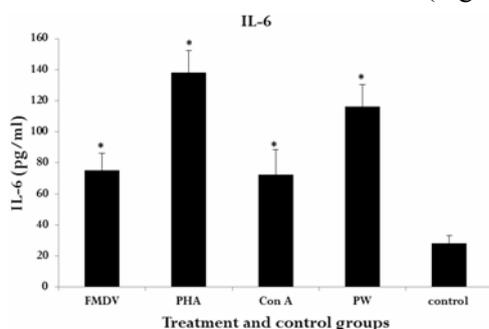


Figure 4. Interleukin-6 production of the stimulated peripheral blood monocyte cells in response to antigens and mitogens at 96 h (or 4 days) after culture in RPMI 1640 at 37 °C in 5% CO₂.

IFN- γ : The results of IFN- γ production in Figure 5 also showed a wide range of values. The median values of IFN- γ production due to the antigen and mitogen stimulatory effects were 88.6 pg/ml for FMDV antigen, 161.14 pg/ml for PHA, 186.14 pg/ml for Con A, 206.42 pg/ml for PW, and 27.51 pg/ml for cell control. The order of preferential mitogen stimulation was PWM>Con A>PHA>FMDV>cell control (Figure 5).

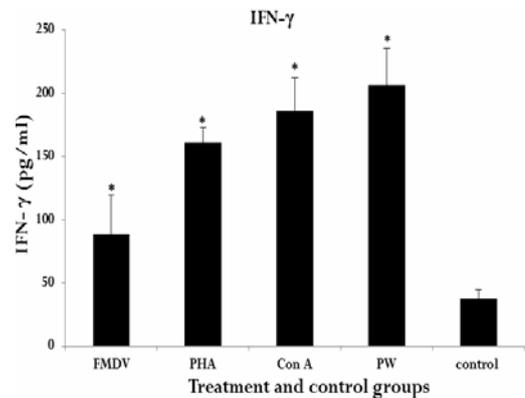


Figure 5. Interferon-gamma production of the stimulated peripheral blood monocyte cells in response to antigens and mitogens at 96 h (or 4 days) after culture in RPMI 1640 at 37 °C in 5% CO₂.

Antigen and mitogen stimulation of anti-inflammatory cytokines. IL-10: The close range of median values of IL-10 production is presented in Figure 6. According to the obtained results, the median values were 50.86 pg/ml for FMDV antigen, 77.46 pg/ml for PHA, 63.78 pg/ml for Con A, 89.44 pg/ml for PW, and 14.3 pg/ml for cell control. The order of preferential stimulation was PWM>PHA>Con A>FMDV>cell control (Figure 6). IL-4: The median values exhibited in Figure 7 show the IL-4 production in response to mitogen stimulation. According to the results, the range of values of these cytokines were 58.86 pg/ml for FMDV antigen, 62.19 pg/ml for PHA, 31.47 pg/ml for Con A, 75.52 pg/ml for PW, and 6.2 pg/ml for cell control. The order of preferential stimulation was PWM>PHA>FMDV>Con A>cell control (Figure 7). IL-5: The results of IL-5 production are presented in Figure 8, demonstrating a close range of values. The median values were 63.18 pg/ml for

FMDV antigen, 88.76 pg/ml for PHA, 59.42 pg/ml for Con A, 96.61 pg/ml for PW, and 7.5 pg/ml for cell control. The order of preferential stimulation was PWM>PHA>FMDV>Con A>cell control (Figure 8).

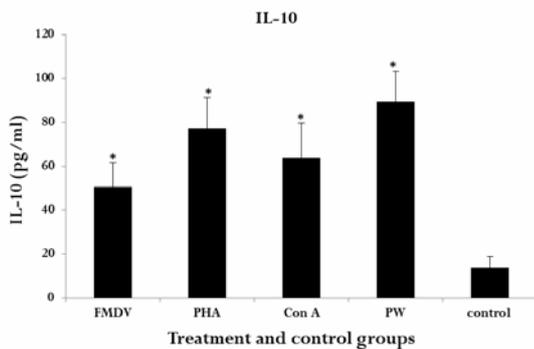


Figure 6. Interleukin-10 production of the stimulated peripheral blood monocyte cells in response to antigens and mitogens at 96 h (or 4 days) after culture in RPMI 1640 at 37 °C in 5% CO₂.

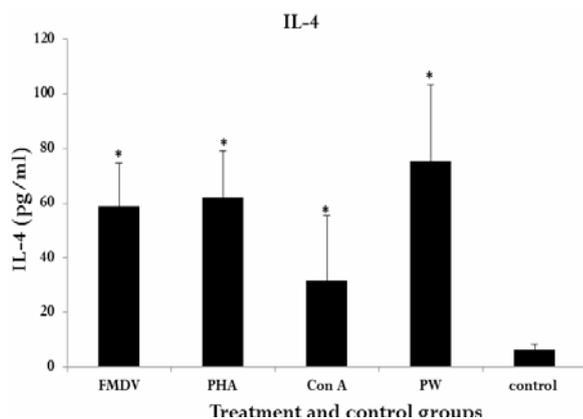


Figure 7. Interleukin-4 production of the stimulated peripheral blood monocyte cells in response to antigens and mitogens at 96 h (or 4 days) after culture in RPMI 1640 at 37°C in 5% CO₂.

Kinetics of antigen- and mitogen-induced IL-17 with IL-2 and IFN- γ production: We investigated IL-17 expression at cytokine level in comparison with IFN- γ and IL-2. The level of IL-17 cytokine secretion increased in all the subjects in the antigen- and mitogen-stimulated cultures, but not in the unstimulated ones. The highest levels of IL-17 production in response to mitogens were observed in PHA-stimulated PBMCs (1422.96 \pm 318.76 pg/ml) and the lowest levels were observed in PWM-stimulated PBMCs (1217.08 \pm 128.95 pg/ml). Moreover, FMDV antigen

stimulated significant levels of IL-17 compared to unstimulated cultures (control group; Figure 2). In contrast to IL-17, IFN- γ and IL-2 cytokine production were evident at all time points considered. The highest levels of IFN- γ and IL-2 production in response to mitogens were observed in PWM-stimulated PBMCs (206.42 \pm 28.34 pg/ml and 252.37 \pm 23.65 pg/ml, respectively) and the lowest levels were observed in PHA-stimulated PBMCs (161.14 \pm 11.87 pg/ml and 188.23 \pm 25.18 pg/ml, respectively). Furthermore, FMDV antigen stimulated significant levels of IFN- γ and IL-2 compared to unstimulated cultures. According to the results presented above, reduced levels of IL-2 and IFN- γ greatly increased IL-17 level and vice versa (Figure 9).

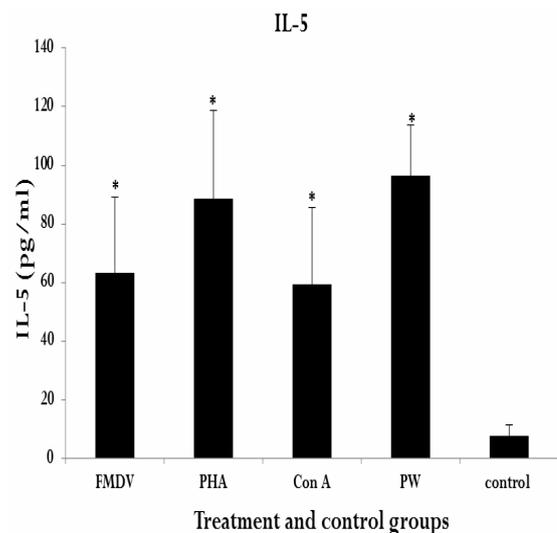


Figure 8. IL-5 production of the stimulated peripheral blood monocyte cells in response to antigens and mitogens at 96 h (or 4 days) after culture in RPMI 1640 at 37 °C in 5% CO₂.

IL-6 and IL-10 production: We also compared antigen- and mitogen-induced IL-17 cytokine production to IL-10 and IL-6 production. IL-10 and IL-6 cytokines were produced by all the antigen- and mitogen-stimulated cultures, and their level was significantly higher compared to those in the unstimulated cultures. The highest and lowest levels of cytokine production in stimulated PBMCs, for IL-10 presented by PWM and Con A were 89.44 \pm 10.09

pg/ml and 63.78 ± 10.13 pg/ml, respectively, and for IL-6 presented by PHA and Con A were 138.38 ± 14.79 pg/ml and 72.5 ± 16.80 pg/ml, respectively.

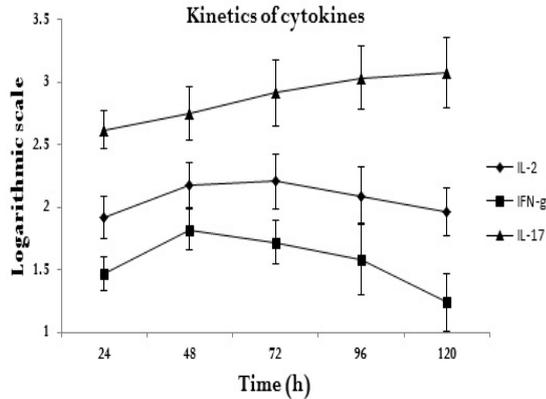


Figure 9. Kinetics of interleukin (IL)-17, IL-2, and interferon-gamma protein secretion over time by peripheral blood monocyte cells, following foot and mouth disease vaccine antigen stimulation. X-axis represents cytokine production, based on log10 and the y-axis represents incubation time.

In contrast to mitogens, production of IL-10 and IL-6 cytokines with FMDV antigen stimulated-PBMCs was significantly increased compared to the unstimulated cultures (50.86 ± 18.32 pg/ml and 75.34 ± 11.21 pg/ml, respectively). This cytokine kinetics demonstrates that the IL-6 production has a direct correlation with Th17 subset stimulation and IL-17 production, whereas when IL-6 cytokine increased or decreased, IL-17 cytokine in turn, increased or decreased. However, this correlation was not observed for IL-10 as an anti-inflammatory cytokine (Figure 10).

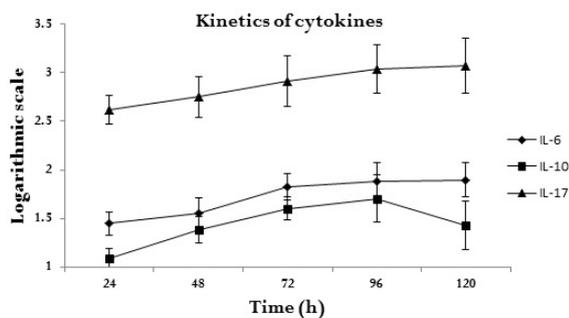


Figure 10. Kinetics of interleukin (IL)-17, IL-6, and IL-10 protein secretion over time by peripheral blood monocyte cells, following foot and mouth disease vaccine antigen stimulation. The X-axis represents cytokine production based on log10, and the y-axis represents incubation time.

IL-4 and IL-5 production: There was not a significant correlation between kinetics of IL-17 cytokine production and IL-4 and IL-5 in mitogen-stimulated PBMCs. In all the subjects, IL-4 and IL-5 cytokines were produced. The highest levels of IL-4 and IL-5 production were observed in PWM-stimulated PBMCs (75.52 ± 28.42 pg/ml and 96.61 ± 7.04 pg/ml, respectively) and the lowest levels were observed in Con A-stimulated PBMCs (31.47 ± 14.51 pg/ml and 59.42 ± 16.01 pg/ml, respectively). Also there was a significant level of IL-4 and IL-5 production in FMDV antigen-stimulated PBMCs compared to unstimulated cultures. A significant correlation was noted between IL-4, IL-5, and IL-17 cytokines. On the other hand, whenever IL-4 and IL-5 cytokines were increased IL-17 cytokine level was enhanced; as well (Figure 11). IL-17 is a cytokine produced by CD4 Th cells and plays an important role in inflammation (Yao et al., 1995). In this study, we investigated pro- and anti-inflammatory cytokines, especially IL-17 production in response to a range of mitogens and antigens.

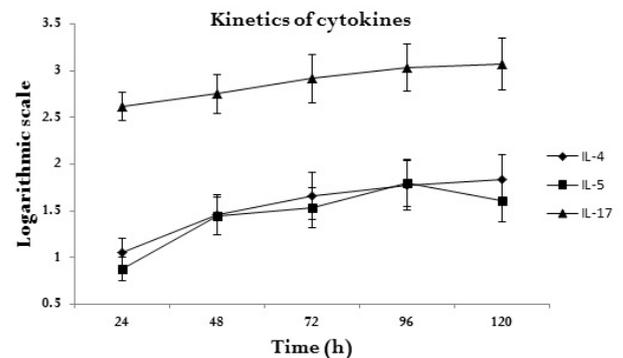


Figure 11. Kinetics of interleukin (IL)-17, IL-4, and IL-5 protein secretion over time by peripheral blood monocyte cells, following foot and mouth vaccine antigen stimulation. The x-axis represents cytokine production based on log10, and the y-axis represents incubation time.

The antigens and mitogens chosen in this study represent a spectrum of exogenous antigens known to stimulate divergent cytokine profiles. PWM has two specific domains available for binding to membrane receptors (Li et al., 1998), and therefore, has a higher

potential for action. This may explain the fact that the mitogenic effect of PWM on mononuclear leukocytes was higher than PHA and Con A, that is, PWM stimulates T cells, particularly helper T cells and B cells (Miller et al., 1991). PHA, in comparison with PWM, has similar effects, but it is less potent in stimulating B cells; Con A is reported to stimulate T cells, particularly cytotoxic T cells (Simon et al., 1986), suppressor inducer T cells (Morimoto et al., 1985), and “virgin” T cells (Miller et al., 1991). In our study, IL-17 was produced at high levels in response to the T cell mitogens; PHA and Con A were produced at low levels in response to PWM mitogens. On the other hand, the highest level of IL-17 was produced in response to PHA stimulation demonstrating that T cell-specific signaling is involved in the production of IL-17. Level of the produced IL-17 in response to the antigen was lower than those produced in response to mitogens, and this low response occurred in the presence of low proliferative responses to the antigen. There was not a significant association between IL-17 production and cellular proliferation, but despite vigorous proliferation to antigen, IL-17 levels produced by the PBMCs were lower than those observed after mitogen stimulation. In all the cases, there was a significant difference between antigen-stimulated and unstimulated PBMCs (Figures 1-a, 2). In the current study, FMDV induced a mixed cytokine profile at cytokine level that was representative of Th2 profile; also, the mitogens induced a mixed cytokine profile. The PHA and PWM mitogens resulted in induction of a response dominated by high levels of IFN- γ , IL-2, IL-6, and IL-17 and low levels of IL-4 and IL-5, demonstrating T cell and B cell stimulation, while the Con A mitogen stimulated a classic type-1 response dominated by production of high levels of IFN- γ , IL-2, and IL-17, whereas PWM mitogen stimulated high levels of classic type-1 cytokine responses (IFN- γ , IL-2, and IL-17) and type-2 cytokine responses (IL-4, IL-5, and IL-10). One of the possible or potential reasons for the high levels of detectable IL-17 protein from peripheral blood lymphocytes in response to mitogens and FMDV

antigen stimulation include the high and rapid generation of Th17 cell clones. Another probable reason may be the large number of special clones of T cells producing IL-17 and the cells producing IL-23 and IL-6 in PBMC (Lenarczyk et al., 2000; Rašková et al., 2005). Whereas, mitogens proliferate the total clones of immune cells such as T cells and dendritic cells (Rašková et al., 2005). A multitude of studies proved that in chronic inflammation, antigen-stimulated dendritic cells and macrophages produce IL-23, which promotes the development of Th17 cells (Iwakura and Ishigame, 2006). IL-23 also acts on dendritic cells and macrophages in an autocrine/paracrine manner to stimulate the generation of pro-inflammatory cytokines such as IL-1, IL-6, and TNF- α (Iwakura and Ishigame, 2006; Cua and Tato, 2010). Our results suggest that these processes are present in mitogens and FMD vaccine-stimulated PBMCs, but do not occur during in-vivo responses. According to the results obtained in the current and other studies, we can conclude that IL-6 potentially initiates Th17 differentiation (Vincent et al., 2013). Furthermore, several researchers showed that TGF- β and IL-6 support the differentiation of IL-17-producing T cells from naive cells and that tumor necrosis factor alpha (TNF- α) and IL-1 β further amplified IL-17 expression (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Mills, 2008). The evidence shows that IL-17 is highly up-regulated at sites of inflammatory tissues of autoimmune diseases and amplifies inflammation through synergy with other cytokines such as TNF- α (Zhu and Qian, 2012). Both Th1 and Th2 subsets negatively regulate Th17 differentiation, that is, the development of Th17 cells from naive precursor cells was potentially inhibited by IFN- γ and IL-4, whereas the committed Th17 cells were resistant to suppression by Th1 or Th2 cytokines. In the absence of IFN- γ and IL-4, IL-23 induced naive precursor cells to differentiate into Th17 cells independent from STAT1, T-bet, STAT4, and STAT6 transcription factors (Harrington et al., 2005; Park et al., 2005). In-vitro re-stimulation of PBMCs of vaccinated calves by FMDV antigens

produced readily detectable levels of IL-17 cytokine. IL-17 may be similar to IL-4 (Kurtzhals et al., 1992) when detectable levels of IL-17 in antigen-stimulated cultures are observed following secondary stimulation with the same antigens.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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