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Potential role of LYN, CCL2, ITGB3 and IL6 genes in the immune response of porcine buccal mucosa cells

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Abstract

The genes considered in this study, namely, *LYN, CCL2, ITGB3* and *IL6* can be related to immune response in porcine buccal mucosa cells primary cultured *in vitro*. These genes are also responsible for, inter alia, cell migration, differentiation, proliferation and apoptosis, intracellular signal transduction, extracellular matrix binding and wound healing. A total of 20 pubertal crossbred Landrace gilts bred on commercial farms were used to obtain buccal mucosa cell cultures, which were harvested on the 7th, 15th and 30th day after initiation of the culture. Expression levels of *LYN, CCL2, ITGB3* and *IL6* were evaluated employing Real-Time Quantitative Polymerase Chain Reaction. All studied genes showed expression. The expression of *CCL2* on day 15 was the highest of all factors measured. The greatest difference between the measurements occurred in gene *IL6,* between 7th and 15th day, while the least difference between the measurements occurred in gene *LYN*, between 7th and 15th day. Moreover, on the 7th day, *LYN* presented the lowest expression among all studied genes. Although *in vitro* conditions are much more controlled than *in vivo* conditions, all the factors that may positively or negatively affect cultured cells still cannot be fully predicted. Nevertheless, *LYN, CCL2, ITGB3* and *IL6* are a valuable starting point for studying further immunological processes in oral mucosal epithelial cells. Given their high regenerative potential, research into them is a highly valuable source of information for future wound healing therapies, where immunological processes should be carefully considered.

Running title: Potential role of *LYN*, *CCL2, ITGB3* and *IL6* genes in the immune response

Keywords: immune response, porcine buccal mucosa, in vitro culture, RT-qPCR

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Introduction

The oral mucosa is an interesting and prominent subject of research due to its large surface and easy isolation. The oral mucosa has numerous functions, the most important of which is to act as a barrier [1]. It prevents harmful factors such as bacteria or toxic substances from entering the tissues. It also protects deeper tissues such as nerves, muscles and fat, which are exposed to mechanical damage when chewing or biting. Three main categories based on function and histology of oral mucosa can be highlighted: specialized, masticatory and lining mucosa [2]. Specialized mucosa can be found in the area of the taste buds localized on the dorsal surface of the tongue. It plays a role in taste perception and overall sensory perception as it contains nerve endings [3]. The next type is masticatory mucosa. This stratified, keratinized squamous epithelium can be found on the hard palate, attached gingiva and on the dorsum of the tongue, too [4]. Finally, the lining mucosa is stratified, but nonkeratinized squamous epithelium and can be found practically all over the oral cavity, excluding abovementioned areas. Three types of lining mucosa can be highlighted: alveolar, labial and buccal mucosa [3]. The interest of this study, buccal mucosa tissue, constitutes inside lining of the floor of the mouth and cheeks [4]. The buccal mucosa has been used extensively in drug delivery and transplant research [5–9]. An especially common replacement for human mucosa is the one obtained from domestic pig, due to the high phylogenetic similarity of both organisms [10,11]. As described above, the oral mucosa mainly acts as a protective barrier in living organisms, therefore strong correlation between this tissue and the body's immune response is assumed. One of the reasons for turning on the mechanisms of cells' immune response may be bacterial contamination [12], which is very common in the cultivation of cells obtained from the oral cavity due to the naturally occurring wide range of microflora. The genes considered in this study were *LYN, CCL2, ITGB3* and *IL6*, respectively. All of them are related to the immune response. In addition to being associated with the immune response, these genes are involved in other biological processes such as cell differentiation [13], intracellular signal transduction [14], extracellular matrix binding [15], positive regulation of cell population proliferation and negative regulation of apoptotic process [16]. The studies of the expression of above stated genes in porcine mucosa are very limited. Our goal was to study the expression of these genes during the *in vitro* primary culture of the porcine buccal mucosa in the context of immune response and cell growth processes. Measurements of expression levels of *LYN, CCL2, ITGB3* and *IL6* mRNA were assessed on cells obtained from the culture on days 7, 15 and 30 of its duration.

Materials and Methods

Animals

A total of 20 pubertal commercially provided crossbred Landrace gilts employed in the study had a mean age of 155 days (range 140 – 170 days), and the mean weight of 100 kg (95- 120 kg). All of the gilts were housed under identical conditions and fed the same forage. All experiments were approved by Local Ethic Committee.

Cell isolation and culture

After slaughter, samples of buccal pouch mucosa were obtained within 40 min and transported to the laboratory. The excised tissue was washed twice in Dulbecco's phosphate buffered saline (D-PBS) (Merck, Darmstadt, Germany). The surface of the buccal pouch was surgically removed using sterile surgical blades. The tissue fragments were incubated with 0.05% collagenase I (Merck, Darmstadt, Germany) for 40 min at 38 °C in a shaking water bath and then were treated witch 0.5% Trypsin/EDTA (Cascade Biologics, Portland, USA) for 10 min. The cell suspension obtained from this digestion was filtered through mesh to remove non-dissociated tissue fragments. Isolated cells were washed three times by centrifugation (10 min at 200 g) with Dulbecco's modified Eagle's medium (DMEM; Merck, Darmstadt, Germany) supplemented with gentamicin (20 µg/mL) and 0.1% BSA. The final cell pellet was resuspended in DMEM supplemented with 10% fetal calf serum (FCS; Merck, Darmstadt, Germany) and 10 U/ mL penicillin G, 10 mg/mL streptomycin, and 25 µg/ mL amphotericin B. Cell viability was 90 to 95% as determined by trypan blue staining (Merck, Darmstadt, Germany). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2. Once the cell cultures attained 70–80% confluency, they were passaged by washing with PBS, digested with 0.025% Trypsin/EDTA (Cascade Biologics, Portland, USA), neutralized by a 0.0125% trypsin inhibitor (Cascade Biologics, Portland, USA), centrifuged, and resuspended at a seeding density of 2x104 cells/cm2 . The culture medium was changed every three days.

Total RNA isolation

After harvesting cells on the 7th, 15th and 30th day of culture, total RNA was isolated. The process of RNA isolation was performed according to modified method of Chomczyński and Sacchi [17]. Briefly, obtained mucosa cells were suspended in 1 ml of monophasic guanidine thiocyanate and phenol solution (TRI Reagent®, Merck KGaA). Next, chloroform was added to separate the phases during centrifugation. The upper aqueous phase, containing isolated RNA, was collected. RNA was extracted with 2-propanol (Merck KGaA, catalog number I9516), added in an amount adequate for 1 ml of TRI-reagent. Finally, RNA was washed with 75%

ethanol, dried and resuspended in 20 µl of pure water. Its purity was estimated using the 260/280 nm absorption ratio (higher than 1.8) (NanoDrop spectrophotometer; Thermo Scientific, ALAB, Poland).

Reverse transcription

RNA denaturation involved the preparation of a reaction mixture consisting of 1 µg RNA, 4 µl 2.5 mM dNTP (Novazym, Poland), 0.5 µl oligo (dT) (Novazym, Poland) and 0.5 µl random hexamers (Novazym, Poland). The mixture was then incubated in a thermoblock for 5 min. at 65 ° C. The next step was the cDNA synthesis which involved the preparation of a reaction mixture by adding 4 µl of 5x concentrated First Strand Buffer (Invitrogen, USA), 2 µl DTT 0.1 M (Invitrogen, USA), 0.5 µl of SuperScriptTM II Reverse Transcriptase reverse transcriptase 200 U / µl (Invitrogen, USA) and 0.25 µl of the ribonuclease inhibitor Rnase OUT 40 U / µl (Invitrogen, USA). The mixture was then incubated in a thermoblock for 50 min. at 37 ° C.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) analysis

1 µL of cDNA obtained from the 7th, 15th and 30th day samples was added to 6 µL pure H_2O, 2 µL Hot Start mastermix QUANTUM EvaGreen® PCR Kit (Syngen Biotech, Wrocław, Poland) and 1 µL of forward and reverse primer mix for amplification. Each sample was repeated in triplicate. One RNA sample of each preparation was processed without the RT-reaction to provide a negative control for subsequent PCR. RT-qPCR was conducted using a LightCycler RT-qPCR detection system (Roche Diagnostics GmbH, Mannheim, Germany), with target cDNA was quantified using the relative quantification method**.**

Ethical approval

The research related to animal use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. Bioethical Committee approval no. 32/2012 from 30.06.2012.

Results

In this research, the relative abundance of *LYN, CCL2*, *ITGB3* and *IL6* mRNA in equal time intervals – 7, 15 and 30 days of buccal mucosa cells *in vitro* cultivation was assessed using the RT-qPCR assay (**Fig 1**).

Fold change of Lyn on 7th day of cell culture is a reference value on the **figure 1**. In genes *LYN and ITGB3* an increase in expression is seen between days 15 and 30 of cell culture of the buccal mucosa cells. In genes *LYN, CCL2* and *IL6* the expression on 30th day was higher than on 7th day.

In *LYN*, the highest expression occurred on 30th day of culture, while the lowest on the 7th day of culture.

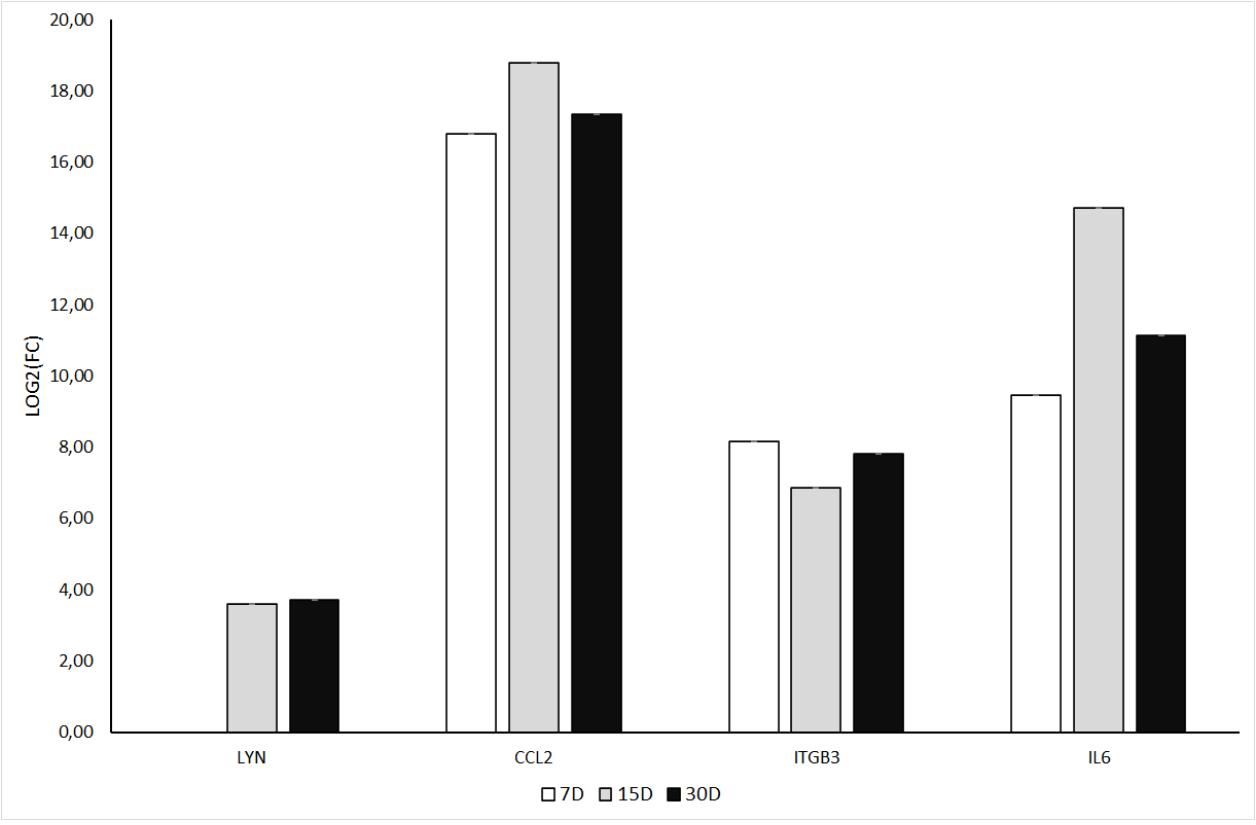


FIGURE 1 Expression of *LYN, CCL2, ITGB3* and *IL6* mRNA in buccal mucosa cells after various periods of in vitro cultivation; the results are expressed in logarithmic fold change scale

The difference in log(FC) level between 7th and 30th day is 3,71. It is the greatest difference between the first and the last measurement assessed in this study.

The expression *CCL2* presented on day 15 was the highest among all the measurements in this study. Its values on days 7 and 15 were also the highest of any other measurements assessed on the reference days among the studied genes.

ITGB3 is the only gene where expression on day 7 was higher than on day 15 or 30 of culture. This gene showed the highest expression on 7th day of culture and the lowest on 15th day. The exponential difference of the highest to the lowest FC value in this case equals 1,29.

IL6 showed the greatest difference in FC value between 7th and 15th day – 5,24. Of all measurements within one gene, only *IL6* and *CCL2* showed the highest expression on day 15 of *in vitro* culture.

Discussion

In the following study, we assessed expression levels of the *LYN, CCL2, ITGB3* and *IL6* genes in the *in vitro* primary culture of porcine buccal mucosa cells in order to broaden the current understanding of immune response and cell proliferation markers in analyzed tissue.

Immunology is one of the most complex biological response and it applies to all tissues of living organisms. The oral mucosa is exposed to numerous pathogens, given that it is the tissue that underlines first section of digestive tract and receives many different stimuli [18]. It should also be remembered that the oral cavity is invariably colonized by bacteria belonging to the natural microflora [19]. Cells of the innate immune system and oral keratinocytes can detect individual pathogen-associated molecular patterns through germline-encoded pattern recognition receptors [20]. Immune response cells, such as lymphocytes produced in the thymus or bone marrow, are not found in the primary *in vitro* culture of mucosal cells. Therefore, we are able to discuss the relationship of the studied genes mostly with keratinocytes and fibroblast present in the buccal mucosa culture.

LYN gene encodes tyrosine-protein kinase Lyn which is mainly expressed in neural and adipose tissue and hematopoietic cells [21,22]. The name "Lyn" is derived from Lck/Yes novel tyrosine kinase. It is linked to numerous biological processes such as innate immune response [23], intracellular signal transduction [24], negative regulation of toll-like receptor 2 signaling pathway [25], positive regulation of cell migration and proliferation [13]. *LYN* was described for the first time by Yamanasi et al. in 1987 after screening a human cDNA library made from placental RNA [26]. The pig model in our article turns out to not to be a widespread subject of research on the *LYN* gene. Nevertheless, the Lyn protein has been shown to be associated with the acrosome reaction of porcine spermatozoa [27] and can partially inhibit Na,K-ATPase activity in lens epithelium [28]. A cascade of signaling events is triggered by *LYN* activation. Among others, kinases such as Syk, phosphatidyl inositol-3 kinase and phosholipase Cγ2 (PLCγ2) are activated. Mentioned above kinases deliver activation signals, which play crucial roles in cell differentiation, Ca^{2+} mobilization and cell proliferation [13]. In this study, we showed that Lyn increased its expression levels over time. This may be due to the association of this gene with the proliferation process of cells which, after attaching to the substrate, began to proliferate increasing confluence.

C-C Motif Chemokine Ligand 2 (CCL2) is a member of the CC subfamily which is characterized by two adjacent cysteine residues [29]. The most described and best understood role of CCL2 so far is the recruitment of dendritic cells, monocytes and memory T cells to sites where inflammation has been triggered by injury or infection [30,31]. As in the genes described below, such a high expression of this gene could have been influenced by bacteria or their remains indispensable in primary cultures, as it has been proven that viral and bacterial infections result in high levels of, among others, CCL2 [32]. Viral infections are of particular importance when it comes to the pathogenesis of asthma, in which research shows that CCL2 plays a significant role [33]. The study showed that human rhinovirus (HRV) infection strongly increases the expression of *CCL2*, which at the same time turns out to be necessary for the initiation of an immune response in this case [33]. An interesting way of research on CCL2 is its role in the wound healing process, due to the fact that it is already known that the administration of anti-CCL2 antibodies reduces scarring. This can be considered not only as a problem in the context of fever, non-healing wounds, but also in the context of the field of aesthetic medicine dealing with excessive scarring or scar removal [34,35]. Pulmonary fibrosis should also be mentioned, and especially interesting studies that have shown a relationship between CCL2 and this disease. While modifications within this gene were able to reduce fibrosis, it appears that complete silencing of the gene causes no protection against fibrosis at all [36]. Interestingly, the authors believe that the reason for the increased *CCL2* gene expression may have been the accidental scratching of the culture during the change of cell culture medium. This way it could have created a gap in the cell layer that was treated like a wound, gradually overgrown, as is the case with the scratch assay technique used broadly for wound healing studies [37].

The next studied gene was *ITGB3*. It encodes protein named integrin beta-3 (β3) or cluster of differentiation 61 (CD61) [38]. Apoptotic cell clearance [39], cell and cell-matrix adhesion, cell migration

[40], extracellular matrix organization [41] and wound healing [42] are just several of the significant number of processes in which *ITGB3* gene is involved. Since CD61 is found mainly on thrombocytes, most research on this gene revolves around blood, blood vessels and the heart [43–45]. There are several studies that indicate the important role ITGB3 plays in the implantation and placentation of a porcine embryo [46,47]. Jaeger et al. were studying gene expression of porcine mammary epithelial cells after challenging it with Escherichia coli and Staphylococcus aureus *in vitro* [48]. The presence of E. coli induced strong and immediate up-regulation of genes encoding cytokines, chemokines and cell adhesion molecules, including ITGB3. This phenomenon was caused because of activating the cells of host defense, mediated by tumor necrosis factor (TNF) and interleukin 1 beta (IL1B) signaling [48]. The highest expression shown by *ITGB3* in our study was on day 7 of the culture. It was probably related to cell adhesion and extracellular matrix organization, as these processes occur most intensively in the first days of culture. However, we cannot completely winnow out even a slight infection, because despite the use of antibiotics in the culture medium, not all bacteria can be eliminated immediately.

The last and the most diversed in terms of expression levels in our study was the *IL6* gene. This gene encodes protein named interleukin 6 (IL6) that mainly acts as both an anti-inflammatory myokine and a pro-inflammatory cytokine [49]. It also takes part in various biological processes, such as acute-phase response [50], defense response to Gram-negative and -positive bacterium [51,52], negative regulation of apoptotic process [53], positive regulation of chemokine production [54] and negative regulation of cell population proliferation [55]. *IL6* is perhaps the most studied gene in the context of the porcine model of all three genes mentioned in this paper. While most of the research refers to inflammation processes [56–58], there are also pregnancy [59,60], xenotransplantation [61,62] and microbiota [63,64] related studies. As mentioned earlier, *IL6* may also be actively involved in the wound healing process. Winkel et al. stated that IL6 in lower doses may serve as effective therapy in healing intestinal injury after ischemia [65]. As epithelial tissues show high regenerative capacity [66], this data may be useful for further research into the wound healing process in the oral cavity. In our study, *IL6* showed the highest expression at day 15 of culture, which may occur for many reasons. The first would be a sudden, even slight, multiplication of bacteria that only reacted late to the antibiotics contained in the culture medium. It can also be related to stages of cell proliferation. If even a minor bacterial contamination did occur during the culture, this result could also be related to the apoptotic processes of *in vitro* cultured cells.

Conclusions

The genes studied in the above work, *LYN*, *CCL2*, *ITGB3* and *IL6*, respectively, may be associated with the immune response of primarily cultured porcine buccal mucosa cells *in vitro*. Although *in vitro* conditions are much more controlled than in vivo, we still cannot predict all factors that may positively or negatively affect cultured cells. In addition to the above limitations, we cannot perfectly reproduce the in vivo conditions as this would require deliberate contamination of the culture, which would undoubtedly interfere with the proper conduct of long-term culture. Nevertheless, these genes are a useful starting point for studying further immunological processes in oral mucosa cells. Given their high regenerative potential, research into them is a very valuable source of information for future wound healing therapies and innovations.

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Conflict of interest
The authors declare they have no conflict of interest.

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