*Exp. Anim. 68(2), 233–239, 2019*

**—Original—**

# Fetal and early postnatal development of the porcine tonsils of the soft palate

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**Abstract:** Tonsils are mucosa-associated lymphoid tissues located at the openings of the gastrointestinal and respiratory tracts, which play a key role in the surveillance of inhaled or ingested pathogens and can concurrently be reservoirs of infectious agents. Therefore, tonsils are important for the immunology and hygiene management of domestic animals, including pigs. However, the process of their fetal developmental has been poorly described, at least in part, because rodents lack tonsils. Therefore, we performed a histological analysis of porcine tonsils of the soft palate from 60 to 100 days of gestation (DG) and from 2 to 14 days post partum (DP). This analysis showed that lymphoid aggregations first appear at DG65, gradually develop during the fetal stage, and expand after birth. In addition, the mRNA expression of chemokine genes involved in lymphoid aggregation and localization was analyzed. *CCL19* expression showed the most marked increase and a sharp peak after birth. *CCL21* expression changed moderately but showed an interesting bimodal pattern. *CXCL13* expression steadily increased throughout the study period. Thus, we demonstrated the mRNA expression of chemokine characteristically changed accompanying tonsillar development.
**Key words:** chemokine, fetal development, pig, tonsil

## Introduction

Tonsils are mucosa-associated lymphoid tissues located at the openings of the gastrointestinal and respiratory tracts [3, 15]. Due to their anatomical position, they are exposed to various external agents, including pathogenic bacteria and viruses, and play a key role in the surveillance of inhaled or ingested microorganisms and in the induction of appropriate immune responses [6, 7]. However, the tonsils are also colonized by numerous bacteria and viruses and can therefore act as a reservoir for infectious agents that do not cause clinical signs to develop, i.e., they permit the establishment of latent infections. Indeed, surveys of the infectious status of pigs are often performed by sampling tonsils of the soft palate, the largest tonsils in the pig.

Histological analyses of the porcine tonsils have been previously performed and have described their characteristic structure [1, 2, 9]. The surface of the tonsils is covered with stratified squamous epithelium, which is perforated at regular intervals by tonsillar crypts, the openings of which are referred to as fossules. In the middle and lower portions of the tonsillar crypts, sporadic M cells and goblet cells are found, which take up foreign antigens and secrete mucus, respectively [1]. The lymphatic tissue of the tonsils has a cellular structure resembling that of lymph nodes, comprising B cell-containing follicles, germinal centers, and parafollicular areas dominated by T cells. Although structural development of the porcine tonsils rapidly proceeds after birth

*(Received 11 September 2018 / Accepted 6 December 2018 / Published online in J-STAGE 14 January 2019)*
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[20, 21], the earlier fetal component of tonsillar development has been poorly described.

Early development of lymphoid tissues, such as lymph nodes and Peyer's patches, has been well documented at the molecular level, especially in rodent models. The interaction of lymphoid tissue inducer (LTi) and lymphoid tissue organizer (LTo) cells *via* cell adhesion molecules and the following induction of chemokines are critical for tissue development [14]. However, these processes have been poorly studied in tonsils because rodents lack them. Murine nasopharynx-associated lymphoid tissue (NALT), which is equivalent to tonsils in other species, has some unique features, such as their development not being RORγt-dependent [4, 8]. However, tonsils are present in most mammals, including humans; therefore, it would be valuable for both academic and clinical purposes to describe their early development at the molecular level.

In this study, we described the fetal and early postnatal development of porcine tonsils of the soft palate, with the intentions of contributing to the improvement of disease control in pigs and refining the use of pigs as model animals for research in the development of mammalian tonsils.

## Materials and Methods

*Experimental animals*

Healthy cross-bred pigs (*Sus scrofa domesticus*) were included that had been conventionally reared at the Institute of Livestock and Grassland Science, NARO. All the sows used in this study had been inoculated with *Bordetella bronchiseptica* and *Pasteurella multocida* combined vaccine (ARBP combined vaccine; Nisseiken, Tokyo, Japan), Swine Erysipelas Vaccine (Nisseiken), Japanese Encephalitis-Porcine Parvovirus Infection Combined Live Vaccine (KM biologics, Kumamoto, Japan), Porcine Circovirus Vaccine (Ingelvac CircoFLEX; Boehringer Ingelheim Vetmedica, St. Joseph, MO, USA), *Lawsonia Intracellularis* Vaccine (Enterisol Ileitis; Boehringer Ingelheim Vetmedica) according to the vaccination program in the Institute of Livestock and Grassland Science, NARO. In addition, the sows which delivered piglets had been inoculated with Transmissible gastroenteritis (TGE) and Porcine epidemic diarrhoea (PED) virus combined vaccine (Nisseiken) in their pregnant stage. All animal experiments were approved by the Animal Care Committee of the Institute of Agrobiological Sciences, NARO.

*Collection of tissues from fetal and newborn pigs*

At 50, 55, 60, 65, 70, 85, and 100 days of gestation (DG), pregnant female pigs (n=2) were sacrificed, and their fetuses were harvested. In addition, piglets (n=4) were sacrificed at 2, 7, 14, and 30 days of postpartum (DP). The tonsils of the soft palate were collected from these fetuses or piglets and used for quantitative RT-PCR and/or histological analyses.

*Hematoxylin and eosin (H & E) staining*

Tonsils of the soft palate (DG60, 65, 70, 85, and 100, and DP2, 7, and 14) were embedded in molds, surrounded with optimal cutting temperature compound, snap-frozen in liquid nitrogen, and then stored at −80°C. Frozen tissues were sectioned at 6 $\mu$m with a cryostat (HM-500; Carl Zeiss Microscopy GmbH, Jena, Germany); then cryosections were air-dried at room temperature and fixed using a mixture of formaldehyde, ethanol, and acetate (20:80:1) for 15 min. After washing with phosphate-buffered saline free of divalent cations [PBS (−)], sections were stained with H & E.

*Immunohistochemistry*

Cryosections were air-dried at room temperature and fixed in ice-cold acetone for 15 min. The sections were then re-dried in air, washed twice with PBS (−), and incubated in PBS (−) containing 10% bovine serum albumin (BSA) for 60 min at room temperature to reduce non-specific staining. The sections were then incubated for 60 min at room temperature with anti-pig CD3 [Abcam Plc, Cambridge, UK ; 1:50 dilution in PBS (−) containing 1% BSA]. After washing with PBS (−), they were incubated with Alexa568-labeled anti-rabbit IgG (ThermoFisher, Waltham, MA, USA) and fluorescein isothiocyanate (FITC) -labeled anti-pig CD21 (SouthernBiotech, Birmingham, AL, USA) for 60 min at room temperature. Sections were then stained with 4',6-diamidino-2-phenylindole (DAPI), washed with PBS (−), and mounted on VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA).

*Total RNA extraction and RT-PCR*

Total RNA was extracted using Sepazol (Nakalai Tesque, Kyoto, Japan), according to the manufacturer's instructions. After being treated with DNase I (Takara Bio, Otsu, Japan) at 37°C for 30 min, RNA was sub-

**Table 1.** Primer sequences used in quantitative RT-PCR

| | forward | reverse | accession No. |
|---|---|---|---|
| *IL2RG* | CTTGGAACAGCAGCTCTGAG | ACCAACAGCCAGAAGTGATC | NM_214083 |
| *CCL19* | GCTAAGCCTCTGGACTTCTC | CAGCCATCTCGAATGAGCAG | NM_001170516 |
| *CCL21* | ATCCCAGCTATCCTGTTCTC | CTTGTCCTTCTTACAGTCCC | NM_001005151 |
| *CXCL13* | GAATGGATGTCCAACCAGAG | CACTGGAGCTGGTAAAGTTG | XM_003129101 |
| *ACTB* | AGGTCATCACCATCGGCAAC | ATCTCCTTCTGCATCCTGTC | XM_021086047 |

jected to first-strand cDNA synthesis at 37°C for 30 min using a PrimeScript RT reagent kit (Takara Bio).

Relative quantification of transcripts of interest was performed by real-time quantitative RT-PCR using a LightCycler (Roche Diagnostics, Basel, Switzerland). PCR amplification was performed in 20-$\mu$l reaction mixtures consisting of 1 $\mu$l cDNA, 0.4 $\mu$M each primer, and 10 $\mu$l SYBR premix Ex Taq II (Takara Bio). The cycling conditions were 95°C for 3 min, followed by 60 cycles of 95°C for 5 s, 55°C for 30 s, and 72°C for 30 s. The primers used are shown in Table 1. Target gene expression was normalized to expression of the beta-actin (*ACTB*) gene.

*Statistical Analysis*

Statistical analyses were performed using Tukey Honestly Significant Differences test (HSD). P values less than 0.05 were considered statistically significant.

## Results and Discussion

We first performed H&E staining to analyze the development of tonsils of the soft palate by characterizing the structural changes and cell aggregations (Fig. 1). The first small aggregations of cells were identified at DG65 (arrows in Fig. 1B), and clear aggregations were observed as early as DG70 (Fig. 1C). At this time, the characteristic structures of the tonsils, such as the stratified squamous epithelium and tonsillar fossules, could be observed (asterisks in Figs. 1 and 2). From this time until birth, the cellular aggregations gradually increased in size and the structure of the crypts concurrently developed (Fig. 1D–F). After parturition, lymphocytes aggregated very rapidly, and the follicular structure became more clearly organized; hence, the whole structure substantially thickened (Fig. 1G).

The colonization and localization processes for the T and B cells, which are the major components of tonsils, were characterized by immunofluorescence using anti-CD3 and CD21 antibodies (Fig. 2). The first positive signals, which were detected at DG65, indicated the presence of a mixture of T and B cells (Fig. 2B). At DG70 and DG85, dense aggregations of B cells surrounded by sparsely distributed T cells were observed (Figs. 2C and D). After this, the T cell-populated area became larger, while the aggregation of B cells continued (Figs. 2G and H). The follicular structure (marked by "f" in Fig. 2) of B cells surrounded by a larger T cell-populated area continued to organize until DP2 (Fig. 2K). During the period from DP2 to DP14, T and B cells rapidly increased in numbers in each area and T cells appeared in the follicles (Figs. 2K and L), which is supporting evidence that there occur interactions between T and B cells which induce immune responses.

Next, we examined the expression pattern of genes that are relevant to the migration and aggregation of tonsillar lymphocytes (Fig. 3). The levels of expression of *IL2RG*, which is widely expressed in immune cells, gradually increased until DG85 and slightly increased more rapidly around birth, which is consistent with the lymphoid aggregation time course shown by the histological analysis (Fig. 3A).

Chemokines such as CCL19, CCL21, and CXCL13 play important roles in the organization of lymphoid tissue, including murine NALT [16, 17]. We hypothesized that these chemokines would also be critical in the development of porcine tonsils and therefore characterized the expression patterns of these chemokines.

CCL19 and CCL21 are expressed in the T-cell areas of secondary lymphoid organs and are primarily responsible for the recruitment of CCR7+ T cells to these areas. *CCL19* showed the most marked change in expression, a 600-fold increase, and a sharp peak after birth (Fig. 3B). This suggests a rapid aggregation and compartmentalization of T cells at this time, which is consistent with the results of the immunohistochemical analyses. Although the change in *CCL21* expression was limited to several-fold, *CCL21* expression demonstrated an interesting bimodal expression pattern (Fig. 3C). An initial transient peak was observed at around DG60, when no

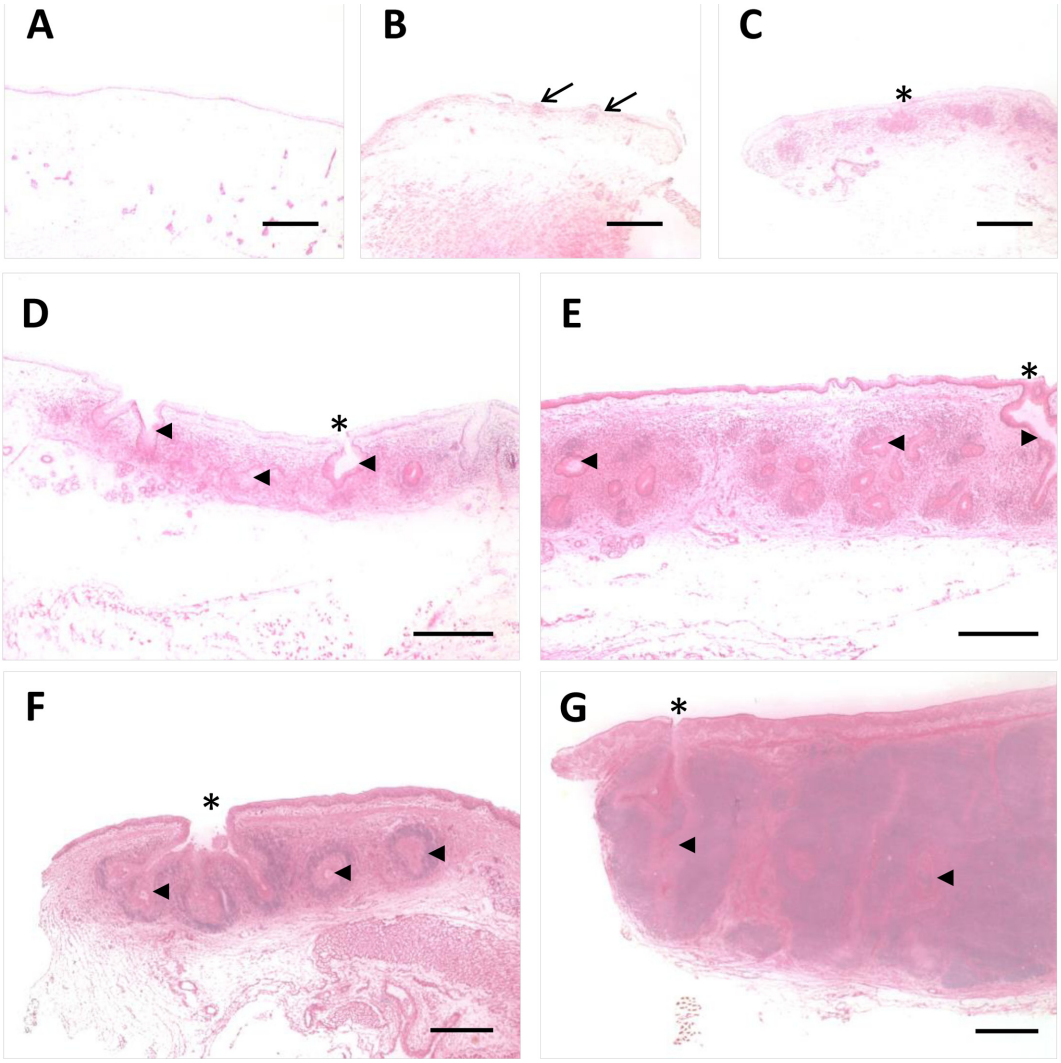


**Fig. 1.** Histological assessment of the development of porcine tonsils of the soft palate. Sections of the tonsils of the soft palate at 60 (A), 65 (B), 70 (C), 85 (D), and 100 (E) days of gestation and at 2 (F) and 14 (G) days postpartum were subjected to hematoxylin and eosin staining. The arrows in (B) indicate primary cellular aggregations. The arrowheads indicate the crypts, and the asterisks indicate the fossules. The scale bars represent 500 $\mu$m.

cellular aggregations were apparent in the putative tonsillar areas, at the time organogenesis process was commencing. The development of secondary lymphoid organs begins with the recruitment of LTi cells into the prospective sites by the chemokines listed above, which are produced by stromal cells [14]. Studies of knockout mice have shown that while CXCL13 plays a critical role in the development of most lymph nodes, facial and cervical lymph nodes can develop in CXCR5-deficient mice expressing CCL21 but lacking CXCL13 signaling [18]. This induction of CCL21 expression suggested that it has a significant role in the recruitment of LTi (-like) cells into designated tonsillar areas and/or functions as a trigger for the initial migration of lymphocytes, thereby substantially contributing to the early phase of tonsil-

**Fig. 2.** Immunohistochemical analysis of CD3 and CD21 expression in the porcine tonsils of the soft palate. The tonsils of the soft palate at 60 (A, D), 65 (B, E), 70 (C, F), 85 (G, I), and 100 (H, J) days of gestation and at 2 (K, M) and 14 (L, N) days postpartum were immunostained using anti-CD3 and CD21 antibodies (A–C, G, H, K, L) and counterstained with DAPI (D–F, I, J, M, N). The arrowheads indicate the crypts, and the asterisks indicate the fossules. Representative follicular structures are labeled with "f." The scale bars represent 500 $\mu$m.

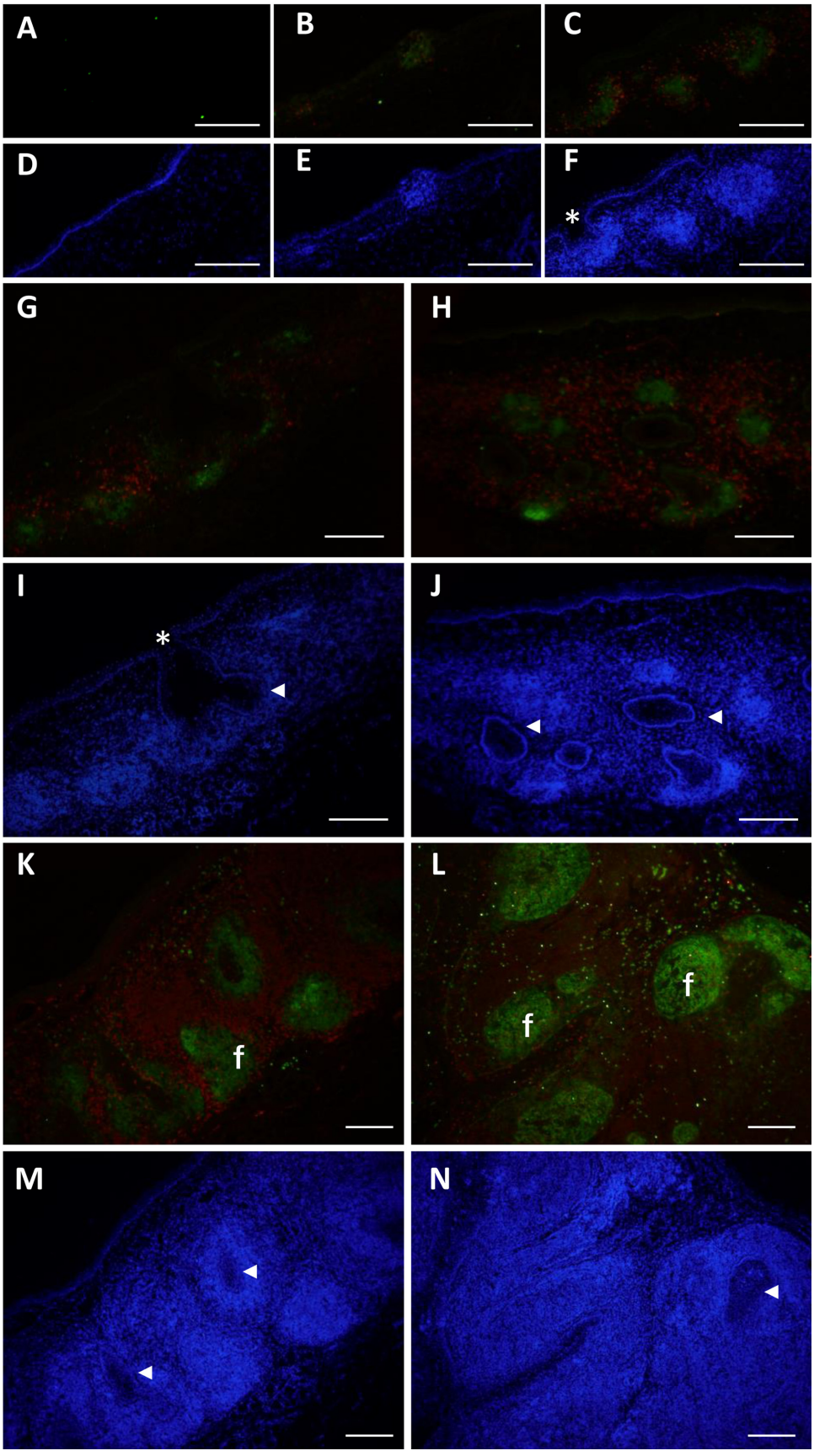


**Fig. 2.**

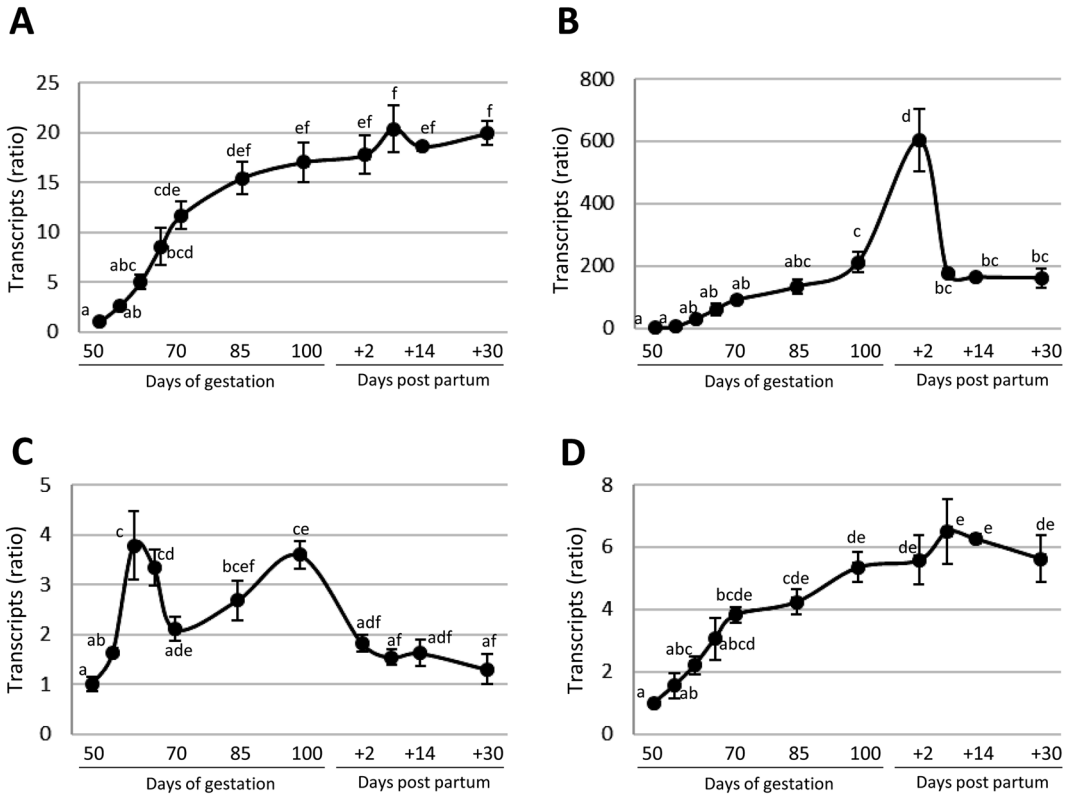


**Fig. 3.** mRNA expression during the development of porcine tonsils of the soft palate. *IL2RG* (A), *CCL19* (B), *CCL21* (C), and *CXCL13* (D) expression levels were measured using quantitative RT-PCR in the tonsils at 50, 55, 60, 65, 70, 85, and 100 days of gestation and 2, 7, 14, and 30 days postpartum. As an internal control, beta-actin (*ACTB*) mRNA levels were measured in each sample. mRNA expression levels were normalized to *ACTB* mRNA levels. The values are means ± SEM (arbitrary units; n=4). Different letters (a–g) indicate values that are significantly different at $P<0.05$ (Tukey's honest significant difference).

lar development. A second peak was identified toward the end of gestation, before the CCL19 surge, suggesting that CCL21 also triggers the following robust aggregation of lymphocytes, especially T cells.

CXCL13 is mainly expressed in the B-cell follicular area and is responsible for the recruitment of CXCR5-expressing B cells [5]. *CXCL13* expression increased throughout the study period, which was consistent with the progressive aggregation of B cells in the tonsils. However, considering that the increase in *CXCL13* expression was limited to six-fold, abundant expression of CCL19 may also be required for B-cell migration, because it has been shown to attract B cells, at least *in vitro* [3].

The pig has been considered as a good model for the study of human tonsils because it has all the four discrete components of Waldeyer's ring (palatine, nosopharyngeal, tubal and lingual tonsils) of human unlike other non-primate animals [12, 13] and also has the deep crypt structures similar to human [1, 10]. The human tonsil development is known to start at 14th week of gestation with infiltration of wandering mononuclear cells and epithelial crypts appears at 16th week [12, 19]. This stage corresponds to 65 days to 70 days of gestation in our porcine observation. Their developmental processes seem comparable, including that follicular structures are formed shortly after birth, although their time courses appear to differ each other, considering the proportions to whole gestational period (human: about 35% past, pig: about 57% past). Furthermore, the chemokines we analyzed (CCL19, CCL21 and CXCL13) are considered to be relevant with the formation of lymphoid tissue also in humans [11]. Thus, the pig can be an appropriate model for the research of human tonsil development.

In summary, we succeeded in outlining the fetal and early postnatal development of the porcine tonsils of the soft palate. Further detailed study is necessary to characterize the expression and function of chemokines and

their receptors and adhesion molecules to better understand the process. The fundamental data presented here should be invaluable for future basic research using pig as a model for mammalian development and for practical research into veterinary applications for use in the animal industry.

## Acknowledgments

The authors would like to thank the staff of the Swine Management Section of the Institute of Livestock and Grassland Science, NARO, for taking care of the pigs and for technical assistance. We also thank K. Iijima for technical assistance.

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