Differential expression of pattern recognition receptors during the development of foetal sheep

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Dear Dr Warr,

Many thanks for the referees comments, they were very helpful. I am glad that they found the paper interesting, of value and worthy of publication, and that by-and-large, their concerns were relatively minor.
In response to their specific comments:

Referee #1:

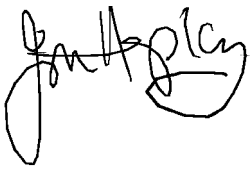
1. The major concern is that the same data were published previously (reference 19). To some extent this is true – we have published sheep skin and spleen PRR transcript levels. However, the data in the two papers come from different animals. In this paper adult animals are specifically being compared with foetal animals – and the adult tissues come from yearling sheep of the same breed as the foetuses. Nevertheless, the data are similar. I think this is valid. Incidentally, reference 19 was in the original version – referring to both methods and adult expression levels.
2. I have improved our description of the source of tissues in the animals– adult tissue comes from exactly the same anatomical sites as foetal tissues.
3. I have further explained the statistics. Because there are so many variables, it would be easy to be utterly pedantic and compare every sample with every other sample. Although that has been done – and an examination of the error bars (standard deviations) in Figs 1 and 2 can give an assessment of significance – to explain it all in the text would be unacceptably tedious. I have focussed on interesting comparisons – especially between foetal and adult samples. I hope this is satisfactory. If required I can give p values for every single comparison – which would be very boring.
4. I have added to the description of the spleen data. However, to generate a separate section comparing skin and spleen is, I think, unnecessary. I have made some comparisons between the two tissues but not in great detail as this is not the main thrust of the paper. Comparing skin with spleen in detail is not particularly informative or for that matter that valid.

Referee #2:

1. All RNA samples were DNAase I treated and RT- controls were always included (both now included in text).
2. Fig 2 is redundant – and has been removed. It is replaced by a single reference (reference 21).
3. I have included some discussion on the sheep/cattle comparison (Menzies & Ingham) – interesting that two closely related species are different.
4. It would be excellent to do analysis of wider gestational ages but unfortunately they were not available. These animals were a serendipitous by-product of a different experiment (acknowledgement to Prof Alan McNeilly – who used their pituitaries). I am unconvinced whether *in situ* data would add much to the general thrust of the paper?

I hope you find these responses and changes acceptable and that the paper is now fit for publication.

Sincerely yours



Differential expression of pattern recognition receptors during the development of foetal sheep

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Abstract

Pattern recognition receptors (PRRs) play a crucial role in the initiation of the adaptive immune response. Immunological competence of foetal lambs occurs progressively throughout gestation and in order to understand the role played by PRRs in foetal immunological competence we quantified transcript expression, in the skin and spleen, of the TLRs, key C-type lectins and CARD15 during the critical second trimester. These data show that lambs express the same spectrum of PRRs as the adult but that the level of expression for most is dependent on developmental age. Key findings include: TLR1 and TLR5 are expressed at high levels in the foetus but are low in the adult; in contrast TLR4, CD14 and CARD15 increase with age. In addition, TLR9 and TLR10 are expressed by the spleen and not the skin while CARD15 is low in the spleen and high in the skin.

Keywords: Pattern recognition receptors; innate immunity; TOLL-like receptors; foetal lambs;

1. Introduction

Innate defence mechanisms provide an essential first line of protection against infection through the recognition of conserved microbial structures by an array of germline-encoded innate pattern recognition receptors (PRRs). PRR engagement alerts the innate immune system to the presence of infection and culminates in the induction of pro-inflammatory mediators and the initiation of the adaptive immune response [1,2].

There are two broad groupings of PRRs. Firstly, lectins that bind pathogens through recognition of carbohydrate moieties and function through complement fixation (soluble collectins), opsonization (mannose receptors) and cell activation/opsonization (C-type lectins). Secondly, the Toll-like receptors (TLRs), engagement of which leads to NF-κB dependent gene activation and the synthesis of effector molecules. This latter definition could also apply to the cytosolic NACHT-LRR proteins (NLRs) including CARD15 (NOD2) [3]. Each TLR has its own array of ligands [4]; however the repertoire of TLR recognition is expanded significantly by heterodimerization [5] or by complexing with co-receptors [6].

Mammalian neonates are known to have a reduced response to specific antigens and an increased susceptibility to bacterial infections and sepsis [7,8]. This increased susceptibility is more pronounced after premature birth where it causes significant morbidity and mortality although species differences exist in the level of immune development at birth [9-11]. This has been partly ascribed to neonates having reduced complement factors, poor phagocytic capabilities [7] and an overall underdeveloped innate immune system and naïve adaptive immune system [12]. Indeed, within sheep the splenic rudiment, containing myeloid and erythroid progenitors appears only by about day 48 [11]; B cells start to appear after day 50 [13] with an expanding T cell population after day 77 [13,14]. The development of the lymphoid system is reflected in the ability of foetal lambs to generate an immune response [15]. However, different antigens are responded to at different time points during gestation. IgM anti-ΦX174 bacteriophage antibodies are produced by 53 days, IgG1 anti-ferritin is

detected by 65 days [12,16] but anti-ovalbumin antibodies are not produced until day 125. Furthermore, before day 75 orthotopic skin grafts are accepted as if they were autologous, but are rejected after day 77 [17]. Interestingly, antibodies to some bacterial antigens, including toxins, are not produced until several weeks after birth [15].

This progressive responsiveness could partly be explained by the sequential expression of PRRs, which are responsible for the initial recognition of the antigen. Information on mammalian PRR ontogeny is scanty; it is possible that the differential pulmonary expression of TLR4 may explain the ontogeny of murine responsiveness to lipopolysaccharide (LPS) [18], but little else is known [7]. To examine the possible contribution of the pattern recognition receptors to the initiation of antigen specific immune reactivity, PRR transcript expression levels were quantified in the spleen - an organ associated with systemic immunity; and the skin, an organ that is in contact with the external environment of the amnion. Tissues were taken from foetal lambs at the critical 60 – 90 day second trimester period and compared with tissues taken from 1 year old adult sheep.

2. Materials and methods

2.1. Animals and tissues

Spleens and thoracic flank skin strips, taken immediately posterior to the shoulder, were collected from foetuses carried by Greyface ewes date-mated with a Texel ram. The ewes were euthanized with intravenous pentobarbitone sodium and the intact gravid uterus removed to sample foetal tissues. The foetuses were estimated gestational age of 60 (*n*=5), 70 (*n*=5), 80 (*n*=5) and 90 (*n*=5) days (all being of second trimester pregnancy - normal ovine gestation 145 – 150 days). Tissues were taken immediately post-mortem, from 1 year old, breed-matched ewes (n=4), from exactly the same site as foetal tissues. All collected samples were immediately placed in five times volume of RNA*later (*Ambion, Huntingdon, UK), and stored overnight at 4ºC; after which the RNA*later* was removed and tissues stored at -80 ºC.

Tissues for histopathology were fixed in 10% formol-saline; 4 μm sections from paraffin wax-embedded tissue were stained with haematoxylin and eosin. Animal experiments were conducted under a valid Animals (Scientific Procedures) Act 1986 Project Licence.

2.2. RNA isolation, cDNA synthesis and quantitative real-time PCR.

Total RNA was isolated from tissues using the RNeasy Mini kit (Qiagen, Crawley, UK); tissue samples were finely chopped and homogenized in 350 μl of lysis buffer. Each sample was diluted with 550 μl of nuclease-free water and digested with 10 μl proteinase K at 20 mg/ml (Sigma-Aldrich, Poole, UK) for 15 min at 55 °C. Genomic DNA was sheared using a 20-g needle. Homogenates were microfuged and RNA purified using Qiagen mini spin columns including DNAase I digestion. RNA samples from the same biopsy were pooled, volumes adjusted to a total volume of 100 μl in nuclease-free water, purified using Qiagen RNA mini spin columns and eluted in 30 μl of nuclease-free water. Total RNA was quantified by spectrophotometry. RNA quality and integrity was confirmed using a RNA LabChip on an Agilent 2100 Bioanalyzer; all samples had an RNA integrity number >7.

For cDNA synthesis, 2.5 μg of total RNA from each tissue sample was mixed with 0.5 μg Oligo$(dT)_{15}$ primer, 5 μl of M-MuLV RT 5x reaction buffer, 1 μl of dNTPs mix (10 mM), 1 μl M-MuLV RNaseH^- reverse transcriptase (Promega, Southampton, UK) and nuclease free water up to 25 μl. The reaction was incubated at 40 °C for 10 min, 42 °C for 50 min and inactivated at 70 °C for 15 min; an RT- control was included in all experiments and never produced signal . The cDNA was diluted four-fold in nuclease free water and stored at -20 °C until used.

Two-step, quantitative real-time RT-PCR (qPCR) for sheep PRRs was performed using a Rotor-Gene 3000 (Corbett Life Science, Sydney, Australia) exactly as described by Nalubamba et al. (2007) [19]. Copy numbers were determined from the Ct values of each sample in comparison to the copy number values assigned from a plasmid DNA standard

using Rotor-Gene analysis software (6.0.34). Data were normalized using β actin and succinate dehydrogenase (SDHA) housekeeping genes; a normalization factor, taking into account the geometric means of both housekeeping genes, was calculated using geNORM [20]. Duplicate PCR reactions were performed for each sample;. Statistical analysis was performed on the data from individual animals. One way analysis of variance and Tukey's multiple comparison tests were used for the pair-wise comparisons, of foetal samples versus adult samples, of the $\log_{10}$ transformed normalized data.

3. Results

3.1. PRR expression in foetal skin

The expression levels of PRR transcripts in sheep skin from four second trimester foetal time points and adults is shown in Fig. 1; which shows transcript copy number in relation to the two housekeeping genes, β actin and SDHA. These data show that different PRRs are present at very different levels in sheep skin, varying from less than 10 copies (e.g. TLR9) to more than 3,000 copies (MyD88). However, foetal skin from all time points tested expressed the same panel of PRR transcripts as adult skin (Fig. 1). The levels of TLR2, TLR3, TLR8 and MyD88 were not significantly different at any time point and TLR9 and TLR10 transcripts were not detectable in skin by qPCR. The pattern of transcript expression of TLR1, TLR5 and TLR7 is similar to the levels in all foetal samples, being significantly greater that in adult skin (*$p \geq 0.05$ for TLR5 and TLR7; **$p \geq 0.02$ for TLR1). Expression of TLR1 and TLR7 were similar at all four foetal time points whereas TLR5 increased by approximately three fold from day 60 to day 90 with the day 70 and day 80 levels being intermediate. TLR4, TLR6, CARD15, CD14 and Dectin 1 also behave in a similar manner with relatively low levels in foetal skin and significantly greater levels in adult tissue (*$p \geq 0.05$ for TLR6, CD14 and Dectin 1; **$p \geq 0.02$ for TLR4 and CARD15). The expression

levels of Dectin-2 were low (<100 copies) but showed an almost linear increase from day 60 to adult.

3.2. PRR expression in foetal spleen

The expression levels of PRR transcripts in spleen are shown in Fig. 2; and as with skin it is clear that there is great disparity in the level of expression of the different PRRs; which vary from <200 copies (TLR5) to >7,000 copies (TLR4). What is also clear is that the magnitude of PRR expression in the two tissues is different; TLR2, TLR6 and Dectin 2 are up to ten fold greater in the spleen than in skin, MyD88 is expressed at about the same level, while the level of CARD15 in the spleen is about ten fold less than in the skin.

The pattern of TLR1 and TLR5 expression is similar in the two tissues, being relatively low at the 60 day gestational time point ($p \geq 0.05$ for TLR1 and TLR5) and at the adult stage ($p \geq 0.05$ for TLR1 but not significant for TLR5) and relatively high at later gestational time points. TLR4, TLR6, TLR7, TLR8, TLR10, CARD15 and CD14 behave in almost exactly the opposite way, with low levels in foetal tissue and high levels in the adult ($p \geq 0.05$ for TLR6, TLR7, TLR8, CARD15 and CD14 for all foetal samples and adult; for TLR4 and TLR10 $p \geq 0.05$ for days 60 and 70 only). Transcript levels of TLR2, TLR3 and MyD88 in the spleen are, as in the skin, approximately the same at all five time points. The expression pattern of the other three PRRs is unrelated to that of the skin; adult levels of Dectin 2 are lower those in the foetus. TLR9 is expressed at relatively high levels in the spleen, with the 80 day time point showing the highest value and Dectin 1 shows no pattern across the five time points.

4. Discussion

The second trimester period of gestation seems to be critical in the development of potentially protective immune responses in lambs [11]. Depending on the nature of the antigen, antibody responses begin by as early as day 53 and T cell responses are active by day 77. This paper begins to examine the contribution of innate mechanisms to the protection of

the foetal lamb. A major part of innate protection is recognition of pathogen-associated ligands by PRRs, which is linked to the initiation of the adaptive immune response. In this study we quantified the expression of a panel of PRRs in the skin and spleen throughout the second trimester of gestation and compare it to the adult pattern. These tissues were chosen because the skin is the interface with the external environment of the amnion and the spleen is largely responsible for systemic immunity. The levels of PRRs in adult sheep tissues have been quantified previously [19] and the data on skin and spleen is similar to that presented here despite the fact that they are of different ages and breeds. However this study is specific comparison of animals of different ages and therefore the adult animals here are one year old and of the same breed as the foetuses. We cannot measure levels of PRR protein because of a lack of specific antibodies but we assume that the relative quantities of receptor and mRNA are linked. This study demonstrates that foetal sheep, even as early as day 60 express the same spectrum of PRR transcripts as the adult but that the level of expression for most is dependent on the developmental age of the animal. However, the relationships are not simply one of a higher level of expression in the older animals.

It is likely that the PRR profile in the tissues reflects their relative expression by the predominant cell types in the tissues [4]. Within the skin the major cell types associated with PRR expression are Langerhans cells (LC), dendritic cells (DC), macrophages and keratinocytes; in addition there will be a contribution from hair follicles and, especially in foetal skin, the large population of infiltrating leukocytes [21]. In the spleen there are major contributions from myeloid and lymphoid cell populations as well as erythroid and myeloid precursors [14]. Minor contributions will also be made in both tissues by epi- and endothelium. Animals at different developmental ages will have variable proportions of these different cells.

There are three main patterns of expression, although not all PRRs have the same pattern in both tissues. The first, where there are no significant changes at any time point; the second, where foetal levels are high and adult levels are low and the third, where foetal levels are low and adult levels are high.

TLR2, TLR3 and TLR8 are the three PRRs with relatively invariant expression levels across the age groups. The probable explanation is that this reflects their expression by keratinocytes [22] and, in the case of TLR2, hair follicles [23]. TLR1, TLR5 and TLR7 have relatively high expression levels in foetal skin and significantly lower levels in adult skin; possibly explained by the fact that foetal skin contains higher numbers of infiltrating leukocytes [21]. The significant level of PRRs expressed in foetal skin could also play a role in the formation of the *vernix caseosa* present on foetal skin at birth and possessing antibacterial properties. Antigenic stimulus may arise from the amniotic fluid in which the foetus is suspended and encourage the formation of the protective layer. The third group, TLR4, TLR6, CARD15, CD14 and Dectins 1 and 2, have relatively low foetal levels and significantly higher levels in the adult. One possible reason is that these PRRs are principally expressed by LC/DC [24,25], which are more abundant and mature in the older animals. The low levels of TLR4 and especially CD14 in the developing foetus may the explain the prenatal hyporesponsiveness to LPS [7], The TLR levels in adult sheep skin reported here and previously [19] vary from those in cattle of similar age [26]; particularly different are TLR4 and TLR6, which are absent in cattle and TLR7, which is very low in sheep. It seems likely that different species are reliant on different panels of innate receptors.

The spleen undergoes major developmental changes in terms of structure and function between 60 and 90 days [14]. Before 64 days it consists of erythroid and myeloid precursors associated with a framework of reticular cells with only small numbers of B cells. Primitive B cell follicles appear by about 80 days, associated by rudimentary periarteriolar lymphoid sheath consisting mainly of CD8+ T cells. The proportion of CD4 and CD8 T cells only approaches the adult ratio after birth. TcR1+ T cells, which constitute more than 50% of peripheral blood T cells in neonatal lambs, do not begin to populate the spleen until after day 113. The pattern of TLR expression in the spleen is surprisingly similar to the skin, given the fact that different cells populate these two tissues. Macrophages and monocytes express all PRRs in this panel; while DC strongly express most but are negative for TLR7 and TLR10 [19]. The different sheep lymphocyte populations express limited panels of PRRs; only B

cells express TLR1 and TLR2, only CD4+ T cells express TLR10 and CD8+ T cells express none of this panel except MyD88. TLR3, TLR5, Dectin 1 and Dectin 2 are not expressed by any peripheral blood lymphocyte population [19].

The levels of expression of most PRRs is generally much higher in the spleen that in the skin; varying from about 2 fold greater for TLR2 to about 50 fold greater for TLR7. Indeed, TLR9 and TLR10 are expressed at significant levels in the spleen but are undetectable in the skin. Strangely, in view of this quantitative differential is the fact that MyD88, the adapter molecule for most TLRs is expressed at similar levels in both tissues. The one PRR that is significantly higher in skin than spleen is CARD15; possibly because it is highly expressed by LC, DC and keratinocytes [27]. This study will contribute towards understanding of the ontogeny of the immune system in sheep and will also form the basis for future research on the role of PRRs in foetal immunology and foetal disease conditions.

Acknowledgements

The work was funded by Association of Commonwealth Universities Academic Staff Scholarship to KSN and by BBSRC Grant 15/S13964. We thank all the Marshall Building staff for care of animals. Access to foetal tissues was courtesy of Professor Allan McNeilly, MRC Reproductive Sciences Unit, Queen's Medical Research Institute, Edinburgh.

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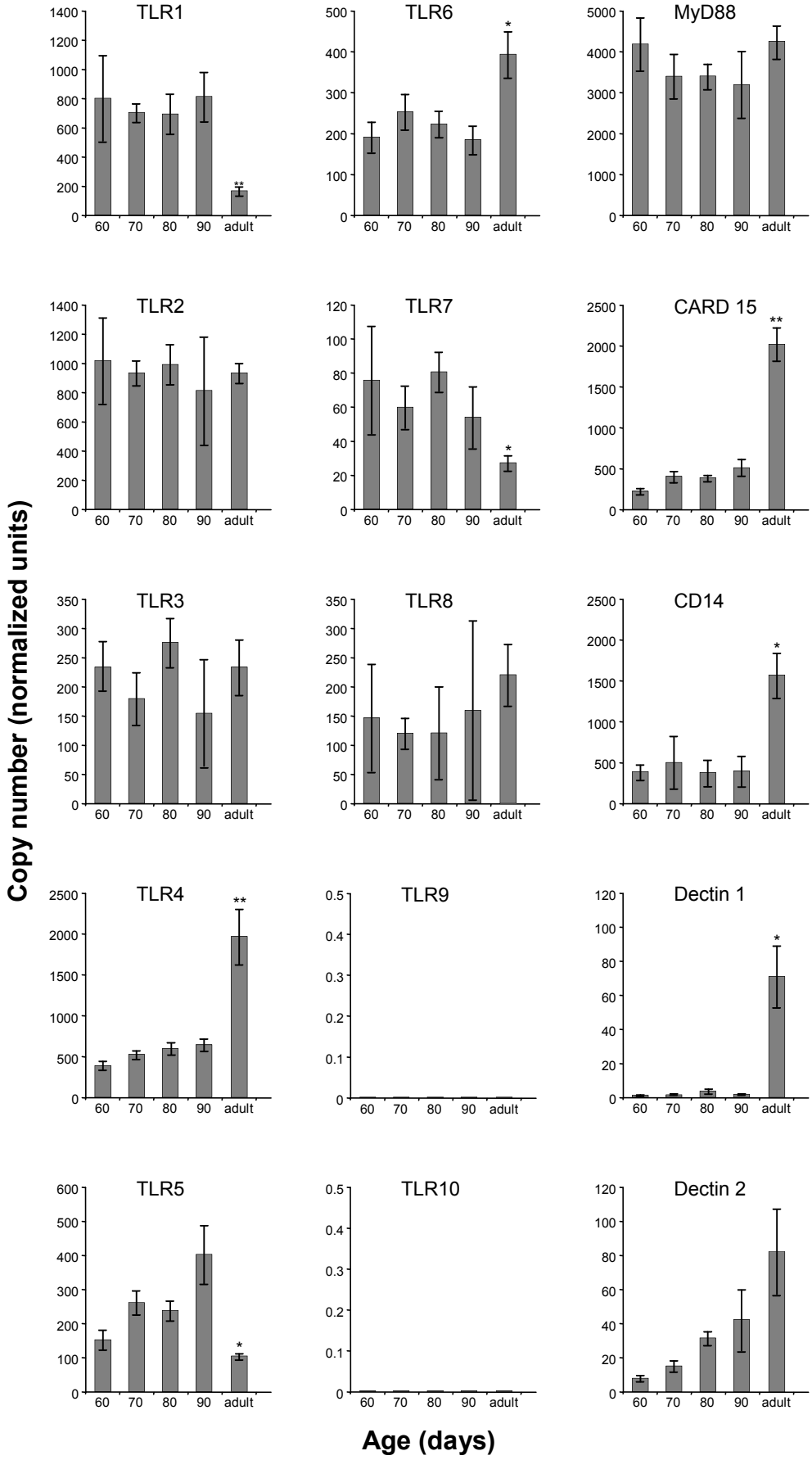
Figure Legends

Fig. 1. PRR transcript levels in skin of second trimester foetal lambs and in adult sheep. Data are transcript copy number ± SD, normalized to SDHA and β-actin; * $p \geq 0.05$, ** $p \geq 0.02$ adult level compared to any foetal sample.

Fig. 2. PRR transcript levels in spleen of second trimester foetal lambs and in adult sheep. Data are transcript copy number ± SD, normalized to SDHA and β-actin; * $p \geq 0.05$, ** $p \geq 0.02$ adult level compared to any foetal sample.

Figure(s)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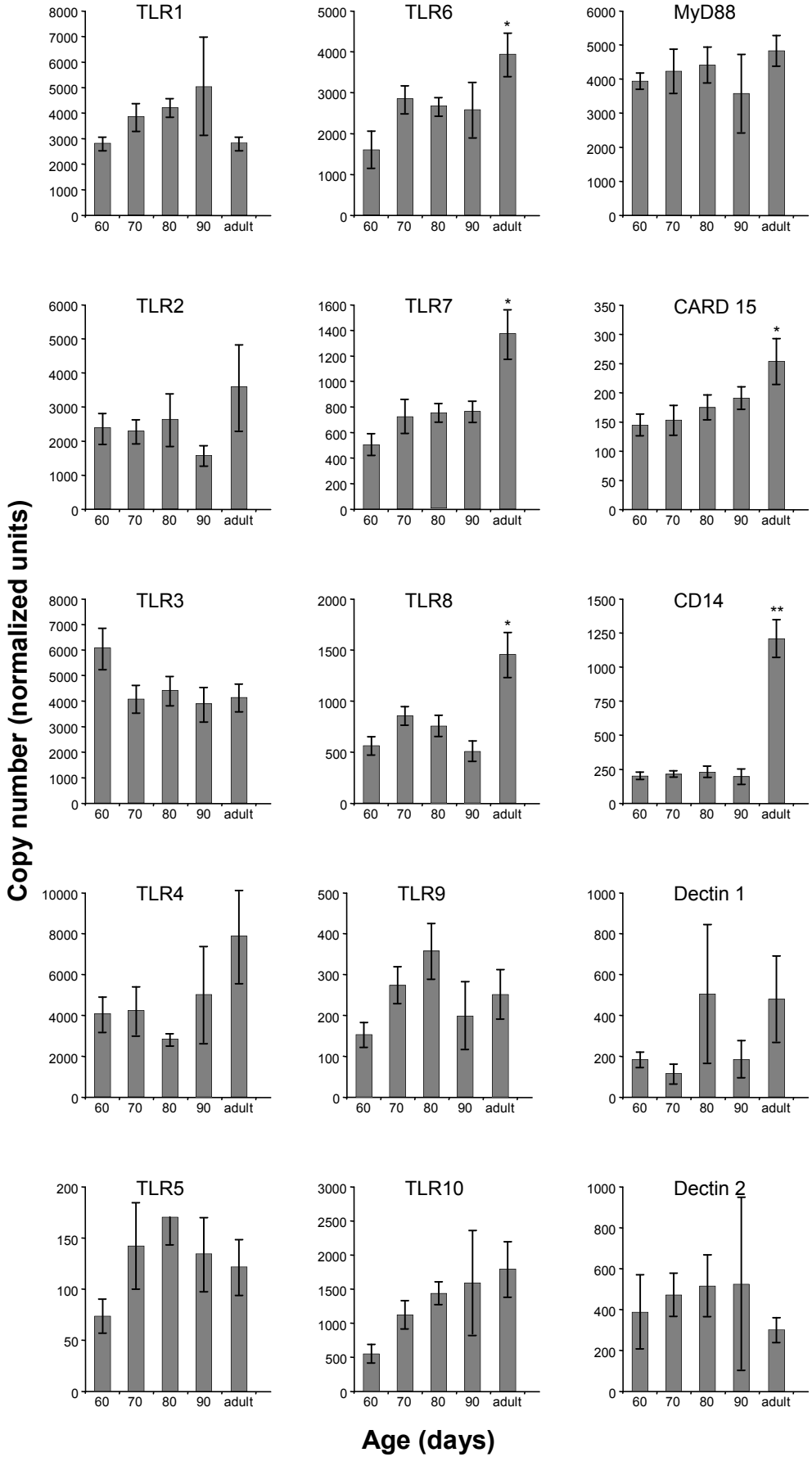
Figure(s)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