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Evolutionary Analysis of the Mammalian IL-17 Cytokine Family Suggests Conserved Roles in Female Fertility

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ABSTRACT

Problem: The interleukin-17 (IL-17) family includes pro-inflammatory cytokines IL-17A-F with important roles in mucosal defence, barrier integrity and tissue regeneration. IL-17A can be dysregulated in fertility complications, including pre-eclampsia, endometriosis and miscarriage. Because mammalian subclasses (eutherian, metatherian, and prototherian) have different related reproductive strategies, IL-17 genes and proteins were investigated in the three mammalian classes to explore their involvement in female fertility.

Method of Study: Gene and protein sequences for IL-17s are found in eutherian, metatherian and prototherian mammals. Through synteny and multiple sequence protein alignment, the relationships among mammalian IL-17s were inferred. Publicly available datasets of early pregnancy stages and female fertility in therian mammals were collected and analysed to retrieve information on IL-17 expression.

Results: Synteny mapping and phylogenetic analyses allowed the classification of mammalian IL-17 family orthologs of human IL-17. Despite differences in their primary amino acid sequence, metatherian and prototherian IL-17s share the same tertiary structure as human IL-17s, suggesting similar functions. The analysis of available datasets for female fertility in therian mammals shows up-regulation of IL-17A and IL-17D during placentation. IL-17B and IL-17D are also found to be over-expressed in human fertility complication datasets, such as endometriosis or recurrent implantation failure.

Conclusions: The conservation of the IL-17 gene and protein across mammals suggests similar functions in all the analysed species. Despite significant differences, the upregulation of IL-17 expression is associated with the establishment of pregnancy in eutherian and metatherian mammals. The dysregulation of IL-17s in human reproductive disorders suggests them as a potential therapeutic target.

1 | Introduction

IL-17 family members—IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (or IL-25) and IL-17F—are potent pro-inflammatory immune mediators that are active as dimers [1, 2]. The IL-17A/F dimer regulates mucosal immune activity through neutrophil recruitment and secretion of matrix metalloproteases, which drive tissue repair [3, 4]. IL-17A/F also manipulates local microbiomes through the induction of antimicrobial peptide secretion by epithelial cells located at mucosal barriers [5]. The roles of the other family members are not as well characterised; however, they show similar pro-inflammatory and immunomodulatory effects [6], as well as mucosal barrier integrity [7–9]. Non-immune-related roles for IL-17s in epithelial repair [10], tissue regeneration [11], keratinocyte proliferation and differentiation [12] and thermoregulation [13] have also been described.

Dysregulated levels of IL-17 cytokines are associated with several inflammatory conditions, including psoriasis, psoriatic arthritis and ankylosing spondylitis [14]. These findings encouraged the development of monoclonal antibody therapies to neutralise IL-17A/F action (Ixekizumab and Secukinumab) or block the receptor (Brodalumab). These therapies help prevent the pro-inflammatory symptomatology linked to psoriasis [15], psoriatic arthritis [16] and multiple sclerosis [17].

The IL-17 family of cytokines differs from other interleukins both in structure and signalling pathways they activate. The IL-17 protein structure incorporates a cystine knot motif, with disulphide bonds between conserved cysteine residues [18]. This cystine knot motif is not found in any other cytokines or chemokines, whose secondary structure is organised in alpha helixes; however, it is a characteristic of certain classes of hormones and growth factors, such as glycoproteic hormones, nerve growth factors and vascular endothelial growth factor [19].

In addition to their involvement in inflammatory conditions, IL-17s likely play a role in female reproduction, as controlled inflammation characterises each stage of successful pregnancy [20, 21]. IL-17A and IL-25 are crucial for the establishment of a successful pregnancy, given their involvement in trophoblast implantation and decidual cell proliferation [22, 23]. The activation of the pro-inflammatory response has been shown to be critical during the window of receptivity in humans [24], confirming that inflammation is not only activated as a mechanism of defence and during pathologic conditions but is also a key player needed for normal physiological processes to occur in the uterus [25, 26].

Therefore, it is not surprising that female fertility complications derive from impaired or exaggerated local immune and inflammatory activity [27–29]. IL-17s, and in particular IL-17A and IL-25, have been linked to other fertility complications such as endometriosis, preeclampsia or miscarriage [30–33]. The activation of the IL-17A pathway and increased systemic and endometrial IL-17A protein levels during the process of implantation in humans is associated with unexplained infertility and unsuccessful implantation [34], suggesting a role in unsuccessful pregnancy. Therapies targeting IL-17 have recently been shown to be effective in controlling pre-eclampsia [33], indicating a role for this cytokine in successful human reproduction.

Given the association of IL-17A with mammalian reproduction, we hypothesised that the evolutionary features of the IL-17 family would track the evolution of reproductive strategies amongst the three mammalian clades—prototherians, metatherians and eutherians. Pregnancy in these clades evolved from a common process in which an early inflammatory reaction is critical for successful embryo implantation. Prototherian mammals resemble the ancestral mammalian pregnancy strategy, in which the implantation step does not occur due to the development of the embryo within the shell coat, thus making prototherian mammals oviparous. Metatherian mammals have a short gestation period (only 15 days in the opossum *Monodelphis domestica*), in which the foetus is retained in the egg coat until day 12.5, when it hatches inside the uterus, and, after a short superficial placentation step, happens parturition of an extremely altricial offspring which continues its development in the maternal pouch. Eutherian mammals display extended pregnancy length, which is characterised by the formation of the placenta, a structure where the nutrients from the mother are delivered to the embryo, which reaches full development towards the end of the pregnancy.

The immune system plays a fundamental role in pregnancy. The involvement of inflammation in the reproduction of prototherian mammals, the most diverged clade of mammals, has not yet been explored. Recent analysis confirmed that the mother provides additional nourishment to the eggs before oviposition, thus confirming that even in such species, maternal–foetal communication occurs [35]. This pro-inflammatory activation is observed in metatherian and eutherian mammals during the placentation stage, after which the eutherian mammals 'added' a prolonged non-inflammatory phase required for foetal tolerance [25, 36], whereas metatherian mammals keep this pro-inflammatory stage and placentation is directly followed by the parturition of an underdeveloped offspring [25].

To better define the roles of IL-17 cytokines in relation to human female fertility, we performed an evolutionary analysis in mammalian subclasses (eutherian, metatherian and prototherian) to understand if the members of the protein family are conserved and, thus, share similar functions in distantly related mammals.

2 | Methodology

Databases: The resources used in this study to retrieve protein and nucleic acid sequences of IL-17 family members were: GenBank and RefSeq databases at the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/), Ensembl genome browser (https://www.ensembl.org/index.html) and the University of California Santa Cruz (UCSC) genome browser (https://genome.ucsc.edu/).

Identification of IL-17 genes: Genes encoding for IL-17 family members were identified in the datasets mentioned above or by performing BLAT searches within the UCSC Genome Browser using human IL-17 genes as a template [37].

Identification of gene conservation using synteny: synteny between mammalian chromosomes was assessed using the following released assemblies: *Homo sapiens* (GRCh38/hg38 Dec. 2013), *Monodelphis domestica* (Broad/monDom5 Oct.

2006), *Notamacropus eugenii* (Meug_1.0, INSDC Assembly GCA_000004035.1 Dec. 2008), *Sarcophilus harrisii* (WTSI Devil_ref v7.0/sarHar1 Feb. 2011), *Ornithorhynchus anatinus* (ASM227v2/ornAna2 Feb.2007) and *Tachyglossus aculeatus* (mTacAcu1.pri Dec. 2020). The synteny tool from Ensembl (https://m.ensembl.org/info/genome/compara/analyses.html) was used to make pairwise whole genome alignments. The UCSC genome browser was interrogated to confirm these findings by searching each IL-17 in the genomic region. IL-17s and their neighbouring genes were summarized in BioRender.com, and a summary table with information on the genomic location can be viewed in Supporting Information S1.

Identification of IL-17 proteins: IL-17 family members protein sequences across several organisms were retrieved from the linked genomic sequences (protein IDs are listed in Supporting Information Table S1). The protein sequences for each IL-17 was subject to a first similarity check step by aligning all the selected mammalian orthologous proteins against the corresponding human IL-17 family member using ClustalW (https://www.genome.jp/tools-bin/clustalw). This allowed us to determine in organisms with poorly annotated proteomes if the protein in question was in fact the most similar to the human one. Sequences were considered for further analysis if they showed a percentage identity higher than 20%, which corresponds to the upper bound of the 'Twilight zone' [38].

Reciprocal best hits: Reciprocal best hits analysis was performed to help assign orthology among mammalian clades [39]. Reciprocal BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) searches against non-redundant protein sequence databases were carried out between human and metatherian or prototherian proteomes and vice versa to identify the highest ranked proteins (Best Hit).

Multiple sequence alignment (MSA): To identify conserved residues and establish phylogenetic evolution among IL-17 isoforms, all IL-17 sequences from included species were used as inputs to generate an MSA using the Muscle algorithm within MEGA version X [40], considering default parameters.

Phylogenetic analysis: The evolution of IL-17 family members was inferred using the MSA and applying the maximum likelihood method, which we have previously used successfully for comparative immunology studies [41–43], specifying the Jones-Taylor Thornton (JTT) matrix-based substitution model and with 100 bootstraps. Gaps in any sequence were included in the analysis and rates among sites were considered uniform. Sequences from *Mus musculus*, *Monodelphis domestica* and *Ornithorhynchus anatinus* were compared with those of humans to find which amino acid residues were different and where are they located in the protein domains.

Protein modelling: The homology modelling of IL-17A and IL-17F was performed using the plugin PyMOD 3.0.2 from the PyMOL visualisation system. The protein sequences from *M. domestica* and *O. anatinus* were aligned with the Protein Data Bank (PDB) entries 4HR9 and 3JVF, corresponding to the human IL-17A and IL-17F crystal structure, respectively, using the template disulphide bonds forming the cystine-knot in MODELLER. The GA341 method was used to generate a score of the percentage sequence identity between the template and model (with a score closer to 1 indicating a good model).

Transcriptomic analysis: Microarray data or RNA-seq raw data were collected from previously published female fertility transcriptomic datasets. Unfortunately, the lack of transcriptome studies from prototherians resulted in the exclusion of this clade from fertility dataset analysis, which focused on therian mammals only. The datasets analysed, which showed IL-17s among the differentially expressed genes (DEGs), comprised human menstrual cycle transcriptomes [44], human fertility complication transcriptomes (unexplained infertility, endometriosis and recurrent implantation failure) [34, 45, 46], mouse pregnancy and placentation transcriptomes [47, 48], as well as metatherian transcriptomes obtained throughout pregnancy [49–52]. The full list of datasets analysed, including those not included in the results, can be found in Supporting Information Table S5.

Microarray transcriptomic analysis was performed using the GEO2R option from the NCBI database, and the normalisation of the data was applied when discrepancies between samples were identified. Limma was applied to compute differences between the control sample group (samples from non-pregnant or post-pregnant or healthy controls) and test sample group [53]. By default, Limma analysis was carried out using Benjamini–Hochberg correction for multiple comparisons and accounting for a false discovery rate. The analysis resulted in a series of tables containing DEGs with their relative $\log_2$ fold change and log10 adjusted p-value.

RNA-seq analysis for the *H. sapiens* unexplained infertility dataset GSE144895 was previously performed [34]. Other RNA-seq datasets from *M. domestica* were analysed using the web-based workflow platform Galaxy. Raw reads were first subjected to quality inspection using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimming/filtering with TrimGalore for working with only high-scored runs. Read mapping and annotation was carried out against the latest genome release Mon.dom5. Reads were mapped using Bowtie2 [54]. Feature counts were used to annotate the coding genes and generate the normalised count matrix for *M. domestica* [55]. *N. eugenii* RNA-seq normalised counts matrix was retrieved directly from GEO dataset. For the metatherian RNA-seq DEG analysis, the limma–voom method was applied, filtering out samples with the sum of counts for all the samples less than 5 [53, 56]. The outcome of the analysis was a list of DEGs with fold change against the control sample, *p* value and adjusted *p* value.

The $\log_2$ fold changes obtained from *Mus musculus* [47] and *Notamacropus eugenii* [51] RNA-seq analysis were also plotted throughout pregnancy stages. Owing to the difficulty in obtaining the factor used to calculate the transcript per million (TPM) of genes expressed throughout pregnancy in *Monodelphis domestica* [57], the data from [52] are presented as TPM.

2.1 | Statistical Analyses

Contingency tables consisting of 'identical'/'non-identical' and 'important'/'non-important' sums of amino acid residues were constructed for each IL-17. These values were then used for Chi-

square testing, with or without Yates correction as appropriate, using GraphPad prism to test the hypothesis that key residues in metatherian and prototherian IL-17s were significantly different from the human ones.

3 | Results

3.1 | IL-17 Genes Are Orthologous Among Mammals

The sequences and location of six members of the human IL-17 family were used to examine synteny (relative genomic position). Human IL-17 family genes are located on different chromosomes, except for *IL17A* and *IL17F*, which both reside on chromosome 6 in humans, on opposite strands and with opposite orientations. The remaining IL-17s are located on chromosome 5 (*IL17B*), chromosome 16 (*IL17C*), chromosome 13 (*IL17D*) and chromosome 14 (*IL25*). All IL-17 genes are organised in three exons, except for IL-25, which comprises two protein-coding exons.

The latest genome releases of metatherians *Monodelphis domestica* (short grey-tailed opossum, Broad/monDom5 Oct. 2006), *Notamacropus eugenii* (Wallaby, Meug_1.0, INSDC Assembly GCA_000004035.1 Dec. 2008), *Sarcophilus harrisii* (Tasmanian devil, WTSI Devil_ref v7.0/sarHar1 Feb. 2011), the prototherians *Ornithorhynchus anatinus* (Platypus, ASM227v2/ornAna2 Feb. 2007) and *Tachyglossus aculeatus* (Australian echidna, mTacAcu1.pri Dec. 2020) deposited in the Genbank, Ensembl and UCSC genome databases were used for these analyses. Pairwise comparison with human genomic loci highlighted the presence of genes predicted to be homologous to the human genes deposited in RefSeq, Augustus and Ensembl. In cases where relevant regions were not yet annotated, UCSC prediction using the human chain-net and comparative genomic tools was applied to infer similarity in the region. The analysis identified the IL-17 genes and the surrounding genomic content in each species selected, confirming the presence of all IL-17 family members (Figure 1A–E and Supporting Information Table S1). Moreover, the flanking genes are conserved in all the species through evolution, particularly in IL-17D and IL-25 flanking regions (Figure 1D and E, respectively), with the same gene orientation, transcription direction and gene size (Supporting Information S1). Therefore, all IL-17 family members can reasonably be defined as syntenic and orthologous, as we see the same number of IL-17s in all mammalian clades, with the same surrounding genes and orientation. This suggests that IL-17s have been inherited vertically during mammalian evolution without involving additional gene duplications.

The orthology of IL-17s among mammals was also confirmed by analysing their protein sequences. Protein sequences of IL-17s in the mammal species under investigation were collected and aligned using ClustalW to confirm they shared at least 20% identity with human IL-17s. The MSA of mammalian IL-17s was performed, allowing for the construction of a phylogenetic tree using the maximum likelihood method, which has been previously shown a powerful tool to reliably establish the evolution of immune molecules [41–43]. This highlighted how each of the IL-17s clusters with orthologues from different species (Figure 2A), with maximal support for all family members except for IL-17A (bootstrap = 67%) and IL-17F (bootstrap = 82%). The branch length on the phylogenetic tree represents the evolutionary distance calculated using the number of amino acid substitutions per site. All the IL-17 family members had similar evolutionary rates among species and family members. In this context, a likely explanation for the weaker support for clearly defined IL-17A and IL-17F clades is the much shorter branch length leading to the duplication event that gave rise to these genes, compared to that of the other family members. This may imply that IL-17A and IL-17F are the most recently diverged genes in the family. In all, these findings indicate a vertical inheritance of IL-17s throughout evolution, confirming their status as orthologous.

Reciprocal best-hits analysis was also performed to characterise relationships among members of the IL-17 family. Pairwise BLAST-P searches of all IL-17s were performed on proteomes from organisms of interest. The top hit results from the searches were examined for reciprocity. If the two proteins are retrieved as the reciprocal best hits, it confirms that they are orthologous and that they share enough percentage identity to be considered to have the same function. Reciprocal best hits analysis in metatherian and prototherian proteomes using human IL-17s as inputs allowed all IL-17s to be identified with at least 50% identity and very low e-values, demonstrating reliable alignment performed by BLAST-P. Similarly, BLAST-P searches using metatherian or prototherian IL-17s against the human proteome showed that the proteins are reciprocal hits, with similar percentage identity and e-values (Supporting Information Table S2).

3.2 | IL-17F in Prototherian Mammals Is Significantly Different from the Human Ortholog in Terms of Amino Acid Composition at Important Residues and 3D Protein Structure

To look at the functional conservation of the IL-17s in mammals in detail, we analysed differences in amino acid sequences between the human IL-17s and corresponding proteins in *Monodelphis domestica* and *Ornithorhynchus anatinus*, as representatives of metatherian and prototherian mammals, respectively. Human IL-17A and IL-17F protein sequences have been well characterised for the residues involved in the cystine–knot formation, dimerization, glycosylation and receptor interaction [58]. The other IL-17 sequences have not been entirely characterised yet; therefore, only the signal peptide sequence, cystine–knot cysteines and glycosylation residues are known. A smaller MSA was performed to identify identical residues between human and *M. domestica* or human and *O. anatinus* (examples for IL-17A and IL-17F are shown in Figure 3A,B). As a control, the same approach was also performed between human and *M. musculus* IL-17s, given that even among eutherian mammals there are differences in terms of invasiveness and type of placenta (Supporting Information Table S3). The non-identical residues were analysed to identify their location and whether they represent a residue known to have key functions in corresponding human IL-17s (colour coded in the MSA and summarised in Figure 3C for IL-17A and IL-17F and in Supporting Information Table S4 for the other IL-17s). Overall, all IL-17s showed a large number of non-identical residues in the N-terminus of the protein and in the signal peptide portion of the sequence. From the first cysteine involved in the knot, the sequences show an increased number of identical

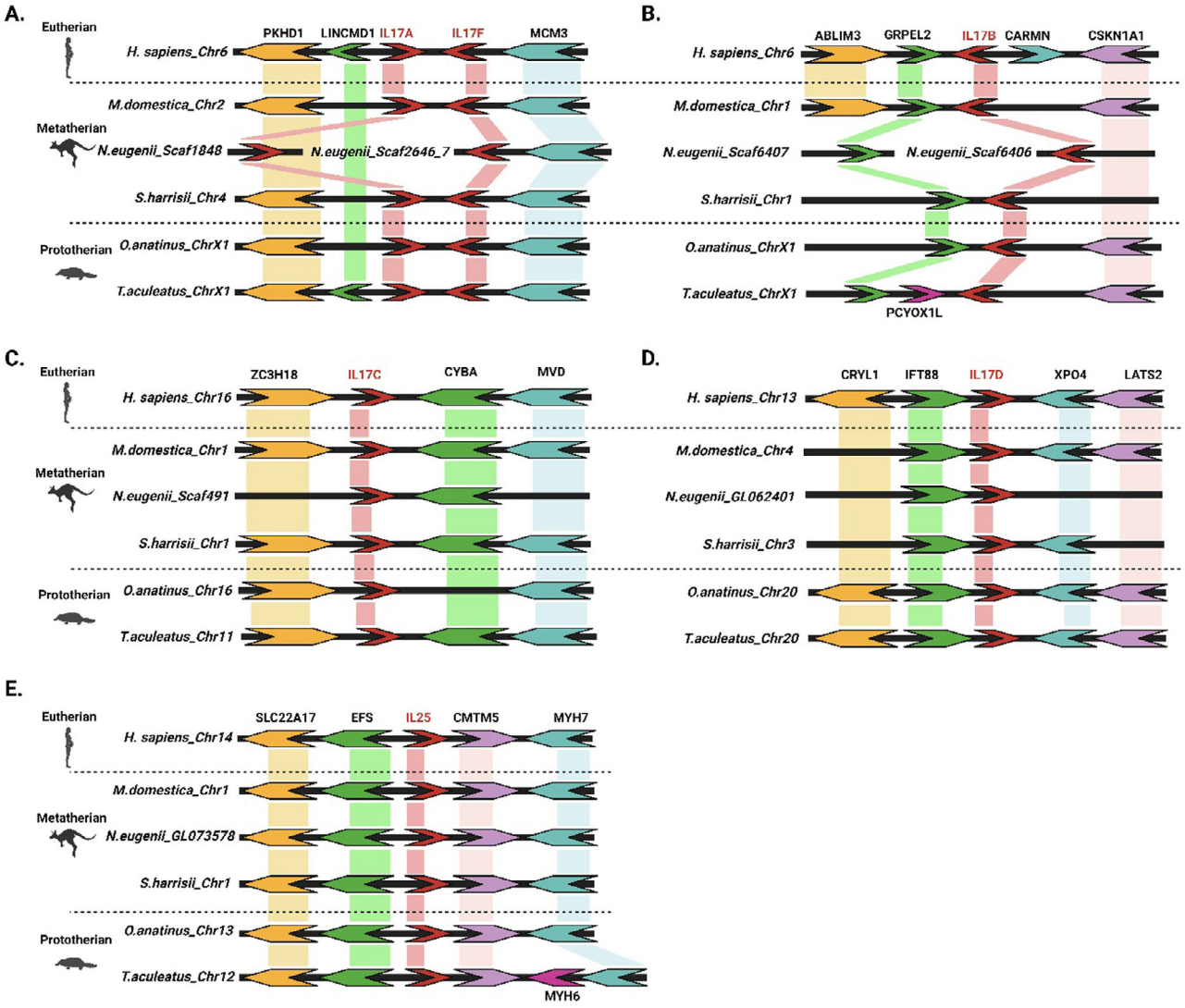


FIGURE 1 | Synteny maps indicating IL-17 gene location in metatherian species, prototherian species and human genomes. Information regarding gene location and surrounding genomic content were retrieved from UCSC, NCBI Genbank and Ensembl and results on the flanking genes and on IL-17 genes can be found in Supporting Information S1. Graphs were generated using BioRender. A. IL-17A and IL-17F synteny maps with flanking genes (PKHD1, LINCMD1 and MCM3); B. IL-17B synteny map with flanking genes (ABLIM3, GRPEL2, PCYOX1L, CARMN and CSKN1A1); C. IL-17C and flanking genes (ZC3H18, CYBA and MVD) synteny map; D. IL-17D synteny map and flanking genes (CRYL1, IFT88, XPO4 and LATS2); E. IL-25 synteny map with flanking genes (SLC22A17, EFS, CMTM5, MYH6 and MYH7).

residues in both metatherian and prototherian clades. Prototherian IL-17s show the highest number of non-identical residues, consistent with prototherians being more distantly related to humans than metatherians. Residues involved in a specific function, for example cysteine of the cystine knot, are conserved in all the IL-17s, apart from *O. anatinus* IL-17F which has two arginine residues instead of the last two cysteines (Figure 3B).

Once identical and non-identical residues were identified, the percentage identity was calculated for the whole protein and key amino acids. In most cases, for example *M. domestica* IL-17A, the percentage identity for the key residues is higher than the percentage identity for the whole protein. This can be explained by the fact that key residues are more conserved than scaffold residues. However, in other cases, such as for *O. anatinus* IL-17A, the percentage identity of the key residues is lower than for the whole protein. Chi-square testing, with or without Yates correction, was carried out to test the hypothesis that key residues were significantly different from the human in the chosen organisms (Figure 3C [highlighted in light blue] and Supporting Information Table S4). From this analysis, only IL-17F and IL-25 from *O. anatinus* were found to have significantly different key residues compared to the human, possibly suggesting that their function might be different in platypus compared to human and other eutherian mammal IL-17s.

We also performed the structural modelling of metatherian and prototherian IL-17A and IL-17F proteins based on published models of human IL-17A and IL-17F crystallised structures deposited in PDB (Figure 4A,B). For both metatherian and prototherian IL-17A and IL-17F, the overall 3D structure is similar to human IL-17s, and the key residues are colour coded according to Figure 3. Both *M. domestica* and *O. anatinus* IL-17A modelling have a GA341 score of approximately 0.9, which indicates a reliable modelling

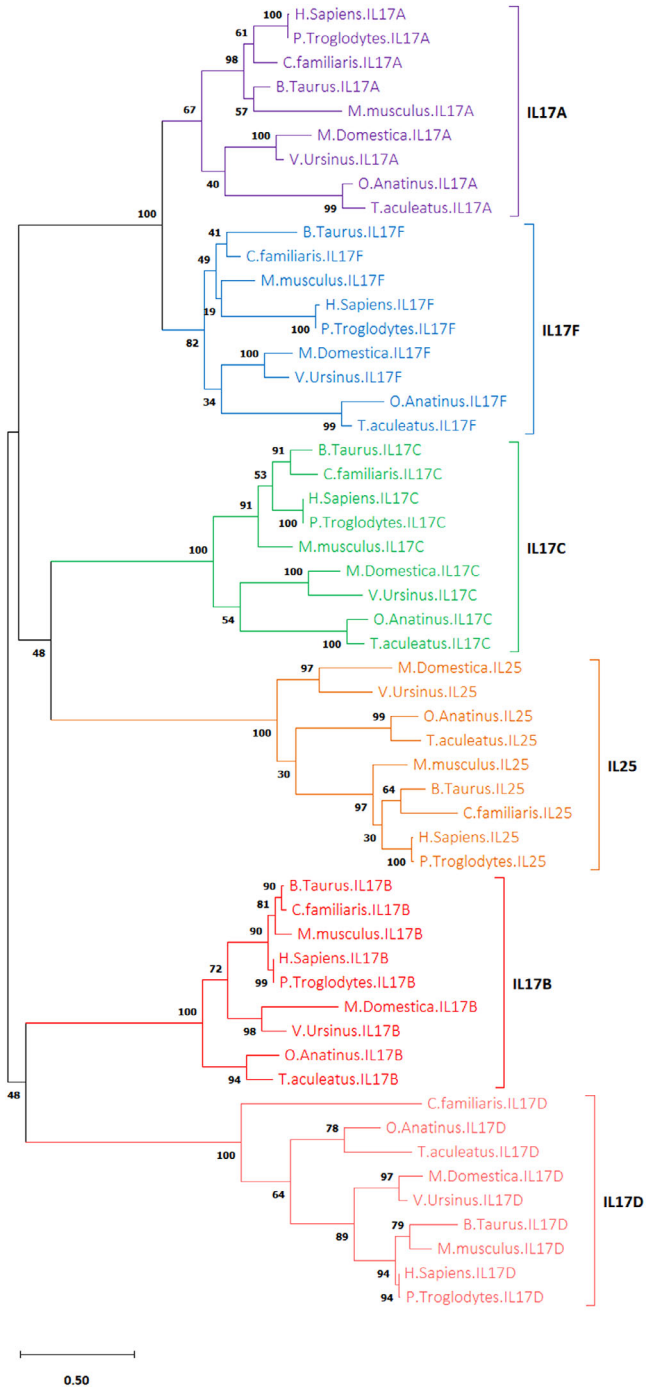


FIGURE 2 | The evolutionary history of IL17s in mammals. A. Phylogenetic tree analysis was conducted in MEGA X using the maximum likelihood method and JTT matrix-based model on IL-17 sequences from several mammalian species. The results from the bootstrap test (100 reiterations) are shown at each node. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Different colours highlight the branch corresponding to each IL17.

(Figure 4D). The only exception is the model of IL-17F from *O. anatinus*, which has a GA341 score of 0.2012, supporting the previous protein analysis, which indicated a significant difference in residues compared to the human IL-17F, including the presence of two arginine residues instead of the cysteine residues involved in the knot. Indeed, if we modify the IL-17F sequence for *O. anatinus* to replace the arginine with cysteine and repeat modelling with human IL-17F, the overall tertiary structure seemed similar to the original platypus IL-17F (Figure 4C); however, the GA341 score improves to 0.7776 (Figure 4D), confirming that the differences in those two residues are critical for a correct protein folding.

Taken together, these findings indicate that all mammalian IL-17s share similar amino acid composition, which might suggest they also have a similar structure and function. IL-17F in prototherians shows significantly different key residues compared to eutherian mammals, suggesting that the protein might have acquired different structures and functions through evolution.

3.3 | IL-17s Are Differentially Expressed in Female Reproduction and Fertility

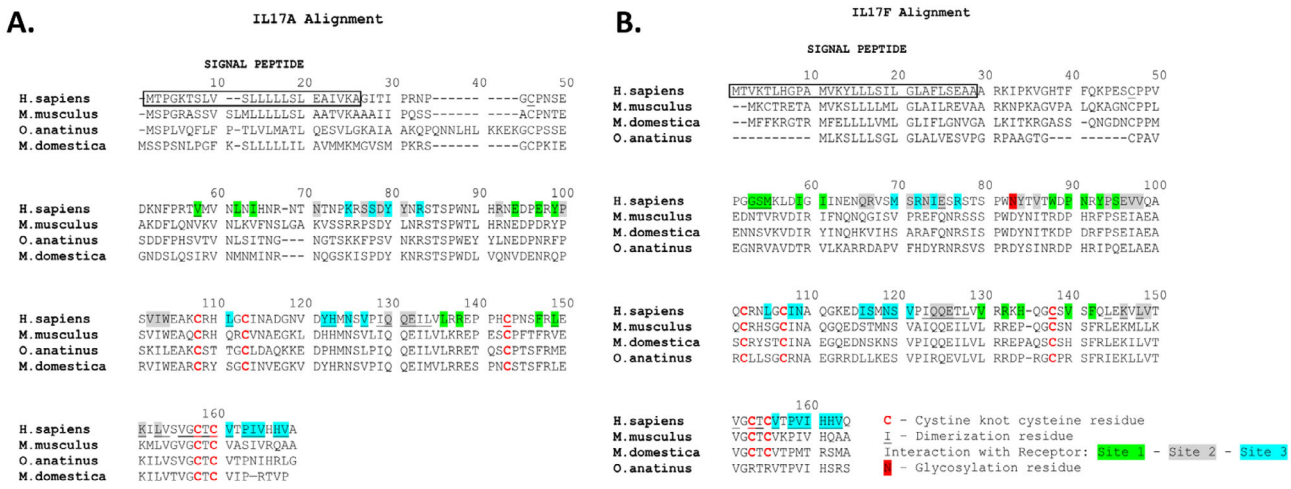
To generate functional insight into IL-17s in mammalian reproduction, IL-17 transcriptomes were analysed in datasets relating to female fertility. We aimed to determine whether there is a correlation between IL-17 expression and female fertility. Several transcriptomics datasets were collected and analysed to obtain data on differential expression of IL-17s shown in Figure 5 and in Table 1 for therian placentation and pregnancy and human fertility.

With regard to the expression of IL-17s during pregnancy, the expression of IL-17s is upregulated during implantation and placentation in metatherian and eutherian mammals. IL-17A is higher during metatherian placentation but is not found in the DEG lists in eutherian datasets, where instead IL-17D is upregulated during placentation (Figure 5A). The log2 fold change values for the IL-17s expression levels retrieved from analysis of pregnancy stages datasets in mouse and metatherian studies were plotted (Figure 5B,C). It seems that IL-17s reach their maximal expression when placentation occurs in each organism, defined by the overview in Figure 5B and represented by the light blue rectangle.

In human fertility datasets, IL-17B and IL-17D are upregulated in most datasets suggesting a possible role in fertility complications (Table 1 bottom section). In physiological conditions, IL-17D is significantly downregulated during the late stages of the menstrual cycle if compared to the proliferative phase.

4 | Discussion

Our findings show the conservation of IL-17 genes and proteins in mammalian clades. Among mammalian clades, there is a certain level of genome conservation: around protein-coding regions, for example eutherian mammals show an average percentage identity of approximately 80%, whereas the percentage identity is lower in metatherians and prototherians, given that divergence happened at around 90 and 140 million years ago, respectively [59]. Previous studies have shown the conservation of proteins in the three clades and a particular interest has been posed for immune-related genes and proteins, such as MHC-locus conservation and antimicrobial peptides in mammals [60, 61].



C.

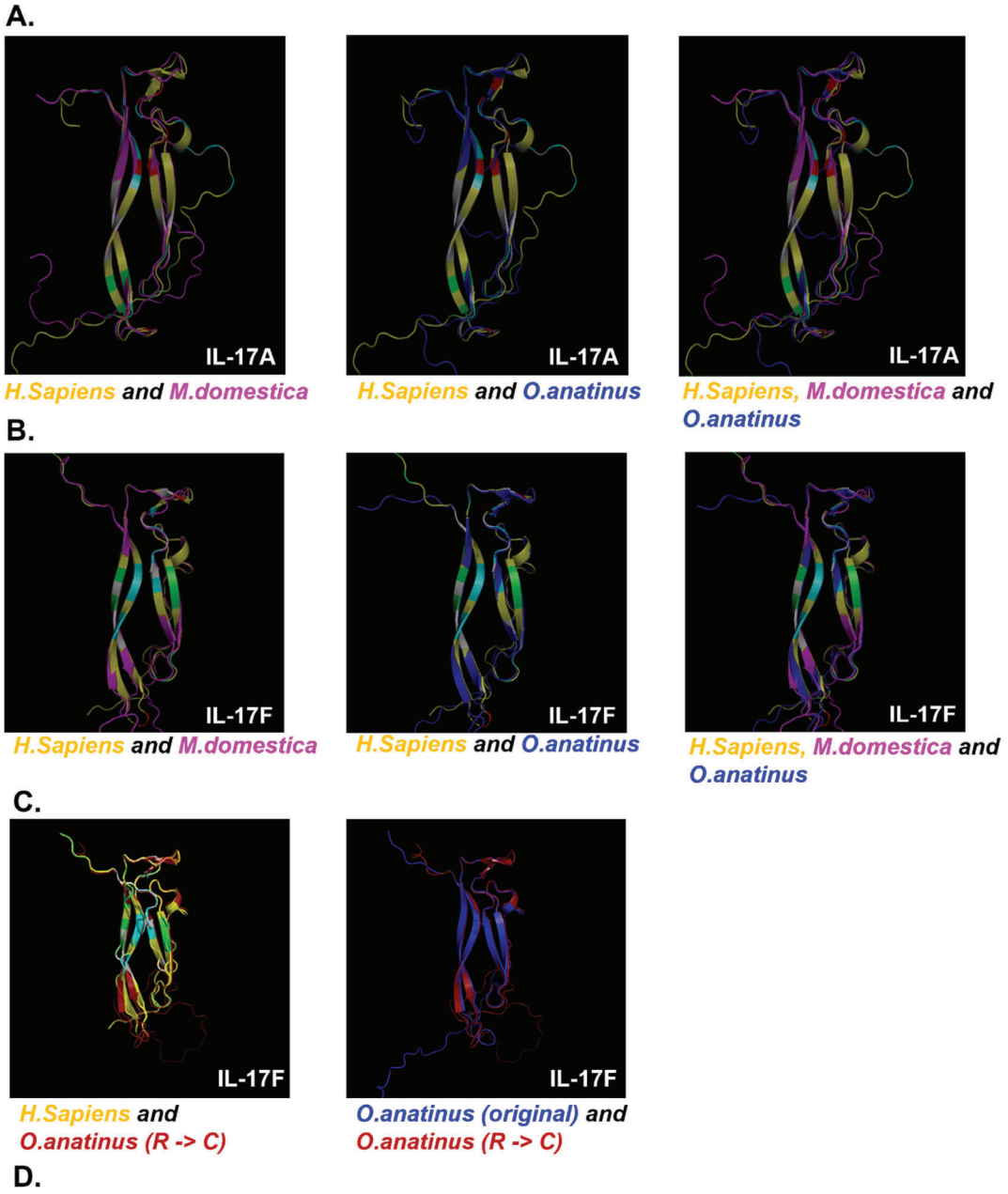
	Specie	Total non-identical residues	Insertion /Gaps	Scaffold residues	Signal peptide	Receptor BS 1	Receptor BS 2	Receptor BS 3	Dimerization core	Glycosylation site	Cystine knot	% identity – total residues	% identity - key residues	P-value (Chi-square test)	P-value (Yates correction)
IL-17A	Mon.dom	68 (153)	NA	37 (79)	16 (23)	6 (10)	2 (12)	6 (15)	6 (25)	NA	0 (5)	55.50%	58.10%	0.53	0.2
	Orn.ana	84 (164)	7	44 (90)	17 (23)	3 (10)	5 (12)	9 (15)	6 (25)	NA	0 (5)	48.70%	47.20%	0.73	0.85
IL-17F	Mon.dom	77 (161)	3	35 (68)	17 (29)	9 (15)	8 (17)	9 (19)	12 (32)	1 (1)	0 (5)	52.10%	54.30%	0.42	0.4
	Orn.ana	81 (144)	19	35 (51)	21 (29)	12 (15)	9 (17)	10 (19)	20 (32)	1 (1)	2 (5)	43.80%	50%	0.026	0.04

FIGURE 3 | Comparison of IL-17A and IL-17F in mammals. A. MSA of IL-17A in eutherian (*H. sapiens* and *M. musculus*), metatherian (*M. domestica*) and prototherian (*O. anatinus*), highlighting the key residues involved in receptor binding, cystine knot or dimerization. B. MSA of IL-17F in eutherian (*H. sapiens* and *M. musculus*), metatherian (*M. domestica*) and prototherian (*O. anatinus*) clades, highlighting the key residues involved in receptor binding, cystine knot or dimerization. C. Non-identical residues between human and either metatherian (*M. domestica*) or prototherian (*O. anatinus*) key IL-17A and IL-17F amino acid residues. The percentage identity was calculated as a percentage of identical residues over the total number of residues either for the whole protein or for the key residues. BS: binding site; Mon.dom: Monodelphis domestica; Orn.ana: Ornithorhynchus anatinus.

TABLE 1 | Involvement of IL-17 family members in human fertility. The table summarises the expression of IL-17s in the human menstrual cycle or in the context of fertility complications. The limma–voom method was applied to obtain differential expression of genes (DEGs) from available datasets using the healthy controls or the early proliferative phase of the menstrual cycle samples as control. The relative expression of the IL-17s is summarised in the table with the arrow defining whether the fold change is increased (↑) or decreased (↓) compared to the control samples. NA corresponds to the absence of IL-17s in the DEG table. The symbol * associated with the arrow means that the adjusted *p* value for that IL-17 is below 0.05.

Source	Type of study	Control sample	IL-17A	IL-17B	IL-17C	IL-17D	IL-25	IL-17F
[44]	Human menstrual cycle transcriptome	Secretory against proliferative phase	NA	NA	NA	↓*	NA	NA
[46]	Human RIF transcriptome	RIF samples against healthy	↑	↑	↑	↓	↓	↑
[34]	Human UI and pregnancy transcriptome	Unsuccessful against successful pregnancies	NA	↑	NA	↑	NA	NA
[45]	Human endometriosis transcriptome	Endometriosis against healthy	Similar	↑*	Similar	↑*	Similar	Similar

Abbreviations: RIF, recurrent implantation failure; UI, unexplained infertility.



D.

Protein	GA341 score
IL-17A *M. domestica*	0.9641
IL-17A *O. anatinus*	0.9992
IL-17F *M. domestica*	0.9192
IL-17F *O. anatinus* (original)	0.2012
IL-17F *O. anatinus* (R -> C modification)	0.7776

FIGURE 4 | IL-17A and F protein structures in mammals. A. Structure superimposition of IL-17A in *M. domestica* (magenta) or *O. anatinus* (blue) based on human (yellow) protein structure was performed using PyMOD plug-in within PyMOL. Matching colour for the key residues (highlighted as in Figure 3) are kept in the 3D structure. B. Structure superimposition of IL-17F in *M. domestica* (magenta) or *O. anatinus* (blue) based on the human (yellow) structure. C. Three-dimensional structure of *O. anatinus* IL-17F modified sequence (maroon) to revert R133 and R135 to cystines compared to the human IL-17F (left) or to original unmodified protein (right) D. Table summarizing the GA341 scores obtained from the modelling of each protein.

A.

Source	Organism	Type of study	Differential analysis	IL-17A	IL17B	IL17C	IL17D	IL25	IL17F
[47]	*M. musculus*	Transcriptome throughout pregnancy	Day 10.5 against post-natal	NA	NA	NA	↑*	NA	NA
[48]	*M. musculus*	Implantation transcriptome	Day 4.5 against day 3.5	↓*	↓*	↓*	↑*	↓*	↓*
[49]	*M. domestica*	Transcriptome throughout pregnancy	Day 13.5-14.5 against non-pregnant	↑*	↑	↑	↓	NA	↓
[50]	*M. domestica*	Transcriptome before and during pregnancy	Day 13.5 against day 7	↑*	↑	NA	↑	NA	NA
[52]	*M. domestica*	Transcriptome throughout pregnancy	Day 13.5 against non-pregnant	↑*	↑	NA	↑	NA	NA
[51]	*N. eugenii*	Transcriptome throughout pregnancy	Day 25 against day 21	↑	NA	↑	NA	↑	↑

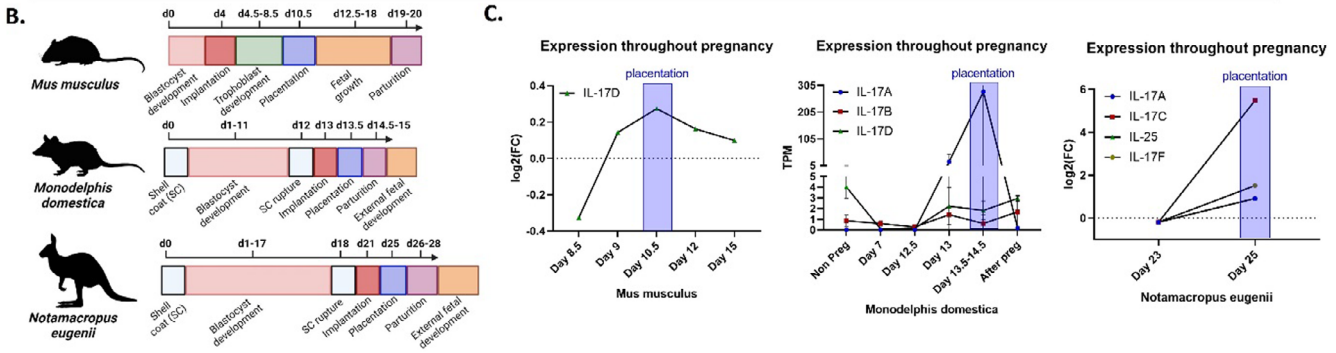


FIGURE 5 | Involvement of IL-17 family members in mammalian pregnancy. A. Table summarising expression of IL-17s in either eutherian or metatherian datasets of pregnancy stages. Differential expression of genes (DEG) was calculated by applying the limma–voom method to the counts retrieved from available datasets using either the non-pregnant, post-pregnant or early pregnancy–stage samples as controls for the analysis. As a result of the DEG analysis, $\log_2$ fold changes and adjusted *p* values are generated for each gene. The relative expression changes of the IL-17s are summarised in the table with the arrow defining whether the fold change is increased (↑) or decreased (↓) compared to the control samples. NA corresponds to the absence of IL-17s in the DEG table. The symbol * associated with the arrow means that the adjusted *p* value for that IL-17 was below 0.05. B. Overview of pregnancy stages in the species analysed (Mus musculus, Monodelphis domestica and Notamacropus eugenii). C. Log_2 fold changes and TPM values obtained from the DEG analysis of two metatherian datasets (Monodelphis domestica and Notamacropus eugenii) and eutherian mammals (Mus musculus) are plotted during pregnancy stages. The light blue rectangle highlights when the placentation process occurs in each animal, recalling the same color-coding in B.

In this study, we highlighted the evolutionary conservation of cytokines belonging to the IL-17 family. The genes encoding these proteins can be found in all mammalian genomes, which have been selected for analysis, and they also show a similar organization of upstream and downstream neighbouring genes. This suggests that they are part of a syntenic block and that also the flanking non-coding regions should have been conserved through evolution, implying that promoter regions and regulatory elements may act in the same way in all the species analysed [62]. Our reciprocal best hits and phylogenetic analyses showed that all IL-17s are also found only as one-to-one orthologs across the mammal species examined and hence radiated from a common ancestor prior to the evolution of mammals [63].

As both IL-17A and F proteins share the same node and their genes both reside in the same chromosome in the three mammalian clades, this suggests that they have evolved from a recent gene duplication event. This finding has been previously described in other reports in which IL-17s were analysed in invertebrates and fishes, pointing out that gene duplication has happened before the mammalian radiation [64, 65]. Furthermore, the concatenated alignment of IL-17s allows us to retrieve a phylogenetic tree in agreement with the mammalian evolution [66], meaning that the overall amount of substitution per site in the protein sequence follows the expected evolutionary history, with prototherian being more distantly related to eutherian and metatherian IL-17s, which are more recently diverged

sister taxa. IL-17s have thus been inherited through purely vertical descent in mammals with these proteins being conserved with at least 50% identity, indicating the conservation of function.

Overall, IL-17s in mammalian clades are not appreciably different from the human proteins, with the exception of *O. anatinus* IL-17F. In this protein, certain key residues are different, especially the amino acids involved in a function. Among these residues, *O. anatinus* lacks the last two cysteines involved in the cystine-knot formation, which has a high impact on the secondary structure formation, as can be noticed by the low GA341 score obtained when modelling the protein based on the human IL-17F. When the two cysteines are introduced back in the sequence, indeed, an increase in the modelling score GA341 is observed, meaning that *O. anatinus* IL-17F has a different structure and, consequently, is likely to have a different function compared to the human IL-17F.

Given the striking difference in the reproductive strategies in the three mammalian clades, we analysed fertility-related transcriptomic datasets to identify any differences in IL-17s that might be linked to the evolution of pregnancy. The analysis of transcriptomic datasets highlights an upregulation of IL-17s in female fertility in both eutherian and metatherian mammals. In particular, IL-17D and IL-17B are upregulated in female fertility complication datasets. Intriguingly, IL-17A and IL-17F transcripts are generally not found (NA) or show similar levels of expression in the fertility complication datasets. This disagrees with other reports that suggest an active contribution of these cytokines in endometriosis [31, 67], suggesting that maybe this difference might be due to the timing of the sample collection for the transcriptomic analysis. Moreover, IL-17B and IL-17D are not known to be involved in fertility-related processes, mainly because the other cytokines of the IL-17 family are not well studied; therefore, it would be helpful to obtain more insights into the expression and function of these cytokines, where we anticipate an unappreciated role in female fertility.

Looking at pregnancy and placentation datasets, a differential involvement of IL-17 family members in different species can be noticed. Unfortunately, the lack of prototherian transcriptome datasets available limits the analysis of potential functional roles of IL-17s in only eutherian and metatherian mammals. Furthermore, the datasets analysed derive from different groups with differences in sampling times, processing and depth of the sequencing (such as choice of microarray or RNA-seq). Due to the nature of sampling, the eutherian mammals dataset analysed consists only of mice pregnancy stages; however, there are slight differences between mouse and human pregnancy, such as length and type of placentation [68], therefore the results of the analysis might not be reflected in human pregnancy. All these considerations, highlight the need for future work around this topic and to build more reliable and inclusive datasets.

From our analysis, we observed that IL-17A is upregulated in Metatherian pregnancy and placentation, whereas in mouse implantation and placentation, we saw an upregulation of IL-17D. The previous analysis showed no major differences in either IL-17A or IL-17D in terms of key residue differences or structure. Therefore, data from additional species are needed to draw firm conclusions on the evolutionary provenance of these differences. A possible explanation for the lack of IL-17A expression in eutherian mammals can be found in the ability of decidualised stromal cells (DSCs) to decrease IL-17A production by local immune cells (T_H17 polarised T-cells) [69], despite other reports suggesting the involvement of DSCs in the recruitment of T_H17 cells and thus the production of IL-17A used as a gradient for trophoblast adhesion [22].

The role of IL-17 in human female fertility is likely to be multifaceted. Recombinant IL-17A was not found to markedly alter the expression of receptivity or decidualization markers in culture models of epithelial or stromal endometrial compartments, respectively [70]. However, the impact of IL-17A on local endometrial immune cell repertoires and endothelial cells during the peri-implantation phase remains largely unknown and warrants further investigation. In the metatherian opossum, IL-17A signalling is activated during the inflammatory attachment reaction [22, 36]. IL-17A blockade in this organism around the attachment phase could help reveal potential redundancies within the IL-17 pathway, for example whether IL-17F, often found to dimerise and synergise with IL-17A [71], can rescue deficiencies in IL-17A signalling.

In both eutherian and metatherian mammals, the involvement of the maternal immune response is pivotal to successful pregnancy. In particular, a pro-inflammatory environment is needed during embryo implantation and placentation; however, the role of IL-17 in these mechanisms is still not well understood. Some reports suggest that IL-17A in eutherian pregnancy is needed for pregnancy establishment [22, 36], whereas a study performed by our group suggests that excessive IL-17A protein levels are detrimental to a successful pregnancy [34]. This is in agreement with other publications in which elevated levels of IL-17A protein have been associated with excessive inflammation leading to fertility complications [30–32]. Little is known about IL-17D, except for the fact that this cytokine seems to be more broadly expressed in the body but with a poor expression in lymphoid and myeloid cells [8]. Similarly to the other IL-17s, IL-17D can induce a pro-inflammatory signature in endothelial cells and in the presence of a viral infection or a tumour, it has been shown to mediate immunosurveillance [8, 72]. However, in the case of liver infection, IL-17D produced by hepatocytes was demonstrated to have anti-inflammatory roles as well. It is shown, indeed, to reduce cytotoxic T-cell response by suppressing dendritic cell activation [73]. These reports demonstrate that IL-17D could exert different and more complex roles depending on the type of stressor or the location where the process occurs.

Further studies are required aimed at unravelling the functions of the least-characterised IL-17 family members to shed light on their non-immunologically related roles, particularly for female fertility.

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Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this article utilises data collected from publicly available databases and datasets.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.