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Original research

Location-specific cell identity rather than exposure to GI microbiota defines many innate immune signalling cascades in the gut epithelium

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ABSTRACT

Objective The epithelial layer of the GI tract is equipped with innate immune receptors to sense invading pathogens. Dysregulation in innate immune signalling pathways is associated with severe inflammatory diseases, but the responsiveness of GI epithelial cells to bacterial stimulation remains unclear.

Design We generated 42 lines of human and murine organoids from gastric and intestinal segments of both adult and fetal tissues. Genome-wide RNA-seq of the organoids provides an expression atlas of the GI epithelium. The innate immune response in epithelial cells was assessed using several functional assays in organoids and two-dimensional monolayers of cells from organoids.

Results Results demonstrate extensive spatial organisation of innate immune signalling components along the cephalocaudal axis. A large part of this organisation is determined before birth and independent of exposure to commensal gut microbiota. Spatially restricted expression of Toll-like receptor 4 (*Tlr4*) in stomach and colon, but not in small intestine, is matched by nuclear factor kappa B (NF-κB) responses to lipopolysaccharide (LPS) exposure. Gastric epithelial organoids can sense LPS from the basal as well as from the apical side.

Conclusion We conclude that the epithelial innate immune barrier follows a specific pattern per GI segment. The majority of the expression patterns and the function of TLR4 is encoded in the tissue-resident stem cells and determined primarily during development.

Significance of this study

What is already known on this subject?

- Several human diseases, such as the different subtypes of IBD, exhibit GI segment-specific pathologies.
- The epithelial layer of the GI tract is equipped with innate immune receptors that sense invading pathogens and trigger inflammatory responses.
- The past decade has witnessed rapid development in the utilisation of stem cell derived organoids as a new model system. Biobanks of organoids now provide an ever-growing range of patient-specific lines.
- Intestinal segment-specific cell identities are encoded in the adult stem cells of epithelia, and this identity is conserved in organoids.

What are the new findings?

- Human and murine GI organoids show expression profiles highly specific to their gastric, small intestinal or large intestinal origin. This conserved tissue identity includes expression of innate immune signalling components.
- Epithelial innate immune signalling is extensively organised in the GI tract. This organisation is also species specific.
- TLR4 senses apical and basal lipopolysaccharide in murine gastric epithelial organoids.
- These results demonstrate that expression of many pattern recognition receptors such as *Tlr4*, as well as the function of TLR4, are intrinsically programmed and independent of contact with microbes.

INTRODUCTION

The epithelial lining of the GI tract physically separates the gut lumen from the body. Although it shields the luminal microbiota from the body's professional immune cells, it must still enable detection of pathogens. To sense microbes, epithelial cells are equipped with pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs).[1 2]

Dysfunction of PRR signalling has been causally implicated in inflammatory diseases, such as IBD and necrotising enterocolitis.[3 4] For example, genetic polymorphisms in PRRs such as nucleotide-binding oligomerisation domain containing 2 (*NOD2*) and *TLR4* are associated with an increased risk of developing IBD.[5 6] Different subtypes of IBD, as well as many other diseases, express segment-specific pathologies, but the molecular mechanisms underlying this phenomenon as well as the contribution of the epithelial cells remain unclear.

Two concepts about epithelial PRR sensing in GI epithelial cells have emerged. First, there is an intuitive understanding that the epithelium needs to minimise inflammation by commensals. This could be facilitated by tolerance in feedback to the microbiota, leading to a certain level of 'blindness' of the epithelium especially towards the lumen of the gut.[1 7] Second, PRR activation is important for homeostatic regeneration and the barrier

How might it impact on clinical practice in the foreseeable future?

- The atlas of gene expression provided here will enable insights into GI segment-specific disease development, leading to a better understanding of the pathologies.
- Since organoid cultures are already used for drug testing, the segment specificity demonstrated here will pave the way for drug development that addresses GI segment-specific diseases.
- The comparison between murine and human GI tract will enable insights into human-specific disease development and may help to explain why some murine models inadequately mimic human disease development ultimately facilitating better drug development strategies.

integrity.[2 8–10] The expression and function of PRR signalling is therefore expected to have a major impact on pathogen sensing and on inflammatory diseases and tissue homeostasis.

The primary response of epithelial cells is particularly difficult to study. Mucosal immune cells express high levels of PRRs and can be highly responsive to stimulation with microbial products. In return, they secrete cytokines such as tumour necrosis factor alpha (TNF-α), which lead to a secondary response of the epithelial cells. It remains a challenge to disentangle this immune cell–epithelial cell interplay in vivo or in ex vivo isolations, where even minor contaminations with immune cells can confound the results by inducing a secondary response, for example, via TNF-α. Alternatively, often used cancer cell lines may have altered signalling pathways. Furthermore, it has been difficult to generate reliable antibodies to TLRs. As a consequence of these technical challenges, the results of expression and functionality of PRRs in the GI tract have been highly contradictive, and our knowledge on whether a given PRR is expressed in the GI epithelium and would activate downstream signalling on recognition of its ligand is limited.[11] Moreover, it remains unclear what initially determines expression and function of PRRs.

Recently, advances in stem cell biology have reopened the chapter of epithelial pattern recognition by adding a central new tool. The GI epithelium harbours tissue-resident adult stem cells, which constantly renew the epithelium during homeostasis.[12] Adult stem cells can be grown in vitro to form three-dimensional (3D) structures called organoids. These are composed of primary, non-transformed cells that can differentiate into the cell types of the specific epithelium.[13–16] They are devoid of immune cells, thus enabling monitoring of the epithelial response alone. They retain tissue characteristics, including regional identity of the GI tract.[17 18] These properties make organoids an ideal tool to study pattern recognition, and several studies already have used organoids to characterise function and effects of specific PRR pathways in the GI epithelium.[16 19–21]

Here we established a biobank of human and murine GI organoids grown from tissue-resident stem cells. Expression profiling has enabled us to generate a gene expression atlas for the different GI tract segments of both species. This revealed striking differences in immune gene expression conserved across individuals. Selecting TLR2, TLR4 and TLR5 as examples, we find that expression is often, but not always, matched by the responsiveness of the respective organoids to stimulation, highlighting the importance of functional testing. We show spatial organisation of innate immune signalling components along the cephalocaudal axis, a large part of which is developmentally determined rather than dependent on the exposure to the gut flora. In summary, our studies demonstrate an unexpected microbiome-independent regulation of immune sensing in primary GI epithelium.

METHODS

Human and mouse tissue material

Human GI tissues were obtained from 18 patients to derive organoid cultures, and from the same patients plus an additional 19 patients to obtain tissue RNA. Tissue RNA was frequently contaminated with RNA from immune or mesenchymal cells, so we selected the best possible isolates. The 21 men and 16 women, aged between 22 and 87 years, underwent sleeve surgery, partial or total gastrectomy for corpus and pylorus samples, Whipple procedure (pancreaticoduodenectomy) for duodenum samples, gastric bypass surgery for jejunum samples or ileocolectomy or haemicolectomy for ileum and colon samples. The procedures took place at the University Hospital of Wuerzburg. online supplementary table S1 contains a list of patient information.

To limit the use of mice in accordance with the 3R principles (Replacement, Reduction, Refinement) we used leftover material of mice that were used for experiments in the respective groups. No additional mice were used for this study. Mouse lines used and providers are named in the online supplement.

A detailed description of material and methods can be found in the online supplement.

RESULTS

Human and murine GI organoids retain the GI segment-specific RNA expression

To better understand the different cellular functions in murine and human GI epithelial cells, we generated a biobank of organoids for the murine and human GI tract. We initiated organoids from six sites: gastric corpus, gastric pylorus, intestinal duodenum, jejunum, ileum and colon, each from three individuals (figure 1A,B). Expression profiles of the different organoids were clustered by tissue of origin (figure 1C,D). Well-known markers for tissue identity, such as the gastric mucin genes *Muc5ac* or *Muc6*, intestinal transcription factors caudal type homeobox 1 (*Cdx1*) and *Cdx2* or region-specific digestion enzymes were expressed as expected (figure 1E), consistent with previous reports demonstrating that tissue identity is encoded in the adult stem cells and maintained in adult stem cell-derived organoids.[18 22]

GI segment-specific expression of innate immune signalling components is species specific

Intrigued by the striking tissue similarity between individuals, we asked which cellular genes are specific for respective regions. To simplify the dataset, we concentrated on three major regions: stomach, proximal small intestine and colon. For this, we combined the data for the two gastric regions corpus and pylorus (= 'stomach'), as well as the two proximal small intestine regions duodenum and jejunum (='prox. int.'). The differentially expressed genes contained genes well known to mark the GI border, *Muc5ac*, *Muc6*, *Cdx1*, *Cdx2* and *villin* (*Vil1*) (figure 2A, black gene names). However, we also found immune-related genes in the differentially expressed groups, such as *Tlr1*, *Tlr2*, *Tlr4, Nod2*, NLR family pyrin domain containing 6 (*Nlrp6*) and many others (figure 2A, blue gene names). Other innate immune-related genes such as *Tlr5* and *Nod2* were not differentially expressed (the complete lists of differentially (twofold and $p<0.05$) and non-differentially expressed genes are shown

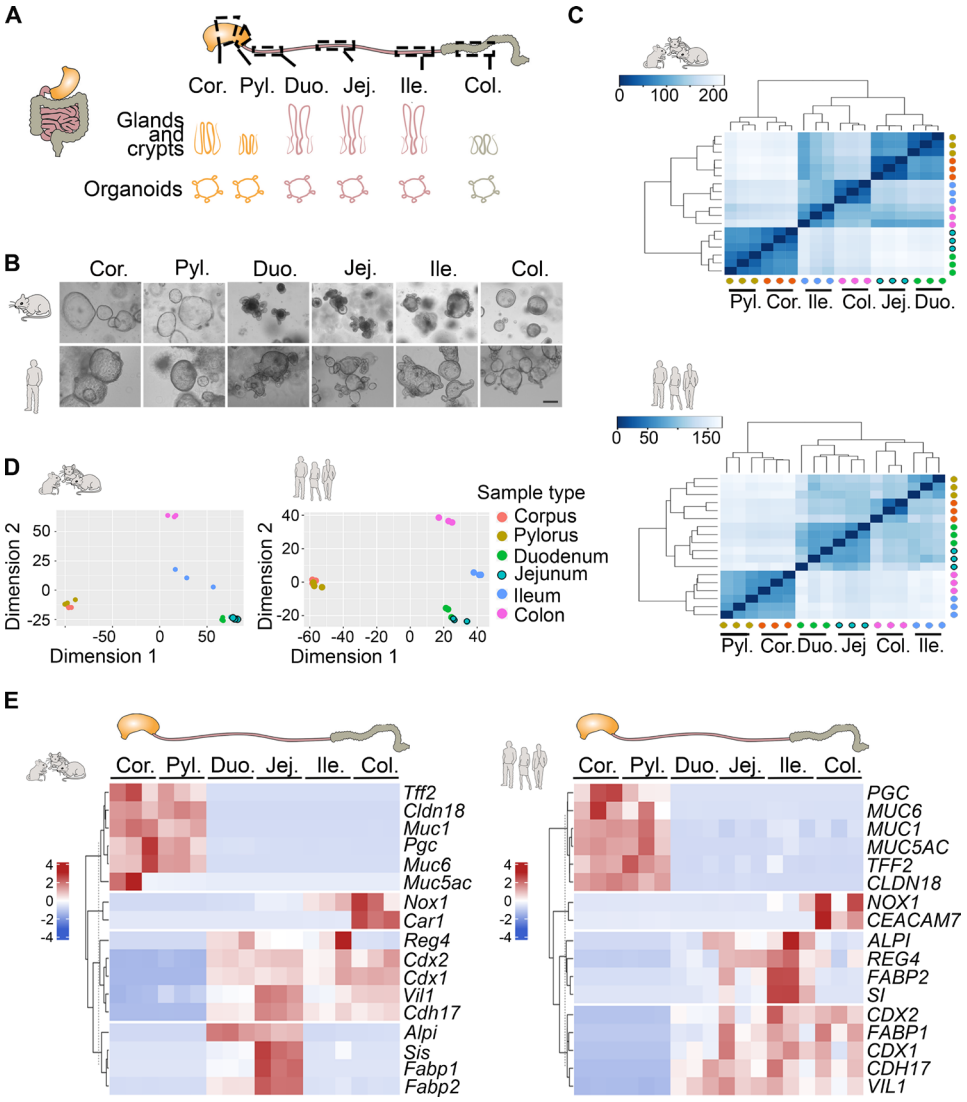

Figure 1 Transcriptome profiling of human and murine GI organoids reveals segment-specific expression of pattern recognition receptor signalling components. Organoids were initiated from three mice or three human donors each from the indicated segments of the GI tract, expanded to a maximum of passage 5, and RNA of each line was subjected to RNA sequencing (one dataset per line). (A) Scheme of setup. (B) Representative light microscope images of mouse and human organoids derived from the indicated segments of the GI tract. scale bar, 200 µm. (C) Hierarchical clustering of total mRNA sequencing. (D) Multidimensional scaling analysis of RNA sequencing, highlighting the closeness of the six gastric organoids and the six proximal intestinal organoids in each species. (E) Heatmaps displaying normalised and scaled gene counts of selected gastric and intestinal cell marker genes through the GI tract for mouse and human organoids.

in online supplementary tables S2 and S3). Gene Ontology (GO) analysis identified the expected GO terms for anatomical and developmental processes and revealed highly significant enrichment of the GO term 'Response to external stimulus', which also contains genes related to innate immunity (figure 2B). Heatmaps of all genes that are included in the GO term 'Response to external stimulus' demonstrated specific expression patterns along the cephalocaudal axis. These were pronounced in the genetically identical laboratory mice and still visible in the more diverse patient samples (figure 2C).

To investigate whether the expression of immune-related genes would follow a gradient along the cephalocaudal axis, possibly mirroring the microbial load, we selected a range of immune-related genes from the GO term 'Response to external stimulus', based on their biological relevance. A heatmap also revealed extensive cephalocaudal organisation of expression of these genes, again more pronounced in mice (figure 2D and online supplementary figure S1). We conclude that expression of immune-related genes in the GI tract is neither uniform, nor follows a single pattern along the cephalocaudal axis. Instead, each segment expresses its specific set of immune-related genes.

Comparison of expression profiles from human and murine organoids revealed that some of the profiles were similar in the two species, such as those for *Nod2*, *Nlrp6* and *Tlr4*, but others were markedly different, such as *Tlr2*, *Tlr5* or *Tlr6* (figure 2D and online supplementary figure S1).

Function of TLR2, 4 and 5 are gut segment specific and species specific

To analyse, whether the RNA expression translated into patterns of functionality, we selected three exemplary TLRs: TLR2 recognises diacetylated or triacetylated lipoproteins, TLR4 lipopolysaccharide (LPS) and TLR5 flagellin.

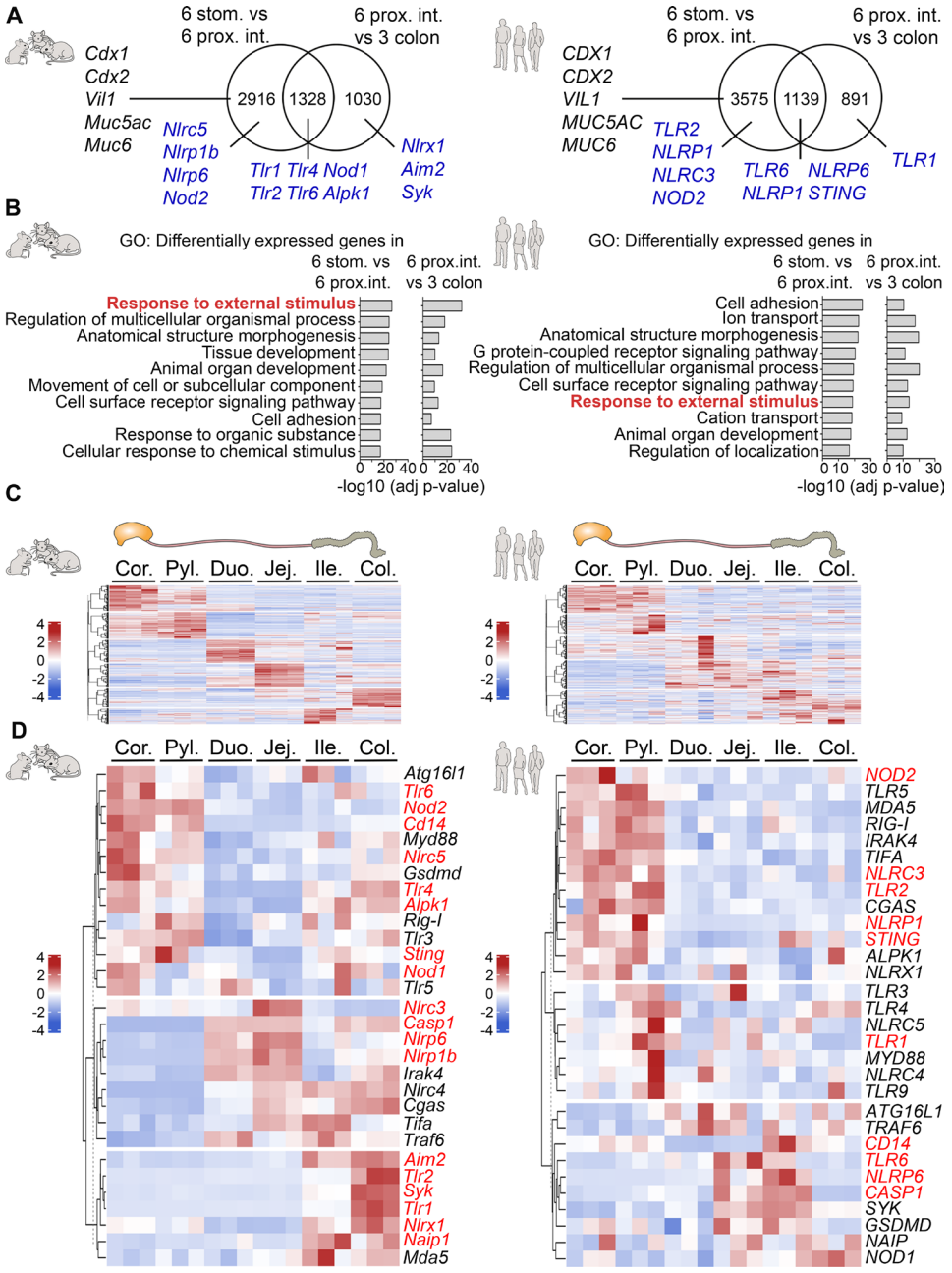

Figure 2 Expression of innate immune signalling components is GI segment specific and species- pecific. (A) Lists of differentially expressed genes were generated, comparing six gastric organoids (combined corpus and pylorus) and six proximal intestinal organoids (combined duodenum and jejunum) or six proximal intestinal and three colonic organoids. Venn diagrams display number of differentially expressed genes ($p<0.05$ and $\log_2$ fold change >±1 (twofold change)). Selected genes chosen for biological interest are listed. Black: genes known for tissue identity. Blue: genes known for immune function. (B) GO enrichment analysis (biological process) comparing six gastric, six proximal intestinal and three colonic organoids. Scores of the top GO terms, indicating the enrichment p value. (C) Heatmap of all genes in GO:0009605 – response to external stimulus (1939 mouse genes, 2012 human genes). (D) Heatmap displaying normalised and scaled gene counts of selected genes from GO:0009605 for mouse and human organoids. Genes identified by the analysis as differentially expressed are marked in red. GO, Gene Ontology.

In the sequencing data, *Tlr4* showed a peculiar expression pattern with high expression in the stomach and colon, but very low expression in the small intestine (figure 2D). This was in principle conserved in humans and mice, and we confirmed this PCR in organoids as well as primary isolated tissue (online supplementary figure S2). However, a closer look at the TLR4 signalling pathway also revealed species specificity. For LPS sensing, TLR4 needs the accessory proteins CD14, MD2 and LBP. RNA-Seq data showed expression of *Cd14*, *Md2* and *Lbp* in mice, but only *CD14* in human organoids (figure 3A,B). This led to the hypothesis, that although TLR4 is expressed in a similar pattern in the two species, LPS stimulation may lead to NF-κB activation in murine gastric, ileal and colonic epithelium but not in human GI epithelium (figure 3A,B, graphic on the right).

To determine whether the TLR4 pathway is functional in the GI epithelium, we assessed expression of IL-8 in humans and its analogue *Cxcl2* in mice, both well-known and reliable markers for inflammation. For practicability, we concentrated on three major GI regions: corpus, jejunum and colon. qPCR analysis showed upregulation of *Cxcl2* in response to LPS, indicating

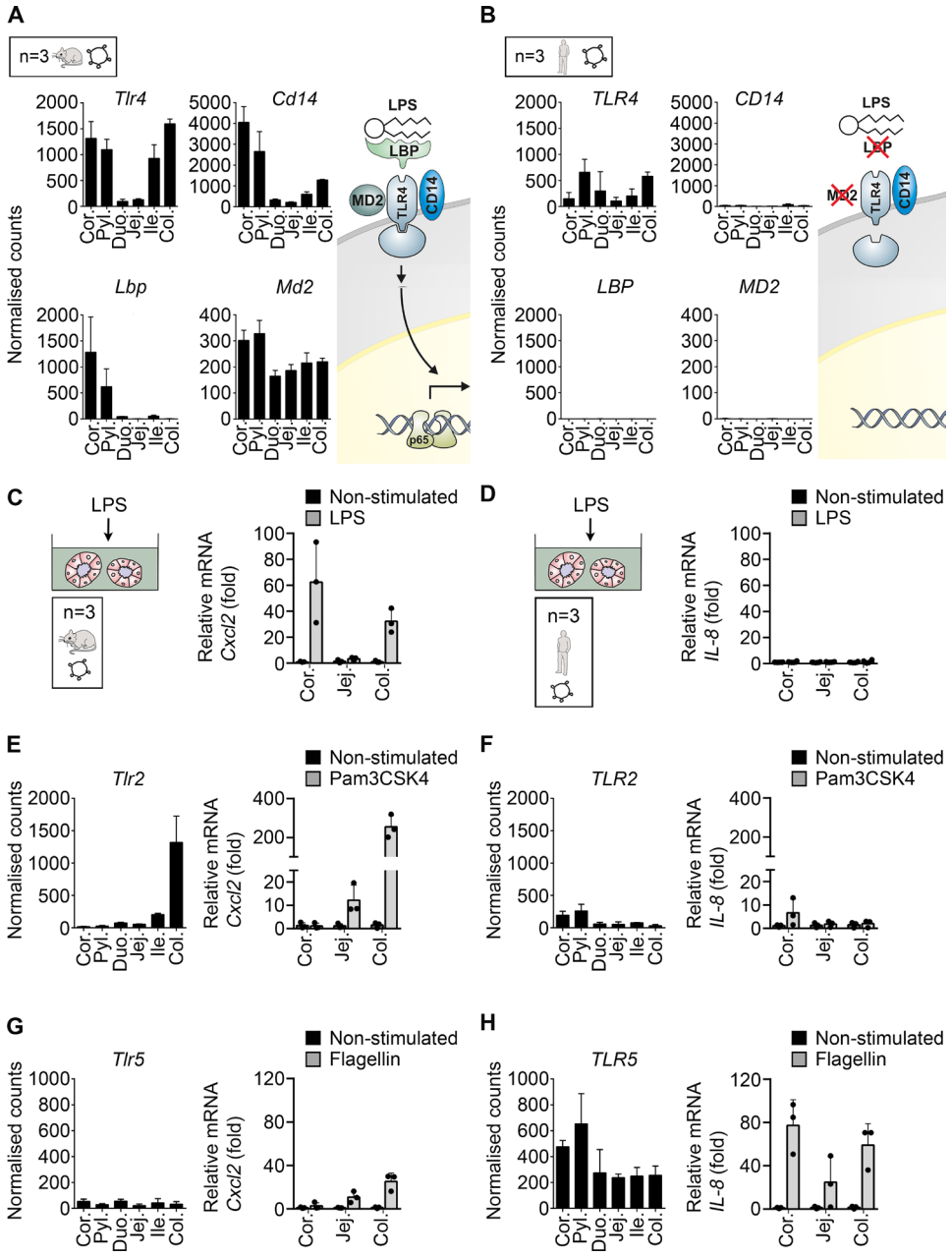

Figure 3 Expression and function of TLR2, 4 and 5 signalling pathways is GI segment specific and species specific. (A and B) Normalised gene counts of *Tlr4, Cd14*, *Lbp* and *Md2* in murine and human organoids. Right: graphical summary of resulting hypothesis. (C and D) Separate lines of organoids from the indicated gut segments of three individual mice (C) or human patients (D) were exposed to LPS 100 ng/mL in the supernatant. After 2 hours, cells were harvested, RNA was prepared and expression of *Cxcl2* (C) or human analogue *IL-8* (D) was determined by qPCR. (E–H) Normalised gene counts of *Tlr2* and *Tlr5* in murine (E and G) or human (F and H) organoids (left panels) and functional test of the respective TLRs using Pam3CSK4 1 µg/mL or flagellin 100 ng/mL (right panels). Organoid seeding, cell processing and qPCR was performed as in C and D. Bars in A and B and E–H (left panels) represent means with SD of normalised gene counts from RNA sequencing of three organoid lines per segment (see figure 1). Normalisation of sequencing data is explained in the online supplementary methods. qPCR results in C–H (right panels), were normalised to *GAPDH* and then to the average of the mock controls. Bars in A–H represent mean with SD of cells from three biological samples (n=3). All results are representative of at least three independent experiments. LPS, lipopolysaccharide.

that murine corpus and colon have functional TLR4 signalling, while jejunum does not (figure 3C), mirroring the expression of *Tlr4*. In human organoids, *IL-8* was not induced, verifying our hypothesis (figure 3D).

For murine *Tlr2*, the sequencing data showed a steep gradient with highest expression in the colon (figure 3E, left panel), while human *TLR2* showed a much flatter gradient at lower expression levels with highest expression in the stomach (figure 3F, left panel). Stimulation with the TLR2 agonist Pam3CSK4 demonstrated that both patterns were generally matched by *Cxcl2* or *IL-8* transcription (figure 3E,F, right panels), but with very low response in the human stomach (figure 3F).

Murine *Tlr5* and human *TLR5* were expressed in all gut segments, with much lower levels in the murine GI (figure 3G, left panel). However, despite the similar gene expression, murine gastric organoids failed to respond to stimulation with flagellin,

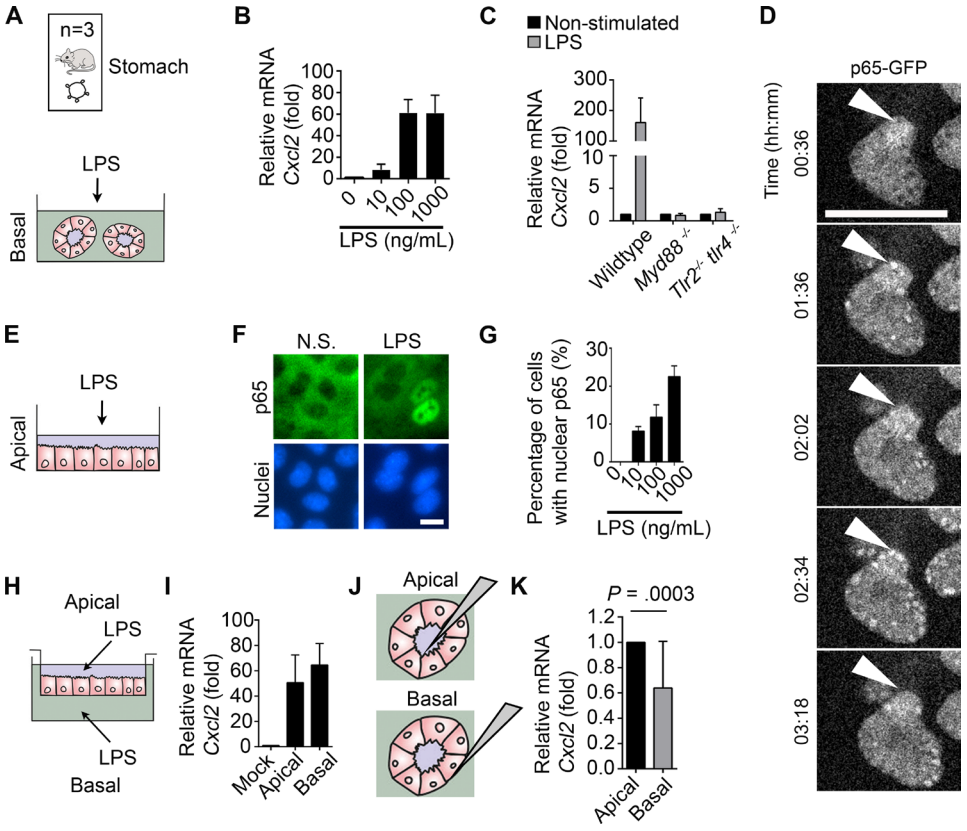

Figure 4 TLR4 senses apical and basal LPS stimulation in murine gastric organoids. (A–C) Organoids from the indicated gut segments of wildtype mice (B), or the stomach of wildtype, *Myd88*$^{-/-}$ or *Tlr2/4*$^{-/-}$ mice (C) were grown in Matrigel. Two hours after exposure to LPS 100 ng/mL in the supernatant, cells were harvested, RNA was prepared and expression of *Cxcl2* was determined by qPCR. (D) Gastric organoids generated from GFP-p65 knockin mice were stimulated with LPS and GFP-p65 translocation monitored by live cell confocal microscopy. Stills of the movie are shown. White arrow points to a single nucleus (see also online supplementary movie and online supplementary figure S5). Scale bar: 100 µm. (E–G) Cells from murine gastric organoids were seeded on conventional cell culture plates to form a two dimentional monolayer of cells, stimulated with LPS at the indicated concentrations for 2 hours, fixed and stained for p65 and nuclei (F). Cells with nuclear p65 were counted and are presented as percent of total cells (G). Scale bar, 10 µm. (H and I) Cells from murine gastric organoids were seeded onto transwells, and 100 ng/mL LPS was added either to the lower or the upper compartment. After 2 hours, mRNA levels of *Cxcl2* were determined by qPCR. (J–K) organoids were microinjected with LPS 100 ng/mL starting concentration either inside the organoid or outside. After 2 hours, cells were harvested, RNA prepared and *Cxcl2* levels quantified by qPCR. Results were normalised to *Gapdh*, and each outside injection was normalised to the paired inside injection on the same plate. Bars in B and G represent means with SD of cells from three mice (n=3). Bars in C and I represent means with SD of technical triplicates. Bars in K represent means with SD of 20 paired samples (apical/basal) in six experiments. Statistical significance was tested with paired two-sided t-test of the underlying ΔΔCq data. All results are representative of at least three independent experiments. LPS, lipopolysaccharide.

while jejunal and colonic organoids did. All human organoids readily responded (figure 3G,H, right panel).

Taken together, RNA expression and functional testing of TLR2, 4 and 5 demonstrated gut segment specific and species specific patterning of PRRs. While the functional data often mirrored the RNA expression data, the occasional absence of this congruency underlines the relevance of functional testing.

Human gastric organoids are refractory to LPS stimulation regardless of apical or basal stimulation

Because of the peculiar expression pattern, the species specificity and general importance of TLR4, we further focused on this gene. Polarisation plays a role in innate immune sensing, since one proposed mechanism to minimise stimulation from the normal (apical) microbiota is to confine pattern recognition to the basolateral or intracellular compartment.[1 7] Organoids allow testing of this hypothesis, as their polarisation is well documented. In 3D organoids grown in extracellular matrix, the apical side of the epithelium faces the lumen of the organoid.[13–16 23] In two-dimensional (2D) monolayers, the apical side faces the lumen of the well.[23–25] This apical-basal orientation was again confirmed in our hands[26] (online supplementary figure S3).

To test, whether LPS could be sensed from the apical side, we compared gastric organoids seeded in 3D to cells from organoids grown in 2D. As positive control, we added TNF-α, a common NF-κB activating stimulus with prime importance in IBD.[6] We observed that only basally administered TNF-α induced *IL-8* as measured by qPCR (online supplementary figure S4A). To exclude an effect of 3D versus 2D growth, we tested apical versus basal stimulation in transwells. Only TNF-α administered to the lower compartment, that is, stimulating the basal side, induced transcription of *IL-8* (online supplementary figure S4B).

We conclude that human gastric epithelium is refractory to stimulation with LPS from both sides. It senses TNF-α only from the basal, not the apical side, which shows that the here-established assays can in principle discriminate between the two sides.

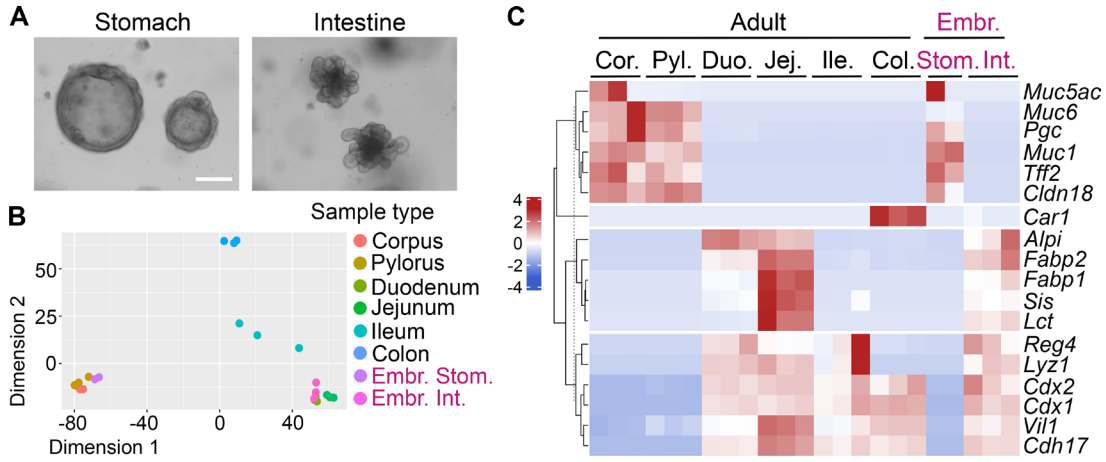

Figure 5 Transcriptome profiling of adult and embryonic mouse-derived GI organoids. Organoids were generated from E16 stomach or proximal intestine and expanded to a maximum of passage 5; RNA of each line was subjected to RNA sequencing (one dataset per line). (A) Representative images of organoids. Scale bar, 200 µm. (B) RNA was isolated from two organoid lines generated from embryonic stomach and three lines generated from proximal intestine (all E16). Transcriptomes were sequenced and compared with transcriptomes of adult-derived organoids. Multidimensional scaling analysis of total mRNA sequencing, highlighting the closeness of the embryo-derived organoids to the adult-derived organoids (data from adult mice also shown in figure 1). (C) Heatmap displaying normalised and scaled gene counts of selected gastric and intestinal cell markers through the GI tract of adult mouse and embryo-derived gastric and proximal intestinal organoids.

Murine gastric organoids sense LPS from both the apical and the basal side

We then assessed whether murine LPS sensing could be restricted to the basal compartment to minimise response to the microbiota. Basal stimulation by addition of LPS to 3D organoids showed that the *Cxcl2* response is concentration dependent, as expected (figure 4A,B).

Because *Cxcl2* can be induced by many NF-κB activators, we confirmed the specificity in two knockout models. Gastric organoids from mice devoid of the TLR adaptor protein *MyD88* or *Tlr2*/*Tlr4* double knockout mice did not upregulate *Cxcl2* in response to LPS measured by qPCR (figure 4C), corroborating that the response to LPS was due to stimulation of *Tlr4*.

To verify the NF-κB signalling in an additional assay, we stimulated gastric organoids from GFP-p65 expressing mice.[27] We observed the expected GFP-p65 nuclear oscillation in response to LPS in live cell microscopy (figure 4D, online supplementary figure S5 and online supplementary movie).

Apical stimulation of organoid-derived cells in 2D (figure 4E) showed nuclear translocation of p65, measured by immunofluorescence (figure 4F). Quantification of cells with nuclear p65 showed dependency on LPS concentration (figure 4G) and time, with a slight tendency for an oscillatory pattern (online supplementary figure S6A and B), similar to previous observations in cell lines.[28] Cells from *Myd88*$^{-/-}$ knockout mice and *Tlr2*$^{-/-}$/*Tlr4*$^{-/-}$ double knockout mice were insensitive to LPS also in this assay (online supplementary figure S6C). To exclude an effect of the 2D or 3D growth conditions, we tested two additional settings. In transwells (figure 4H), addition of LPS either to the upper or the lower compartment both induced upregulation of *Cxcl2* (figure 4I). Similarly, LPS microinjected to either the apical or basal side of organoids in 3D (figure 4J), stimulated both sides (figure 4K). We conclude that murine gastric TLR4 can sense both basally and apically administered LPS.

Tissue identity is already encoded in tissue-resident stem cells in the embryo and retained in embryo-derived organoids

Intrigued by the distinct patterning of PRR expression, which did not follow the density of microbial colonisation, and the apical LPS sensing, which would in principle allow sensing of gut luminal bacteria, we hypothesised that PRR expression could be defined independently of contact to the microbiota. To address this question, we searched for a model that had never been in contact with microbial products. We could not turn to germ-free mice, because their food still contains LPS after sterilisation. Therefore, we generated organoids from murine embryos, which—because of the sterile prenatal environment—were never exposed to compounds of GI bacteria.[29] As the embryonic GI tract is very small, we did not separate it into six regions, but only grew organoids from stomach and proximal intestine. Organoids grown from embryonic tissue phenotypically resembled adult organoids (figure 5A). Gene expression profiles of embryo-derived and adult organoids clustered according to their site of origin demonstrating regional identity (figure 5B). Tissue-specific markers expressed in embryo-derived organoids confirmed regional identity and showed similar expression patterns as their adult counterparts (figure 5C).

Many immune-related genes, including *Tlr4*, are developmentally and not environmentally defined

We then asked, which of the genes that characterise the GI border in the adult-derived organoids would do so also in the embryo-derived organoids. For this, we compared the differentially expressed genes between embryonic stomach and proximal small intestine to their adult counterparts (the complete lists of differentially (twofold and $p<0.05$) and non-differentially expressed genes are shown in online supplementary table S4). The majority of genes marked the border in both the embryo-derived and the adult-derived organoids. Within the list of commonly regulated genes, the expected well-known genes for the GI border were present, such as *Cdx1*, *Muc5ac* or *Lyz1*. In addition, we found *Tlr4* and many other PRRs (figure 6A). Other PRRs marked the GI border either only in the adult-derived or only in the embryo-derived organoids (figure 6A). GO analysis of the 2988 common markers again highlighted the GO term 'Response to external stimulus' (figure 6B). Clustering based on all genes in this

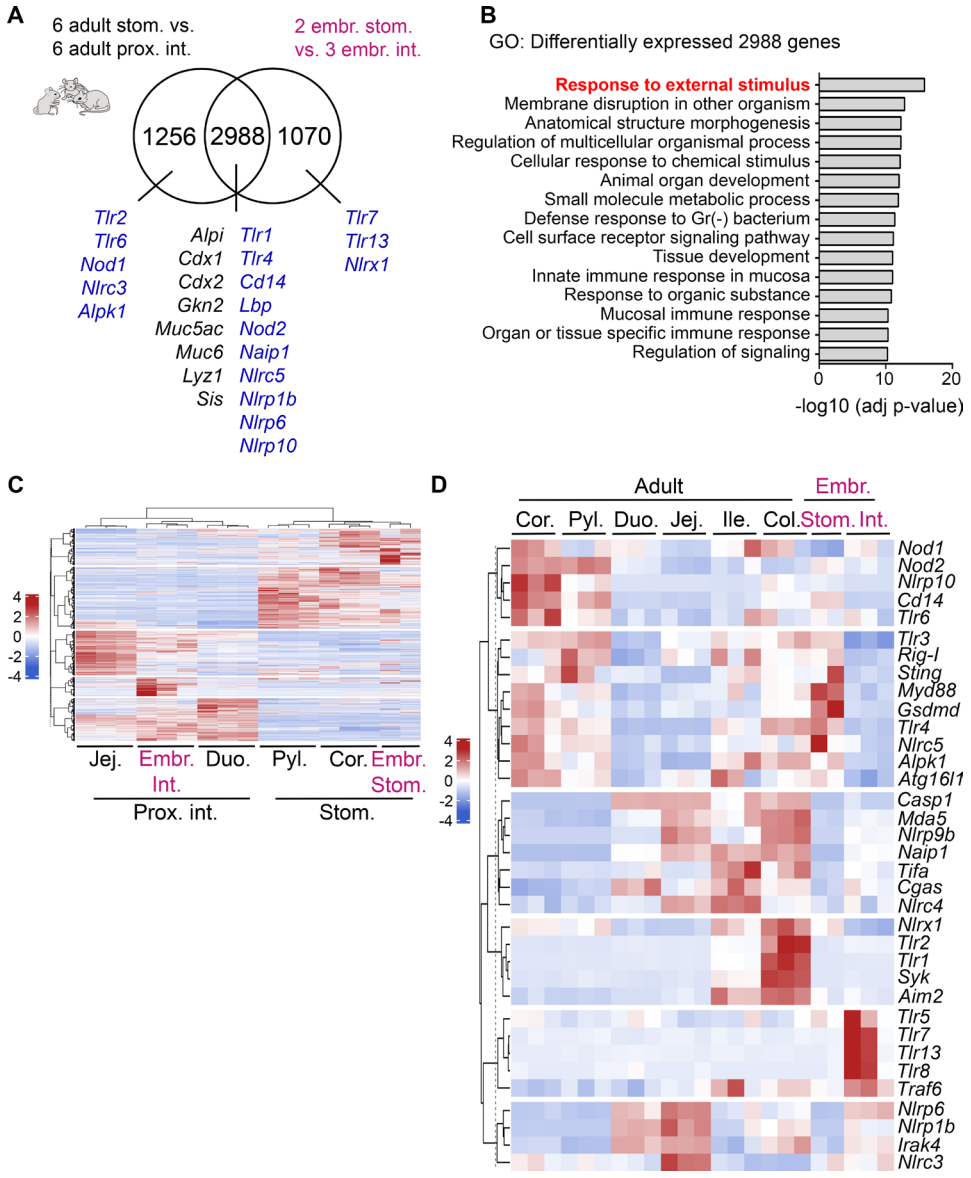

Figure 6 Expression of innate immune signalling components is segment specific in murine embryo-derived organoids. (A) Lists of differentially expressed genes were generated, comparing six gastric organoids (combined corpus and pylorus) and six proximal intestinal organoids (combined duodenum and jejunum) or two embryonic gastric organoids and three embryonic proximal intestinal organoids. Venn diagrams display number of differentially expressed genes ($p<0.05$ and $\log_2$fold change >±1 (twofold change)). Selected genes chosen for biological interest are listed. Black: genes known for tissue identity. Blue: genes known for immune function. (B) GO term enrichment analysis (biological process) of 1626 overlapping adult versus embryonic differentially expressed genes. Scores of the top GO terms, indicating the enrichment p value. (C) Heatmap of all genes in GO:0009605 – response to external stimulus (1974 genes). (D) Heatmap displaying normalised and scaled gene counts of selected genes from GO:0009605 for adult and embryonic mouse-derived organoids. GO, Gene Ontology.

GO-term placed the embryonic proximal intestinal organoids between adult duodenal and jejunal organoids, and embryonic gastric organoids next to the adult gastric organoids (figure 6C). A heatmap depicting the same selected immune signalling components as above revealed that most of the genes including *Tlr4* were similarly expressed between adult-derived and embryo-derived organoids, but some PRRs, like *Tlr5* or *Nod2*, were expressed differently in embryo-derived organoids compared with their adult counterparts (figure 6D, online supplementary figure S7). We conclude that the patterning of expression of most but not all PRRs and immune signalling components is already encoded in the tissue-resident stem cells of the embryo.

To analyse, whether this expression patterning also translates into functional protein, we again focused on TLR4. Expression levels of *Tlr4* in the RNA-Seq were highly similar between adult and embryo-derived organoids (figure 7A). Conventional PCR confirmed that in all embryo-derived organoid lines, *Tlr4* was expressed in the stomach and not in the intestine (figure 7B). Exposure of organoids to LPS and subsequent qPCR of *Cxcl2* demonstrated that the embryo-derived proximal intestinal organoids did not react to LPS, while the embryo-derived gastric organoids expressed *Cxcl2*, thus fully resembling their adult counterparts (figure 7C). We conclude that the expression of many immune-related genes as well as the function of TLR4 are defined developmentally rather than environmentally.

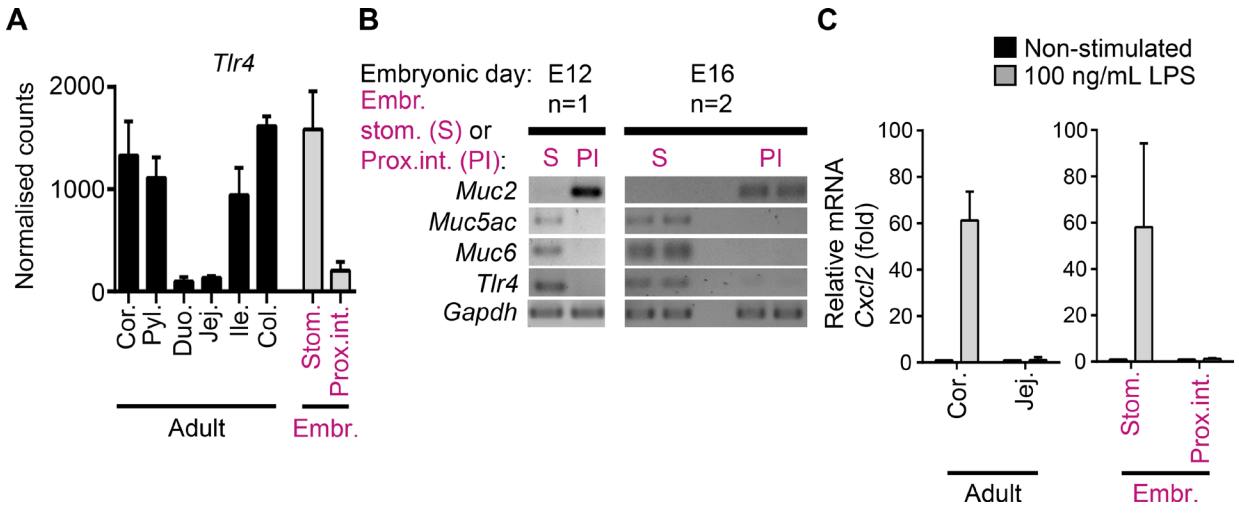

Figure 7 *Tlr4* expression and function are mainly developmentally rather than environmentally programmed. (A) Normalised gene counts of *Tlr4* in murine organoids. Data from RNA-Seq are shown in figures 1 and 4. Bars represent mean with SD of three embryonic proximal intestinal organoids or two lines for embryo-derived gastric organoids. (B) RNA was prepared from embryo-derived organoids. Expression of indicated genes was assessed by conventional PCR. Mucus genes are marker genes for stomach or proximal intestine. Each lane shows PCR results from the indicated organoid lines, n=3 stomach and n=3 proximal intestine. (C) Organoids in culture were exposed to 100 ng/mL LPS for 2 hours, cells were harvested, RNA was prepared and expression of *Cxcl2* was quantified by qPCR. Results were normalised to *Gapdh* and then to the paired non-stimulated control. Bars represent means with SD of three organoid lines for adult murine corpus organoids and three technical replicates for embryo-derived organoids. Results are representative of at least three independent experiments. LPS, lipopolysaccharide.

DISCUSSION

Pattern recognition and innate immune signalling play a crucial role in inflammatory responses and tissue homeostasis. Here, we provide a gene expression atlas for human and murine GI epithelial organoids. The data indicate a vast extent of species-specific regional patterning of PRR signalling components throughout the GI tract. This regional patterning as well as the apical LPS sensing by TLR4 prompted us to test whether pattern recognition may be regulated independently of contact with microbial products. Transcriptional profiling in embryo-derived organoids showed that while expression of some PRRs may depend on environmental cues as expected, an unexpectedly large part of segment-specific expression of PRR signalling components is indeed independent of prior contact with microbial products. Taking TLR4 as one example, functional experiments show that sensing of LPS by TLR4 is also determined already prior to birth and therefore independent of feedback from microbiota. We conclude that the expression of a large part of epithelial innate immunity is developmentally defined and conserved in tissue-resident stem cells.

The extent of patterning of innate immune signalling components seems surprising at first. However, patterning is a universal developmental process and defines tissue identity. This regional identity is to some extent already encoded in the tissue-resident stem cells of the embryo and retained in organoids.[22] Our results regarding the patterning of innate immune signalling components are in line with previous studies, which analysed RNA from primary cells for expression of *Tlr4*[30] or expression of transgenes in *Tlr2*, *4*, *5*, *7* and *9* reporter mice.[21] Our data indicate that these TLRs and expression of many immune-related genes are part of this tissue identity. At this stage, we can only speculate about the evolutionary reason for this. We hypothesise that the need for specialised innate immune recognition is also evolutionarily determined by the complex environment, such as the presence of physical or chemical barriers, of each site.

One example for the location-specific function of PRRs is TLR4. The data regarding localisation of TLR4 have been heterogeneous, owing to difficulties with TLR-specific antibodies. To circumvent these problems, Price and colleagues[21] used staining of genetically introduced HA-tags in reporter mice for *Tlr4*. The cephalocaudal expression pattern identified here matches the expression of the reporter gene. Furthermore, Price and colleagues found localisation at both the apical and the basolateral surfaces. Here, we add the functional data for TLR4, finding that apical and basal stimulation of murine gastric organoids induced an NF-κB response. This also includes the possibility that LPS could be taken up by the cell to stimulate intracellular TLR4.[31] While this shows the general capability of epithelial cells to mount an inflammatory response to apical and basal LPS, it cannot be concluded that the luminal LPS induces constant inflammation in vivo. We have only addressed one inflammatory response gene, and it is possible that apical and basal stimulations induce additional different transcriptional responses, regulating the overall outcome. In addition, we hypothesise that in vivo, the mucus in the stomach and the colon may pose a diffusion barrier to keep luminal LPS at a distance from epithelial TLR4. Indeed, in the colon, LPS is present in a steep gradient with high luminal and very low mucosal concentrations.[32] Also, gastric mucus and colonic mucus share physical properties and are both firmly attached to the epithelium, while the small intestine has a different, easily removable mucus.[33] Therefore, we speculate that in the stomach and colon, but not the small intestine, apical LPS sensing indicates a breach of the mucus barrier. Low levels of LPS diffusing through the barrier at physiological conditions may induce the often proposed, low level of 'physiological inflammation', important for epithelial homeostasis.[9–11 34 35] Future studies are required to verify these speculations.

A further surprising finding from our study was the species specificity of patterning. It must be taken into account that the subjects the organoids were generated from were very different: laboratory mice had identical genotype, diet and probably also a highly similar gut microbiome, while the patients differed in these respects. It is likely that environmental factors also

influence the expression patterns, and the higher diversity of expression patterns in the diverse human cohort can be taken as an indication for this. Beyond this, however, some PRRs, such as TLR1, 2 and 6, showed strikingly species-specific expression along the cephalocaudal axis. Also, the functionality of the TLR2, 4 and 5 signalling cascades indicated species specificity. Again, we can only speculate about the reasons. It may be environmental, as mentioned above. It is also possible that for each species, tissue-specific sensors are beneficial in general, but which ones are present is not important. For example, a species would benefit from a sensor indicating a breach of the mucus barrier, because this would enable sensing of pathogens that come in close proximity of the epithelium. It may not matter which of several possible sensors indicates such a threat. While TLR4 could assume this function in the mouse, another PRR could assume it in humans.

It has been hypothesised that some PRRs are regulated by feedback to stimulation by the microbiota at birth.[21 36] To address this hypothesis, we used embryo-derived organoids. Importantly, previous studies demonstrated that embryo-derived organoids may mature over time in culture,[22 37–39] so we did not aim to address at which developmental step PRR expression is defined. However, because the embryo is sterile, we used embryo-derived organoids to define the contribution of the GI flora to PRR patterning. Comparing embryo-derived with adult-derived organoids, we also find that some innate immune-related genes are differentially expressed, leaving open a possible role of environmental influence and further development during or after birth. However, the expression of the majority of innate immune-related genes, including *Tlr4*, was patterned independent of contact with bacteria at birth. Regarding TLR4, embryo-derived murine organoids show the same activation pattern as adult-derived organoids, with functional TLR4 signalling in the stomach, but not in the proximal intestine. These data support developmental definition of a large part of pattern recognition.

Taken together, data presented here support the concept of strategic expression of PRRs in specific segments of the GI tract. We hypothesise that a large part of this compartmentalisation is regulated by the general developmental processes that define tissue identity. Future studies have to determine the molecular basis for the intricate regional activity of PRRs in the GI tract.

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