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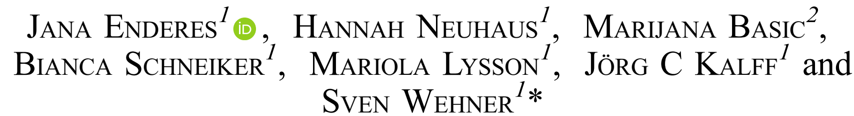
Microbiota-dependent presence of murine enteric glial cells requires myeloid differentiation primary response protein 88 signaling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Enteric glial cells (EGCs) were shown to maintain the barrier integrity and immune homeostasis of the bowel. Postnatally, EGCs develop from progenitor cells located in the myenteric plexus and are continuously replenished through adulthood. Both, murine EGC development and replenishment were shown to depend on the microbiome. The underlying mechanisms are still unknown, and we hypothesized that the myeloid differentiation primary response protein 88 (Myd88) or toll-like receptor signaling pathways may be involved. Adult and neonatal C57BL/6 wild-type (wt) and Myd88$^{-/-}$ mice were housed under specific pathogen-free (SPF) or germ-free (GF) conditions. GF mice were further conventionalized by gavaging stools from, and cohousing with, SPF mice having intact microbiomes. The small bowels were harvested at various time points, and immunohistochemistry and qPCR analysis of EGC markers in the muscularis externa and mucosa were performed. In wt mice, after conventionalization, the glial cell-specific markers, glial fibrillary acidic protein (GFAP) and S100 calcium-binding protein β (S100β), were upregulated in the mucosa and muscularis externa. In Myd88$^{-/-}$ mice, this upregulation did not occur. Importantly, GFAP (only in the mucosa) and S100β (in both the mucosa and muscularis externa) were significantly reduced in conventionalized Myd88$^{-/-}$ mice compared with the conventionalized wt mice. In neonatal mice, the gene expressions of *GFAP* and *S100β* increased between the day of birth (P0) and postnatal day 15 (P15) in the mucosa and muscularis externa of both wt and Myd88$^{-/-}$ mice. Notably, in the mucosa but not the muscularis externa, at P15, the gene expressions of *GFAP* and *S100β* were significantly reduced in Myd88$^{-/-}$. Our data demonstrated that postnatal development and replenishment of EGCs require intestinal microbiota and depend on Myd88. The specific upstream mechanisms may involve toll-like-receptor recognition of the microbiota and will be the subject of further research.

Keywords. Enteric glial cells; microbiota; Myd88

Abbreviations: CONV-D, conventionalized; EGC, enteric glial cell; GDNF, glial cell line-derived neurotrophic factor; GF, germ-free; GFAP, glial fibrillary acidic protein; IL, interleukin; IL-1R1, interleukin 1 receptor 1; MC, tunica mucosa; ME, tunica muscularis externa; mEGC, mucosal enteric glial cells; Myd88, myeloid differentiation primary response protein 88; P0–P15, postnatal day 0–15; SPF, special pathogen-free; S100β, S100 calcium-binding protein β; TLR, toll-like-receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β; Wt, wild type.

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1. Introduction

As peripheral glia cells, enteric glia cells (EGCs) are a component of the enteric nervous system and are grouped together with enteric neurons primarily as so-called intraganglionic EGCs, which are found in the myenteric plexus and deep myenteric plexus (Rühl 2005). There are also extraganglionic EGCs, which can be found primarily in smooth muscle layers and in the tunica mucosa (Gulbransen and Sharkey 2012). EGCs play a decisive role in maintaining gut and immune homeostasis (Neunlist *et al.* 2014) and in the regulation of gastrointestinal motility (Grubišić and Gulbransen 2017). Furthermore, they actively participate in inflammatory processes in the gastrointestinal tract through the secretion of cytokines, such as interleukin (IL)-1β and IL-6 and chemokines such as monocyte chemoattractant protein (MCP)-1 (Stoffels *et al.* 2014; Grubišić and Gulbransen 2017) and CXCL10 (Progatzky *et al.* 2021). EGCs were shown to maintain barrier integrity (Clayburgh *et al.* 2004; Savidge *et al.* 2007; van Landeghem *et al.* 2009; Flamant *et al.* 2011), which is disrupted in several gastrointestinal diseases (Bush *et al.* 1998; Cornet *et al.* 2001). In the neonatal setting, barrier integrity is known to stabilize only during the course of the early postnatal phase, and commensal bacteria seem to induce this maturation process (Patel *et al.* 2012).

Murine EGCs start to develop prenatally (Sasselli *et al.* 2012), and their development continues into the postnatal phase. On embryonic day 11.5 (E11.5), glial progenitors can be detected in the foregut and midgut. On E14.5 and E16.5, EGCs start to express the glia cell-specific markers S100β and GFAP, respectively, and are found exclusively in the tunica muscularis (ME). In contrast to the prenatal development within the ME, the appearance of EGCs in the tunica mucosa (MC) occurs postnatally and continues during adulthood by continuous renewal from precursor cells located in the myenteric plexus (Kabouridis *et al.* 2015). This gradual appearance of EGCs in the tunica mucosa depends on the intestinal microbiota (Kabouridis *et al.* 2015), which simultaneously colonize the gut within the postnatal phase (Schaedler *et al.* 1965; Tanaka and Nakayama 2017). However, the underlying molecular mechanisms still remain unknown. Luminal pathogens as well as commensal bacteria can be recognized via toll-like receptors (TLRs) (Rakoff-Nahoum *et al.* 2004), all of which, except TLR3, signal via the myeloid differentiation primary response protein 88 (Myd88). In this study we showed that the postnatal development and replenishment of murine EGCs require intestinal microbiota and depend on the adaptor molecule Myd88. Detailed knowledge about these mechanisms is relevant to the proper control of barrier integrity, maturation, and intestinal inflammation, and is crucial for host defense in early life.

2. Methods

2.1 *Animals*

Wild-type (wt) C57BL/6 (Janvier, Saint-Berthevin Cedex, France) and Myd88$^{-/-}$ mice (Hou *et al.* 2008) (Jackson Laboratories, Charles River, Sulzfeld, Germany) were kept under specific pathogen-free (SPF) conditions in the animal housing facility of the University of Bonn (Germany). The mice were acclimatized and cohoused with littermates in groups of up to 5 animals for 1 week after transportation from the vendor. Animal experiments were performed once with the indicated numbers of animals. The mice had free access to a standard diet chow and water *ad libitum*. The experiments were performed with female mice at the age of 8–12 weeks, weighing 20 to 25 g.

Adult GF C57BL/6JZtm (GF wt) and B6.129P2-*MyD88*^{tm1Aki} (GF Myd88$^{-/-}$) male and female mice were obtained from the Institute for Laboratory Animal Science and Central Animal Facility (Hannover Medical School, Hannover, Germany). Breeding of GF mice was performed in plastic film isolators (Metall+Plastik GmbH, Radolfzell-Stahringen, Germany) located in a room with a controlled environment and 12 h light/dark cycles. GF mice received pelleted 50 kGy gamma-irradiated feed (V1124-927, ssniff Spezialdiäten GmbH, Germany) and autoclaved water *ad libitum*. Routine microbiological monitoring of GF mice did not reveal any evidence of infection with common murine pathogens or contaminants (Mähler *et al.* 2014; Nicklas *et al.* 2015). For the experiments, the mice were shipped in airtight Hannover gnotocages to preserve their gnotobiotic status (Basic *et al.* 2021). Breeding was accompanied by genotyping of the mice, confirming the Myd88 knockout.

Mice pups from our own breeding were sacrificed by cervical dislocation at the indicated time points between P0 and P15. All animal experiments were performed according to the German Protection of Animals Act (TierSchG) and were approved by the governmental authority of North-Rhine Westfalia (LANUV).

2.2 *Conventionalization*

Mice were shipped to our laboratory under GF conditions and were either sacrificed upon arrival or

Table 1. Primer sequences, primer assays, and TaqMan probes used for quantitative RT-PCR

	Primer sequences (Metabion)	
Gene	Forward	Reverse
GFAP	ACATCGAGATCGCCACCTAC	CCTTCTGACACGGATTTGGT
S100β	TGGTTGCCCTCATTGATGTCT	CCCATCCCCATCTTCGTCC
GDNF	CAGTGACTCCAATATGCCTGA	CCGCTTGTTTATCTGGTGAC
IL-1R1	GAAAAGTGCTAGAACATCCTTTGAG	GTACCAATGGAGGCCAGAAG
TLR2	GCTTCACTTCTCTGCTTTTCGT	CACATGACAGAGACTCCTGAGC
TLR4	GAAACTCAGCAAAGTCCCTGAT	TTGTTTCAATTTCACACCTGGA
TLR5	ATCTGTGAGACACCCCTTGC	TTGAGGATCCAGGGAATCTG
Gene	QuantiTect Primer Assay ID (Qiagen)	
IL-1α	QT00113505	
Gene	TaqMan Probes Assay ID (Applied Biosystems)	
IL-1β	Mm00434228	
GAPDH	Mm99999915_g1	

conventionalized over a period of 4 weeks by gavaging 100 µL of stools from, and cohousing with, SPF littermates, harboring a complex intestinal microbiota. Stools were gavaged on day 1 and 8 after arrival, and had been freshly prepared beforehand by thoroughly mixing 1 mL of PBS with two stool pellets from SPF littermates. Conventionalization was checked once a week by smearing stools on a nutrient agar plate (Becton Dickinson GmbH, Heidelberg, Germany). The mice had free access to an autoclaved standard diet chow and autoclaved water *ad libitum*. The experiments were performed with male and female mice at the age of 8–12 weeks, weighing 20 to 25 g.

2.3 *Immunohistochemical analysis*

For immunofluorescence staining of GFAP, cross sections of the ileum were fixed in acetone at −20°C for 10 min, permeabilized with 0.2% Triton X-100/PBS for 15 min, blocked with a blocking solution containing 3% BSA, 0.2% Triton, and 1% penicillin/streptomycin for 1 h, and incubated with primary GFAP IgG at a dilution of 1:600 at 4°C overnight. The secondary antibody was used at a 1:800 dilution and incubated for 1 h at room temperature. Nuclei were stained by Hoechst (Sigma), 3 µg/mL. Microscope images were taken using a TE2000 Nikon microscope at 200× magnification.

2.4 *Gene expression analysis*

Small bowels were harvested, and the ME was separated from the MC. Preparation of the small bowel of mice pups required precise preparation under a microscope. Total RNA was extracted from ME or MC using the RNeasy Mini Extraction Kit (Qiagen, Hilden, Germany) followed by treatment with deoxyribonuclease I (Ambion, Austin, USA) and reverse transcribed to complementary DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). The expression of messenger RNA (mRNA) was quantified in triplicate by a real-time reverse transcriptase polymerase chain reaction (RT-PCR). Gene expression data were normalized to the housekeeper gene *GAPDH*. For primer sequences, primer assays or TaqMan probes were used (table 1). Quantitative polymerase chain reaction was performed with SYBR Green PCR Master Mix (Applied Biosystems) or TaqMan Gene Expression Master Mix (Applied Biosystems).

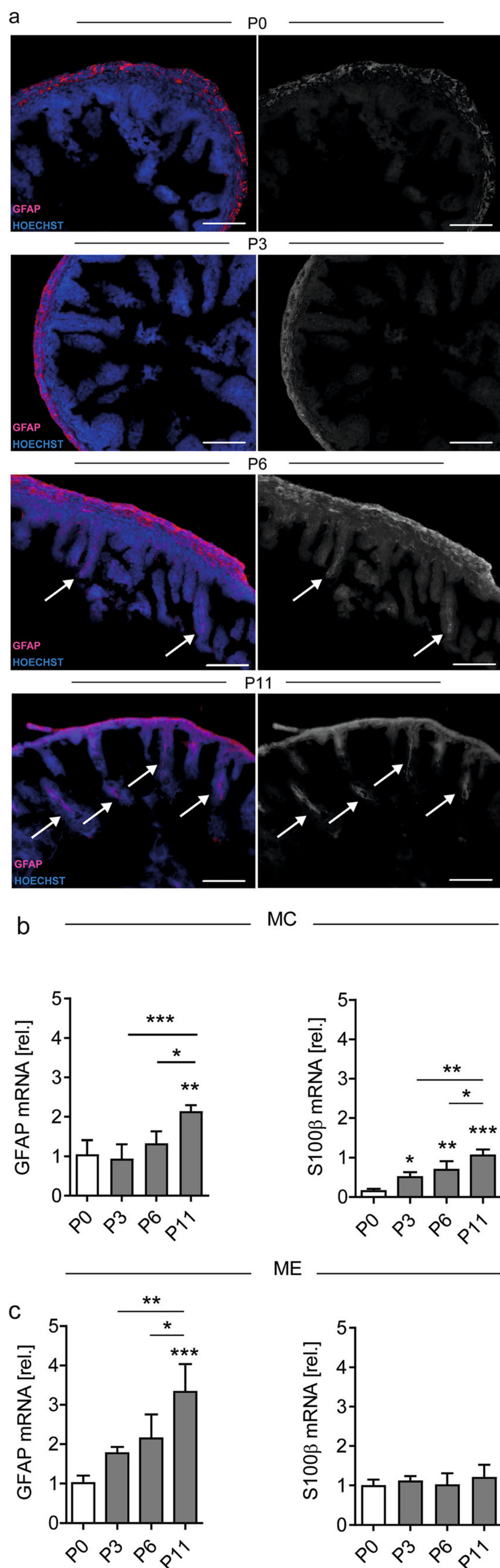
2.5 *Statistical analysis*

Statistical analysis was performed using GraphPad Prism Version 8.4.3 for Windows software (GraphPad Software, San Diego, CA). Data were analyzed by two-way ANOVA or ordinary one-way ANOVA with Tukey's *post hoc* test. The data are shown as means±SD.

3. Results

3.1 *Quantification of the postnatal development of EGCs*

Kabouridis *et al.* (2015) showed that murine EGCs in the mucosal villus-crypt region developed postnatally between the day of birth (P0) and postnatal day 10



◄**Figure 1.** The postnatal development of EGCs. C57BL/6 mice pups were sacrificed at the indicated time points ranging from immediately after birth (postnatal day 0; P0) up to postnatal day 11 (P11). The small bowel was harvested and the tunica mucosa (MC) was separated from the tunica muscularis (ME) by precise preparation under a microscope. (**a**) Immunohistochemistry of cross sections of the ileum was performed, and the cross sections were stained for GFAP (red) and Hoechst (blue). The arrows indicate the EGCs. Microscope images were taken using a TE2000 Nikon microscope at 200× magnification (scale bar 100 μm). *GFAP* and *S100β* gene expression levels in the MC (**b**) or the ME (**c**), respectively, in the postnatal phase between P0 and P11, were analyzed. Samples were normalized to the ME on the date of birth (n=6). Samples were analyzed by one-way analysis of variance with Tukey's *post hoc* test, and the results are shown as means±SD. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ versus the corresponding control group or between indicated groups.

(P10). First, in order to obtain more precise information about the time of EGC appearance in the early postnatal phase, we performed immunohistochemical staining against GFAP of small bowel tissue samples at day P0 and at days P3, P6, and P11 (figure 1a), and we were able to confirm the gradual development of EGC appearance. Furthermore, we believed that transcriptional measurements within pooled tissue samples taken along the small bowel may provide more precise quantitative information than immunohistochemical cross sections. Therefore, we isolated mucosal-free ME samples and ME-free mucosal samples from the small bowels of mice on P0 and P3, P6, and P11 by precise mechanical separation under a preparation microscope. The tissues were minced and mixed, and analyzed for *GFAP* and *S100β* gene expression (figure 1b–c). In the MC, we observed a gradual increase in both GFAP and S100β mRNA expression in the postnatal phase between P0 and P11 (figure 1b). S100β mRNA levels already increased on P3 ($p \leq 0.05$) and gradually increased thereafter (P6: $p \leq 0.01$; P11: $p \leq 0.001$), while GFAP was only significantly increased on P11 ($p \leq 0.01$). In the ME, we observed a continuous increase of GFAP at days P3, P6, and P11 ($p \leq 0.001$), while S100β was not affected (figure 1c). These findings are in line with our immunohistochemical staining and the studies of Kabouridis *et al.* (2015) and show that EGC markers gradually increase in the MC in the early postnatal phase. The increase in postnatal GFAP expression in the ME indicates that beyond the mucosal changes, EGC still undergo maturational changes or increase in numbers also in the ME, a finding not observed before.

3.2 *Postnatal development of EGCs depends on Myd88*

We next aimed to confirm if the presence of EGC depends on the detection of bacteria via TLRs. As most TLRs, except TLR3, signal via the adaptor molecule Myd88, we compared postnatal gene expression of *GFAP* and *S100β* on P0 and P15 between wt and Myd88$^{-/-}$ mice (figure 2a). In this experiment we again observed a strong upregulation of GFAP and S100β between P0 and P15 in both the MC and ME (GFAP MC: $p \le 0.001$; GFAP ME: $p \le 0.001$; S100β MC: $p \le 0.001$; S100β ME: $p \le 0.001$) (figure 2b–c). However, the upregulation on P15 was strongly diminished within the MC of Myd88$^{-/-}$ mice (GFAP MC: $p \le 0.01$; S100β MC: $p \le 0.001$). In contrast, GFAP

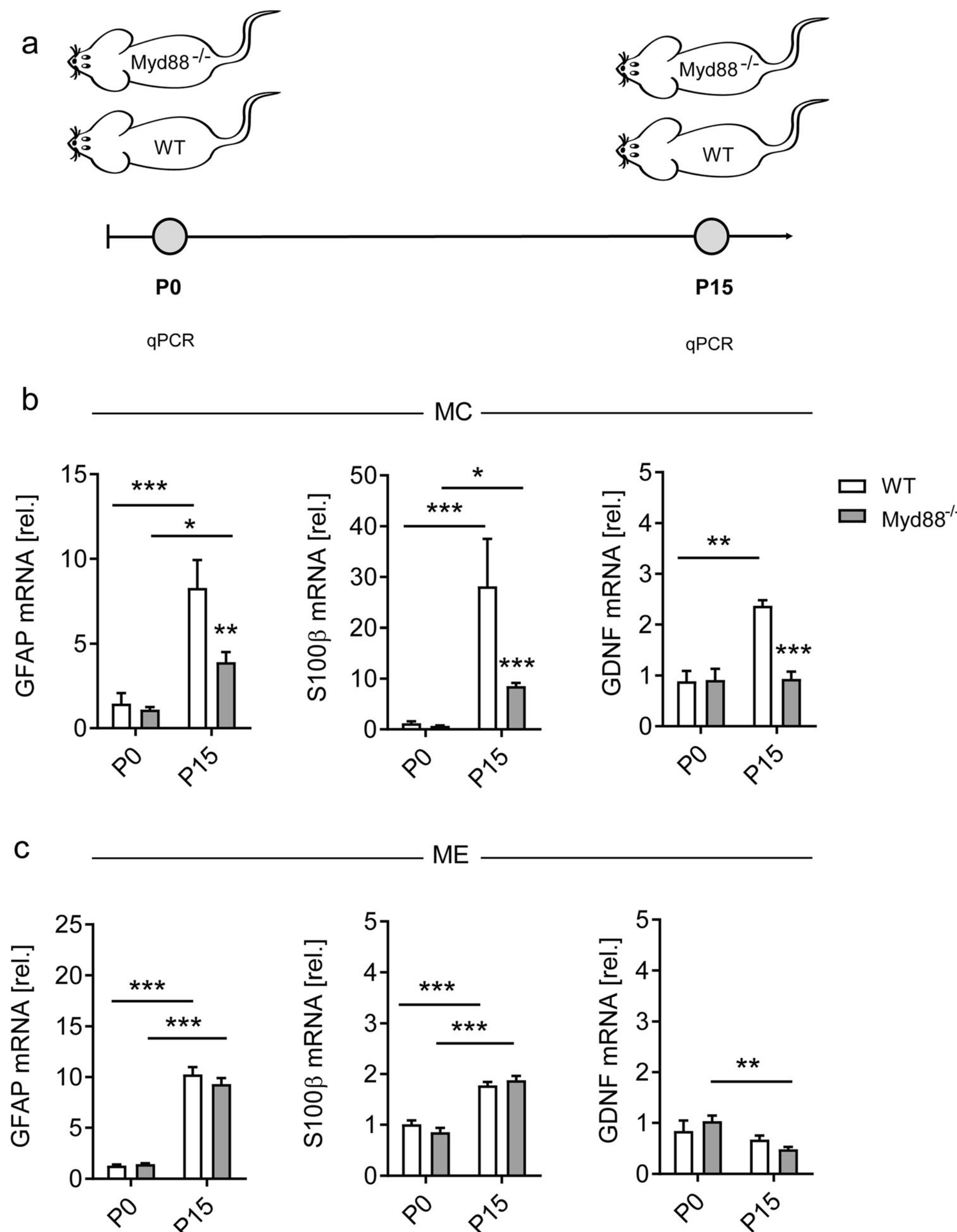


Figure 2. The postnatal development of EGCs depends on Myd88. C57BL/6 wild-type (wt) or Myd88$^{-/-}$ mice pubs were sacrificed at the indicated time points, either right after birth (postnatal day 0; P0) or on postnatal day 15 (P15) (**a**). The small bowel was harvested, and the tunica mucosa (MC) was separated from the tunica muscularis (ME) by precise preparation under a microscope. *GFAP*, *S100β*, and *GDNF* gene expression levels in the MC (**b**) or the ME (**c**), respectively (n=4–11), were analyzed. Samples were analyzed by two-way analysis of variance with Tukey's *post hoc* test, and the results are shown as means±SD. *$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$ versus the corresponding control group or between indicated groups.

and S100β mRNA levels within the ME did not differ between wt and Myd88 mice on P15. Further analysis revealed that expression of glial cell-derived neurotrophic factor during the early postnatal phase is also Myd88-dependent (figure 2b–c). Taken together, these data indicate that the microbiota-dependent development of EGC in the MC depends on Myd88.

3.3 *Replenishment of EGCs in adult mice depends on Myd88*

We next aimed to confirm if the presence of EGC indeed depends on the microbiome and on Myd88 signaling by using germ-free mice. Adult GF wt or GF Myd88$^{-/-}$ mice were housed under GF conditions and were either sacrificed upon arrival or conventionalized over a period of 4 weeks (figure 3a). In the MC of GF mice, GFAP and S100β mRNA levels did not differ between GF wt and GF Myd88$^{-/-}$ mice (figure 3b–c). Conventionalized (CONV-D) wt mice showed increased GFAP ($p \leq 0.01$) and S100β ($p \leq 0.001$) mucosal mRNA levels compared with GF wt mice (figure 3b). In the ME, we also observed an upregulation of GFAP and S100β in CONV-D wt mice compared with GF wt mice (GFAP: $p \leq 0.01$; S100β: $p \leq 0.001$) (figure 3b). Notably, the CONV-D-induced induction of *GFAP* and *S100β* gene expression was much stronger in the MC than in the ME, indicating that the presence of EGC indeed depends on microbial-triggered Myd88 signaling, which more strongly affects mucosal EGC.

3.4 *Innate immune receptors during postnatal Myd88-dependent EGC development*

In a final experiment, we aimed to analyze the transcriptional changes of the receptors signaling upstream of Myd88. Therefore, we measured the mRNA levels of the three representative TLR receptors, TLR2, TLR4, and TLR5, in wt and Myd88$^{-/-}$ mice at P0 and P15. These receptors detect bacterial surface molecules and signal exclusively via Myd88. Furthermore, we measured the transcript levels of the IL-1α and IL-1β receptor IL-1R1, known to signal via Myd88 and to be expressed in the MC (figure 4). IL-1R1 mRNA was upregulated in wt animals ($p \leq 0.01$) between P0 and P15, and there was a strong downregulation on P15 between wt and Myd88$^{-/-}$ animals ($p \leq 0.001$). Interestingly, both IL-1R1 ligands IL-1α ($p \leq 0.05$) and IL-1β ($p \leq 0.001$) and mRNA were also downregulated on P15 between wt and Myd88$^{-/-}$ animals (supplementary figure 1). At the receptor level, TLR5 mRNA, but not TLR2 and TLR4, were upregulated in wt animals ($p \leq 0.01$) at P15 compared with P0. In contrast, TLR5 was strongly downregulated in Myd88$^{-/-}$ ($p \leq 0.001$) compared with wt mice at P15. TLR2 and TLR4 were, surprisingly, increased in Myd88$^{-/-}$ at P15 compared with P0 but wt mice were not. Together, these data indicate that IL-1R1 and TLR5, rather than TLR2 and TLR4, might play a role in the Myd88-mediated microbiota-dependent postnatal development of EGCs.

Our study adds to previous findings and confirms a role of the microbiota in postnatal mucosal EGC development. Furthermore, our data indicate that mucosal EGCs develop linearly within the early postnatal period and depend on Myd88 signaling. Although further work is needed to identify the signaling pathways upstream of Myd88, the potential targets could be TLR5 and IL-1R1.

4. Discussion

Immunohistochemical studies have demonstrated that mucosal EGCs develop postnatally and that the postnatal development of EGCs as well as the replenishment during adulthood depend on intestinal microbiota (Kabouridis *et al.* 2015). In this study, we delivered further supportive evidence of this postnatal development and its dependency on the microbiota, and provided evidence on the role of Myd88 signaling.

The underlying molecular mechanisms of the interaction between EGCs and the microbiome remain unclear. Several potential mechanisms might contribute, of which one is the detection of bacterial molecules via TLRs. All TLR members signal via the downstream adapter molecule Myd88 except TLR3, which signals exclusively via the TIR-domain-containing adapter-inducing interferon-β (TRIF). Herein we demonstrated that Myd88$^{-/-}$ mice showed a reduced postnatal induction of EGC marker genes *GFAP* and *S100β*. Importantly, Myd88 deficiency almost completely stopped the microbiota-triggered induction of both genes during conventionalization of GF mice with a complex enteric microbiota from SPF housed mice. This is in line with previous work (Kabouridis *et al.* 2015) and emphasizes the gradual microbiota-dependent migratory process of mEGCs from the myenteric plexus to the top of the villi and further supports the role of commensal bacteria and their interaction with the mucosal layer as the first line of host defense.

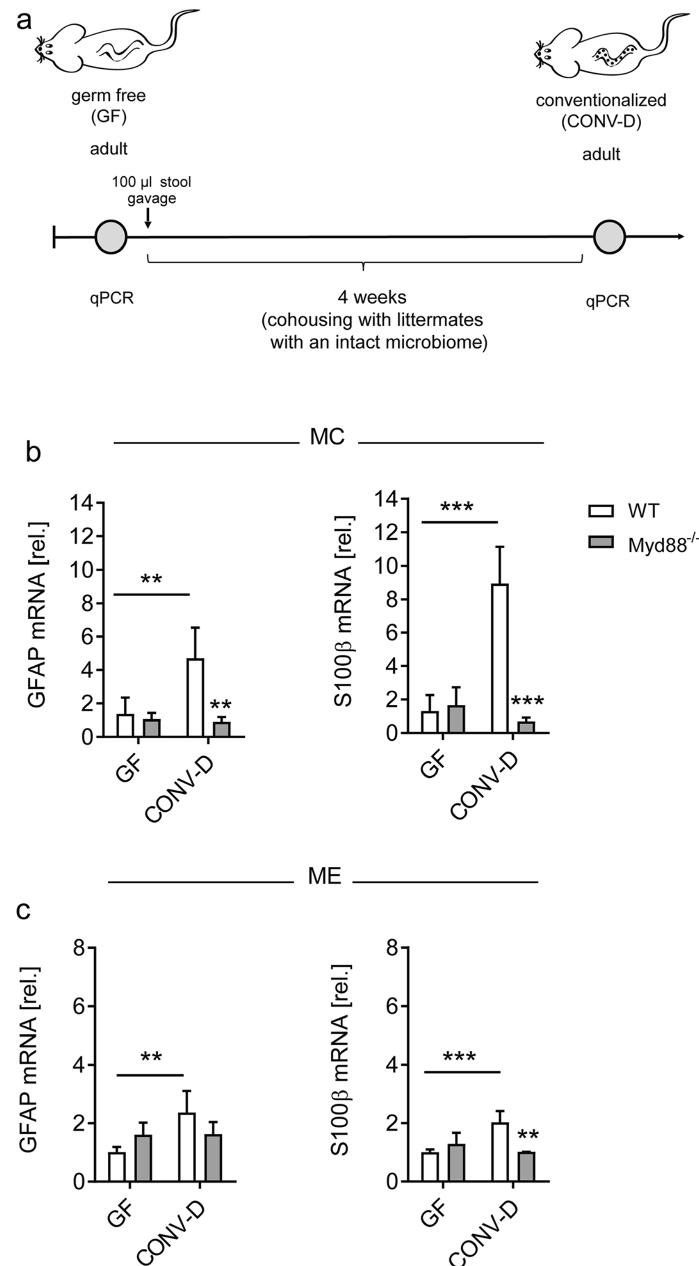


Figure 3. The replenishment of EGCs in adult mice depends on Myd88. Adult C57BL/6 wild-type (wt) or Myd88$^{-/-}$ were housed under germ-free (GF) conditions or conventionalized (CONV-D) by gavaging stools from, and cohousing with, specific pathogen-free littermates, harboring complex enteric microbiota (**a**). The small bowel was harvested, and *GFAP* and *S100β* gene expression levels in the MC (**b**) or the ME (**c**), respectively (n=3), were analyzed. Samples were analyzed by two-way analysis of variance with Tukey's *post hoc* test, and the results are shown as means±SD. **$p \leq 0.01$, ***$p \leq 0.001$ versus the corresponding control group or between indicated groups.

Myd88 is a downstream adapter molecule of several innate immune receptors, including TLRs, IL-1R1, and the IL18 receptor. TLRs recognize pathogen-associated molecular patterns that originate from luminal microbiota. Numerous cell types express TLRs, for instance, enterocytes (Frosali *et al.* 2015), but also EGCs (Barajon *et al.* 2009). Therefore, it is possible that bacteria directly signal via EGCs, and EGCs have already been shown to detect microbial signals via Myd88 and thereby regulate barrier integrity by contributing to the secretion of the neurotrophic factor IL-22 via innate lymphoid cells (Ibiza *et al.* 2016). However, direct interaction between bacteria and EGCs would require an immature intestinal epithelium. Indeed, the intestinal epithelial barrier undergoes a maturation process within the first 2–3 postnatal weeks

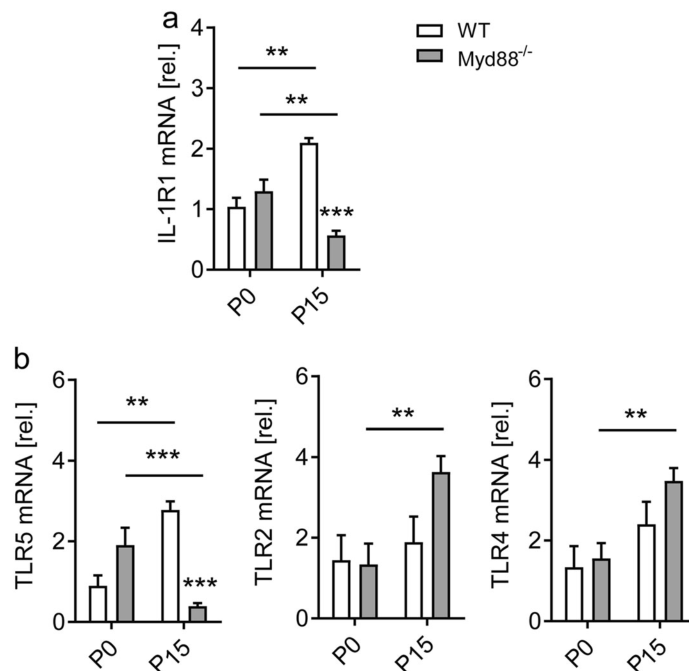


Figure 4. Innate immune receptors during postnatal Myd88-dependent EGC development. C57BL/6 wild-type (wt) or Myd88$^{-/-}$ mice pups were sacrificed at the indicated time points, either immediately after birth (postnatal day 0; P0) or on postnatal day 15 (P15). The small bowel was harvested, and after having separated the tunica muscularis from the tunica mucosa, **(a)** *IL-1R1* and **(b)** *TLR5*, *TLR2*, and *TLR4* expression levels were analyzed (*n*=4–11) in the latter. Samples were analyzed by two-way analysis of variance with Tukey's *post hoc* test, and the results are shown as means±SD. $^{**}p\leq0.01$, $^{***}p\leq0.001$ versus the corresponding control group or between indicated groups.

and is accompanied by an increase of claudin expression, and this mechanism is known to be Myd88-dependent (Patel *et al.* 2012).

An immature barrier is also believed to be the origin of sepsis and/or necrotising enterocolitis in pre-term children (Niño *et al.* 2016; Weström *et al.* 2020) and barrier disturbances are seen in various chronic diseases during adulthood such as Crohn's disease, ulcerative colitis, celiac disease, and irritable bowel syndrome (Fukui 2016), and also in acute disease like post-operative ileus (Stoffels *et al.* 2014; Stein *et al.* 2018; Hupa *et al.* 2019; Schneider *et al.* 2021). In these inflammatory conditions, barrier breakdown is accompanied by glial cell activation and proliferation, showing a direct correlation between the disrupted barrier, the presence of bacteria, and the actions on EGCs.

Given that the microbiome affects mucosal EGC development in an Myd88-dependent manner, two main questions remain to be answered: First, does Myd88 signaling occur directly in EGC or in other intestinal cell types, and then indirectly affects EGC development? Second, do upstream-acting receptors contribute to the Myd88-dependent effects? Support for indirect action comes from the observation that enterocytes express TLRs and are involved in the interaction between the microbiota and EGCs. TLR expression along the intestinal epithelium is well described. Our data indicate that TLR5 might also play a role in the early postnatal phase in regard to the microbiota-dependent development of EGCs. Interestingly, a recent publication showed an increased expression of TLR5 on intestinal epithelial cells over the first 2 weeks after birth (Fulde *et al.* 2018); expression levels returned to baseline at 3 weeks after birth, and this postnatal expression of TLR5 was shown to influence the composition of adult microbiota. However, TLR5 is also expressed in EGCs, and in direct comparison with TLRs 1, 2, 3, 4, 7, and 9, it is the highest expressed TLR (Turco *et al.* 2014). Therefore, a direct role of TLR5/Myd88 signaling in EGCs can also be hypothesized. However, the question whether TLR5 plays a key role in mucosal EGC development needs to be determined in future studies.

Apart from TLR pathways, IL-1R1 and IL-18R also signal via Myd88. Since we had demonstrated in a previous study that IL-1R1-mediated pathways were involved in EGC activation during postsurgical intestinal inflammation (Stoffels *et al.* 2014), we analyzed *IL-1R1*, *IL-1α*, and *IL-1β* gene expression. As gene expressions of all analyzed molecules increased between

P0 in wt but not in Myd88$^{-/-}$, maturation of the IL-1R1 pathways seems also to be under control of Myd88 pathways. These pathways might be either controlled by a feedback loop or by microbiota signaling TLRs.

Finally, our data indicate that the microbiota not only affect EGC development but also induce functional changes that might affect barrier function. EGC expression of GDNF in the intestinal mucosa during the postnatal phase was diminished in Myd88$^{-/-}$ mice. As GDNF stabilizes the epithelial barrier (Meir *et al.* 2015) and it was recently shown that EGCs secrete GDNF and confirmed its barrier supportive role in immature but not mature intestinal epithelial cells (Meir *et al.* 2019), GDNF might be considered a signaling link between enterocytes and EGCs during microbiota-dependent development of EGC in the early postnatal phase.

5. Conclusion

Our study uncovered Myd88-mediated signaling as a crucial pathway for the early postnatal development as well as replenishment of EGCs within the mucosa. Although further studies are needed, the specific upstream mechanism may involve TLR5 recognition of bacteria. These results provide solid groundwork for further projects that involve the postnatal maturation of the microbiota especially in terms of an abnormal colonisation during the postnatal phase and a possible impact on the immune system. A relevance of these pathways might be of particular interested for disease occurring during early life, i.e. sepsis and necrotizing enterocolitis, which are a major cause of preterm infant morbidity and mortality.

Author contributions

JE, HN, BS, and ML performed the research; JE and SW designed the research; JE analyzed the data; JE prepared the manuscript; JE, JCK, and SW critically read the manuscript; MB contributed to gnotobiotic experiments. All authors carefully read the manuscript and approved the final version of the manuscript.

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Declarations

Conflict of interest SW and JCK receive royalties from Wolter Kluwer for contributions to the postoperative ileus section of the UpToDate library. All other authors declare no competing interests.

Ethics approval All animal experiments were performed according to the German Protection of Animals Act (TierSchG) and were approved by the governmental authority of North-Rhine Westfalia (LANUV).

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