Bacterial Stimulation of the TLR-MyD88 Pathway Modulates the Homeostatic Expression of Ileal Paneth Cell α -Defensins

A. Menendez^a B.P. Willing^a M. Montero^b M. Wlodarska^a C.C. So^a
G. Bhinder^b B.A. Vallance^b B.B. Finlay^a

^aMichael Smith Laboratories, The University of British Columbia, and ^bDivision of Gastroenterology, BC's Children's Hospital and Child and Family Research Institute, Vancouver, B.C., Canada

Key Words

Defensins • Toll-like receptors • Intestinal microbiota • Paneth cells • Antimicrobial peptides • Cryptdins

Abstract

Paneth cell α -defensins are antimicrobial peptides involved in the control of the intestinal microbiota and immunological homeostasis. In mice, they are encoded by multiple, highly homologous genes (*Defa*). The transcriptional activity of ileal *Defa* genes was studied in response to pharmacological and genetic perturbations of the intestinal environment of C57BL/6 mice. *Defa* gene transcription was sensitive to oral antibiotic administration suggesting that commensal microbes regulate *Defa* expression. Ileal microbiota analysis showed that decreased transcription of *Defa* genes correlated with depletion of *Lactobacillus*. *Defa* expression was partially restored in vivo by lactobacillus administration to antibiotic-treated mice. *Defa* transcripts were less abundant in ex vivo, microbiota-free intestinal explants but recovered after explant exposure to UV-killed bacteria, Toll-like receptor (TLR)-2 or TLR4 agonists. Genetic deficiency of several TLRs or MyD88 led to dramatic drops in *Defa* transcription in vivo. These results show that Paneth cell *Defa* genes are regulated by commensal bacteria through TLR-MyD88 signaling and

provide a further understanding of the dysregulation of intestinal homeostasis that occurs as a result of imbalances in the populations of commensal bacteria.

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Introduction

Together with other antimicrobials, enteric α -defensins are secreted into the lumen of the mammalian small intestine [1]. They are thought to defend against pathogenic insults as suggested by in vivo studies, where gain of a human defensin 5 (HD5) in a transgenic mouse model improved protection from enteric *Escherichia coli* and *Salmonella* infections whereas deficiency of Mmp7 (the enzyme responsible for processing cryptdin precursors to their mature form) correlated with increased susceptibility to those infections [2, 3]. In addition, recent evidence indicates that enteric α -defensins participate in the control of intestinal homeostasis by modifying the commensal microbial composition and in turn, influencing the immunological landscape of the intestinal mucosa [4].

A.M. and B.P.W. contributed equally to this work.

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Dr. B. Brett Finlay
Michael Smith Laboratories, The University of British Columbia
301-2185 East Mall
Vancouver, BC V6T 1Z4 (Canada)
Tel. +1 604 822 2210, E-Mail bfinlay@interchange.ubc.ca

In the healthy adult gut, enteric α -defensins are produced exclusively by Paneth cells in the crypts of the small intestine [1]. In mice, enteric α -defensins, the cryptdins, are encoded by a group of highly homologous genes (*Defa*), located on the defensin gene cluster on chromosome 8. The repertoire of C57BL/6 mice includes more than 20 potentially active *Defa* genes and numerous pseudogenes [5, 6]. In addition, the C57BL/6 *Defa* genotype is different from that of other laboratory mouse strains; more than 10 transcriptionally active genes identified in other mouse lines have not been found in C57BL/6 and several genes are unique to this mouse strain [5, 6].

Expression analyses of *Defa* genes have been of limited use in understanding α -defensin biology in mouse models of enteric diseases. A major problem is the high gene sequence homology, which makes PCR primer design challenging and clouds the validity of microarray data. This fact, combined with the incomplete knowledge of the strain diversity in the *Defa* gene pool, has led to the generation of a significant amount of data that require reinterpretation in light of the recent C57BL/6 *Defa* genotyping [5, 6]. Other than an involvement of the β -catenin pathway [7, 8], very little is known about the control of intestinal α -defensin expression. Previous studies on the role of bacteria in cryptdin synthesis have produced conflicting results [9–12]. Here, we studied the transcriptional activity and regulation of ileal *Defa* genes in adult C57BL/6 mice by qPCR. Two major, unique aspects of this work are the generation and validation of primers specific for the *Defa* gene repertoire of this mouse strain, and the analysis of the majority of the known genes of C57BL/6 mice. We found significant variation in the steady-state *Defa* gene expression, which was responsive to antibiotic-induced perturbations of the intestinal environment. Most interestingly, *Defa* transcriptional activity correlated to specific changes in the composition of the intestinal microbiota and was affected by genetic deficiencies in the TLR-MyD88 signaling pathway. Collectively, these findings show that Paneth cell *Defa* gene expression is controlled by the intestinal commensal microbiota through the TLR-MyD88 pathway.

Materials and Methods

Animals

Eight- to twelve-week-old, wild-type C57BL/6 and congenic sex- and age-matched *MyD88*^{-/-}, *Tlr2*^{-/-}, *Tlr4*^{-/-}, *Tlr2*^{-/-}/*Tlr4*^{-/-}, *Tlr9*^{-/-} or *Nod2*^{-/-} mice were raised in the same room in the animal facility of the Child and Family Research Institute. All knockout mouse strains were backcrossed onto a C57BL/6 ge-

netic background between 6 and 10 times and housed in the same room as wild-type C57BL/6 for a minimum of 3 years. Animal protocols for this study were approved by the University of British Columbia's Animal Care Committee. Antibiotics were administered in the drinking water for 4 days at 450 mg/l streptomycin or 750 mg/l metronidazole. Mice were sacrificed and tissue samples of the terminal ileum were taken approximately 1 inch from the ileocecal junction for RNA purification, analysis of microbiota and microscopy.

Ileal Explants

Ileal explants were prepared essentially as described [13]. Briefly, the distal region of the ileum was resected and flushed with sterile PBS. The tissue was opened longitudinally and washed repeatedly with sterile PBS to eliminate the remaining intestinal content and immediately placed in DMEM/10% FCS/100 μ g/ml streptomycin/100 U/ml penicillin. The tissues were divided into 2-mm-long sections in sterile conditions and incubated at 37°C in humidified 5% CO₂ atmosphere. After 1 h, the media were replaced and the samples were incubated for an additional 2 h in the same medium. Thereafter, the explants were transferred to fresh media containing *E. coli* lipopolysaccharide (LPS) (Sigma), Pam2CSK4, Pam3CSK4 (InvivoGene, <http://www.invivogen.com/>, cat. No. tlrl-pm2s and tlrl-pms) or UV-killed *Lactobacillus johnsonii*, *Lactobacillus reuteri* or *E. coli* (all previously isolated from the intestinal commensal population of experimental animals) and incubated for 16 h at 37°C in a humidified 5% CO₂ atmosphere.

Expression Analysis

Sections and ileal explants were collected in RNA Later (Qiagen) and total RNA was isolated using the RNeasy kit (Qiagen). cDNA was prepared from 1 μ g of RNA using the Quantitech Reverse Transcription kit (Qiagen). Quantitative PCRs (qPCRs) were done on an Applied Biosystems 7500 using the DyNamo SYBR Green PCR Kit from New England Biolabs. The qPCR primers and conditions for the *Defa* genes are shown in table 1, the primers for the housekeeping gene 36B4 [ribosomal protein, large, P0 (*Rplp0*), NM_007475.5] were TCTGGAGGGTGTCCGCAAC (forward) and CTTGACCTTTTCAGTAAGTGG (reverse); the primers for matrix metalloproteinase 7 (*Mmp7*) were CACTCTAGCTCATGCCTTCGC (forward) and GGTGGCAGCAAACAGGAAGTTC (reverse). Efficiencies for the 36B4 PCRs were 1.9055 at 58°C, 1.9333 at 60°C and 1.8544 at 62°C. qPCR specificity was verified by cloning of PCR products using TOPO TA 4.0 vector and *E. coli* TOP 10 chemically competent cells (Invitrogen, Carlsbad, Calif., USA) and sequencing of 10–20 independent clones. Relative expression data were generated using the ddCt method, corrected for primer efficiencies according to Pfaffl et al. [14]. Relative expressions from intestinal explants were normalized against the relative expression of villin (expressed exclusively in the epithelial cell layer) as an indication of epithelial survival. The data were normalized for each sample using the formula $\text{Rel Exp}_{(\text{gene of interest})} / \text{Rel Exp}_{(\text{villin})}$.

Microbial Analysis

Total bacteria were enumerated by SYBR green DNA staining. Ileal contents were homogenized in 1 ml of PBS and then fixed in 3.7% formalin. Fixed samples were diluted and applied to 0.2 μ m Anodisc 25 filters (Whatman International Ltd.), then stained

Table 1. The gene composition of each amplification group as defined in this work

Genes included in group	Group name	Remarks
<i>Defa3</i> and OTTMUSG00000019892	Defa3	Gene duplication, identical genes and protein products
<i>Defa5</i> -related genes*	Defa5	Four genes differing in one or two nucleotides in the pre- or pro- regions but producing identical mature proteins, identical PCR products
<i>Defa20</i> ** and OTTMUSG00000019860	Defa20	Gene duplication, identical genes and protein products
<i>Defa21</i> and <i>Defa22</i>	Defa21/22	Two different genes, amplified together, different PCR products
<i>Defa23</i> and OTTMUSG00000019762	Defa23	Gene duplication, identical genes and protein products
<i>Defa24</i> and OTTMUSG00000019742	Defa24	Two different genes, amplified together, identical PCR products
<i>Defa25</i> and OTTMUSG00000019857	Defa25	TATA box-less promoters
<i>Defa26</i> and OTTMUSG00000019784	Defa26	Two different genes, identical mature proteins, identical PCR products
<i>Defa4</i>	Defa4	Gene not present in C57BL/6 mice

With the exception of *Defa4*, gene ID numbers are from the Vertebrate Genome Annotation (VEGA) database (<http://vega.sanger.ac.uk/index.html>) following Amid et al. [5].

The VEGA database ID numbers are *Defa3* (OTTMUSG00000019782), *Defa20* (OTTMUSG00000019856), *Defa21* (OTTMUSG00000019489), *Defa22* (OTTMUSG00000019763), *Defa23* (OTTMUSG00000019488), *Defa24* (OTTMUSG-00000019980), *Defa25* (OTTMUSG00000019700) and *Defa26* (OTTMUSG00000019889). *Defa4* is NCBI NM_010039.

* *Defa5*-related genes include OTTMUSG00000018258, OTTMUSG00000019785, OTTMUSG00000018259 and OTTMUSG-00000019924.

** The names of the *Defa* genes as used here correspond to and substitute former designations such as Cryp (e.g. *Defa3* is Cr3 or Cryp3, or Defcr3) and so on, with the exception of *Defa20*, which has been also called Cr4-C57B6 or Crp4 (B6a) [10, 34] and *Defa21/22*, which have been called Crp4 (B6b) [34].

with SYBR green (Invitrogen), diluted 1:400 in PBS, for 15 min. Filters were mounted on glass slides with 30 μ l of antifade solution. Images were collected on an Olympus IX81 microscope from 3 randomly chosen fields for each sample and cell counts were averaged. Microbial composition was assessed in tissue samples collected from the ileum by terminal restriction fragment length polymorphism analysis (T-RFLP) and cloning and sequencing. Total DNA was isolated from ileal tissue using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions, with the addition of a 60-second bead-beating step at level 5 on a FastPrep-24 (MP Biomedicals, Solon, Ohio, USA). Bacterial 16S rRNA genes were PCR amplified using broad-range primers 8F [15] 5'-end-labeled with 6-FAM, and 926r [16] and subjected to digestion with *Hae*III. Fragment length determination of 6-FAM-labeled products was performed on an ABI 3730 capillary sequencer (Applied Biosystems) and electrophoregrams were processed using GeneMarker (State College, Pa., USA). For the phylogenetic identification of terminal restriction fragments of interest, pooled PCR-amplified products from each treatment group were cloned into the pCR[®] 4 TOPO vector and transformed into *E. coli* TOP10 chemically competent cells (Invitrogen) and 64 clones per treatment were sequenced. Sequences were classified using the naïve Bayesian rRNA classifier in Ribosomal Database Project [17]. Selective culture of lactic acid bacteria was performed on Rogosa agar under anaerobic conditions.

Microscopy

Tissue sections were prepared and stained with hematoxylin-eosin (HE) by WaxIt Inc. (Vancouver, B.C., Canada). HE images were taken in an Olympus VS110 using the VS-ASW software. Immunofluorescent Ab localization was performed on paraffin-

embedded tissue sections (5 μ m) after paraffin removal with xylene and rehydration by sequential immersion in 100, 95, and 75% ethanol and water. Antigen retrieval was performed in 10 mM citric acid pH 6.0 at 100°C for 30 min. Immunostaining was performed using a rabbit anti-human lysozyme (Dako A0099, 1:600). Fluorescence was visualized using a Zeiss AxioImager 2 and processed with the AxioImager software.

Data Analysis

Data were analyzed with GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, Calif., USA). Statistical comparisons were done with Mann-Whitney A tests with 95% confidence intervals. Sequence alignment and the phylogenetic tree were done using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

Results

Differential Expression of *Defa* Genes in the Terminal Ileum of C57BL/6 Mice

A careful analysis of the published work on cryptdin gene expression reveals that (1) frequently, primer sets designed to amplify one particular *Defa* gene are potentially able to cross-amplify others; this problem is often aggravated by failure to report the PCR conditions and lack of product sequence verification, (2) in vivo expression data for genes not yet found in C57BL/6 mice are

Table 2. The primers set for the C57BL/6 *Defa* genes qPCR

Gene group	Primers sequences	Product length, bp	qPCR annealing, °C	qPCR efficiency
<i>Defa3</i>	F: GAGAGATCTGGTATGCTATTG R: AGCAGAGTGTGTACATTAAATG	98	62	1.956
<i>Defa5</i>	F: TCAAAAAAGCTGATATGCTATTG R: AGCTACAGCAGAATACGAAAAG	106	58	1.982
<i>Defa20</i>	F: GAGAGATCTGGTATGCTATTG R: AGAACAAAAGTCGTCCTGAG	86	62	2.125
<i>Defa21/22</i>	F: GAGAGATCTGATCTGCCTTTG R: CAGCGCAAAAAAGGTCCTGC	87	64	2.079
<i>Defa23</i>	F: GAGAGATCTGGTATGCTATTG R: AGCAGAGCGTGTATATTAAATG	98	62	1.376
<i>Defa24</i>	F: GAGAGATCTGGTATGCTATTG R: AGCAGAGCATGTACAATAAATG	98	62	1.556
<i>Defa25</i>	F: GTGAAGATCTGATATGCTATTG R: ACCAGAGCATGTACATTAAATG	99	58	1.980
<i>Defa26</i>	F: ATTGTAGAAAAAGAGGCTGTAC R: AGCAGAGTGTGTACATTAAATG	81	62	1.802
<i>Defa4*</i>	F: TGAGAGGTTTGTATGCTATTG R: AGCAGTACAAAAATCGTATTCC	90	60	1.992

Reaction cycling conditions were: one enzyme activation step at 95°C for 10 min followed by 40 cycles of 95°C, 30 s, annealing for 30 s at the specified temperature and extension at 72°C for another 30 s. Fluorescence readings were taken during the extension time.

* Not present in C57BL/6 mice.

often provided in studies using this mouse strain (e.g. *Defa1*, *Defa4* and *Defa10*), probably as a result of the primer design problem mentioned above, (3) in most studies, only one or two *Defa* genes are analyzed; however, generalized conclusions about *Defa* expression are made. To avoid these inconsistencies, we designed a novel set of primers for the detection of relative amounts of multiple *Defa* transcripts by qPCR. The high homology of the *Defa* genes prevented the generation of individual primer pairs; instead, the genes were clustered in subgroups based on coding sequence homology and phylogenetic distance of their promoters (online suppl. fig. 1; for all online supplementary material see www.karger.com/doi/10.1159/000341630). Six subgroups were formed including two genes each, coding for identical or quasi-identical mature protein products (table 2). An additional subgroup was created with the four *Defa5*-related genes and another one with *Defa4*, a gene cloned from 129SvJ mice, which has not yet been found in C57BL/6

mice. PCR conditions for specific amplification (table 1) were carefully developed, and were validated by sequencing of cloned PCR products generated with each primer mix. Specific amplification of expected targets was obtained and verified for all primer mixes. Expression of *Defa3*, *Defa5*, *Defa20*, *Defa21*, *Defa22*, *Defa23*, *Defa24* and *Defa26* was confirmed in C57BL/6 mice (fig. 1) whereas transcripts for *Defa25* were undetectable, consistent with the absence of a TATA box in its promoter [5]. *Defa4* transcripts were not detected in C57BL/6 but were readily detected in BalbC and 129SvJ mice (online suppl. fig. 2). The results in figure 1 show that the homeostatic transcript levels of independent *Defa* genes are dissimilar in vivo.

Oral Antibiotic Treatments Modify the *Defa* Expression Pattern

Clinical use of antibiotics is frequently associated with the expansion of opportunistic pathogens, such as vanco-

Fig. 1. Paneth cell *Defa* genes are not equally expressed. Ileal transcript levels of *Defa* genes in C57BL/6 mice, relative to the transcript levels of the *Rplp0* gene (defined as 1, dashed line). Open bars represent the overall relative expression for each subgroup. Solid bars represent relative expression of each individual gene within a subgroup (this was arbitrarily calculated by dividing the

overall relative expression of the subgroup by the number of genes within the subgroup; this correction assumes equal expression between the members of each independent subgroup; 2 genes for *Defa3*, 20, 21/22, 23, 24 and 26, and 4 genes within the *Defa5* group). Data shown are medians and ranges from the average of two qPCRs; n = 5 animals per group.

Fig. 2. Transcript levels of *Defa* and *Mmp7* genes in the ileum of antibiotic-treated mice, relative to untreated animals. The graph shows medians and ranges, the table shows the Mann-Whitney test p values with respect to the untreated controls; * p < 0.05: significant differences. Results shown are one representative experiment of three; n = 4 females per group.

mycin-resistant enterococcus [18] and *Clostridium difficile* [19]. This is thought to result from alterations of the commensal intestinal microbiota and perturbations of mucosal immune function such as disrupted mucus layer and imbalances in immune cell populations [reviewed in 20]. To determine the susceptibility of *Defa* expression to antibiotic treatment, independent groups of mice from the same supplier (Jackson) and same housing facility were administered streptomycin or metronidazole, which are known to target distinct bacterial groups (aerobes and

anaerobes, respectively). Oral streptomycin treatment for 4 days resulted in decreased expression of several *Defa* genes (fig. 2) whereas metronidazole induced moderate expression increases that reached statistical significance only for *Defa3* (fig. 2). Expression of *Mmp7* (which in intestinal epithelial cells is stimulated by bacteria [21]) was unaffected in response to antibiotic administration (fig. 2). Interruption of streptomycin treatment led to a gradual increase in the *Defa* gene transcripts that were the most clearly affected by treatment (online suppl. fig. 3).

Fig. 3. Changes in microbial populations associated with antibiotic treatment. **a** Average *Hae*III-digested T-RFLP profiles of ileal 16S rRNA gene populations of mice untreated, treated with metronidazole or streptomycin, and 2 or 4 days after suspension of streptomycin treatment. Each bacterial phylotype/TRF peak is represented by a different color; peaks of major interest are marked by hatched bars (TRF232 red and TRF329 blue). * $p < 0.05$: significant differences. **b** Total (SYBR staining) and culturable (Rogosa agar) bacteria in ileal contents, data are means $\pm$ SE.

To identify changes in bacterial groups that correlated with changes in *Defa* expression, the ileal microbial populations of untreated and antibiotic-treated mice were quantified and characterized by T-RFLP and cloning/sequencing of 16S rRNA genes (fig. 3a). Streptomycin reduced total luminal bacteria in the ileum (fig. 3b) whereas metronidazole depleted specific bacterial populations. Based on the respective effects of these antibiotics on *Defa* expression, we aimed to identify candidate microbes that were depleted by streptomycin, unaffected by metro-

nidazole and that recovered after streptomycin removal. Only two TRFs, TRF232 and TRF329, fit these criteria. Clone libraries revealed that TRF232 represents a yet uncultured *Turicibacter* (accession No. EF406422) whereas TRF329 represents members of the genus *Lactobacillus*. The clone library generated from metronidazole-treated mice was nearly exclusively represented by sequences >99% identical to *Lactobacillus johnsonii* and *Lactobacillus reuteri*. Changes in the lactobacillus populations of streptomycin-treated mice were confirmed by plating/

Fig. 4. a Oral administration of heat-killed commensal bacteria to streptomycin-treated wild-type C57BL/6 mice partially restores ileal *Defa* expression in vivo; the data shown are *Defa* transcript levels relative to untreated animals. The table shows the Mann-Whitney test p values with respect to the streptomycin-treated animals; * $p < 0.05$: significant differences. **b** Exposure to commensal bacteria partially restores *Defa* expression in ileal explants from wild-type C57BL/6 mice. Results shown are relative *Defa* expressions normalized to relative villin expression for each sample ($RelExp\ Defa/RelExp\ villin$), as a correction for epithelial survival of the individual explants. The graphs show medians and ranges. Results shown are one representative experiment of two; $n = 4$.

isolation on Rogosa agar (fig. 3b) and identification by sequencing of the 16S rRNA gene. These results show that the presence of lactobacilli associates with homeostatic *Defa* transcriptional activity whereas lactobacillus depletion by antibiotic treatment correlates with a drop in ileal *Defa* gene expression.

The contribution of lactobacilli to the regulation of *Defa* expression was confirmed by introducing heat-killed *L. johnsonii* and *L. reuteri* to the drinking water of streptomycin-treated mice. The introduction of 10^8 lactobacilli resulted in a partial rebound in the expression of *Defa5*, *Defa20* and *Defa23* whereas a commensal heat-killed *E. coli* did not have the same effect (fig. 4a). Intriguingly, the expression of *Defa3* and *Defa24*, which reacted rather poorly to streptomycin administration, was significantly reduced by the administration of the heat-killed commensal *E. coli*. Since the in vivo antibiotic treatments did not completely eliminate the ileal micro-

biota, we decided to test the effect of lactobacilli and *E. coli* in cultured intestinal explants. In spite of the limitations inherent in tissue explant culture (such as progressive dying), ileal explants are efficiently devoid of commensals and therefore useful to study their effects on epithelial function. The explants were exposed to UV-killed bacteria for 16 h and *Defa* expression measured by qPCR. As shown in figure 4b, exposure to bacteria induced the transcription of *Defa* genes, demonstrating that in this model, bacterial stimulation participates in the control of *Defa* gene expression.

Homeostatic *Defa* Expression Requires TLR-MyD88 Signaling

Mice with genetic deficiencies in bacterial sensing (*Tlr2*^{-/-}, *Tlr4*^{-/-}, *Tlr9*^{-/-}, *Tlr2/4*^{-/-} or *MyD88*^{-/-}) and C57BL/6 wild-type controls were tested for ileal transcript levels of *Defa* (all the animals came from colonies

Fig. 5. TLR2, TLR4 and MyD88 deficiencies cause a drop in the ileal transcript levels of *Defa* genes. The graph shows medians and ranges; the table shows the Mann-Whitney test p values with respect to the C57BL/6 wild-type (wt) animals; * $p < 0.05$ and ** $p < 0.01$: significant differences. Results shown are the average of two qPCRs, $n = 6-9$ males per group.

housed at the CFRI). As shown in figure 5, mild to significant reductions in expression were observed in *Tlr2*^{-/-} mice, most notably for *Defa20* and *Defa23*. *Tlr4*^{-/-} mice showed a more moderate impact, with significant changes only for *Defa3* and *Defa24* although reductions were also observed for other genes. *Defa3* and *Defa20* transcription was affected in *Tlr9*^{-/-} animals, but the rest of the genes were insensitive to TLR9 deficiency. However, the *Tlr2*^{-/-}/*Tlr4*^{-/-} double-deficient mice displayed very dramatic reductions in transcript levels for most genes. Deficiency of the downstream adaptor molecule MyD88 also resulted in marked decreases in *Defa* transcription (fig. 5), in a manner not associated with either gross histological alterations of the intestinal epithelium, reduced number of Paneth cells or reduced levels of lysozyme transcripts (online suppl. fig. 4). Moreover, the expression of *Defa20* was measured in a small group of littermates, co-housed *MyD88*^{-/-} ($n = 3$) and *MyD88*^{+/-} heterozygous ($n = 2$) mice derived from heterozygous parents to ensure that differences in expression were not a result of separate housing. The observed differences in

transcript levels were consistent with mice housed independently, suggesting that disparities in *Defa* expression are associated with genotype rather than environment (not shown). In general, transcript levels of *Defa* in *MyD88*^{-/-} mice were very similar to those of the *Tlr2*^{-/-}/*Tlr4*^{-/-} double knockouts, indicating that the TLR-MyD88 pathway is important for homeostatic expression of *Defa* genes (fig. 5).

In contrast to TLR deficiency, lack of NOD2 had no significant effect on *Defa* expression, as compared to sex- and age-matched C57BL/6 wild-type controls from the same supplier and housed in the same facility (online suppl. fig. 5). Different from *Defa* genes, *Reg3β* and *Reg3γ* expression was insensitive to TLR deficiencies although it was decreased in *MyD88*^{-/-} mice (online suppl. fig. 6). Our results suggest that TLR2- and TLR4-mediated signaling is a fundamental mechanism for the maintenance of homeostatic expression of *Defa* genes in the intestinal epithelium of adult mice.

The involvement of TLR2 and TLR4 signaling in *Defa* expression was verified ex vivo using ileal explants. Ileal

Fig. 6. Treatment with TLR2 and TLR4 agonists (Pam2CSK4, 10 ng/ml; Pam3CSK4; 100 ng/ml and LPS, 10 ng/ml) restores *Defa* expression in ex vivo ileal explants from wild-type C57BL/6 mice. Results shown are relative *Defa* expressions normalized to relative villin expression for each sample (Rel Exp *Defa*/Rel Exp *villin*), as a correction for epithelial survival of the individual explants. The

asterisk marks the lack of detectable *Defa23* transcripts in the untreated explants. Results shown are one representative experiment of two; n = 4 or 5 explants per group. Pam2CSK4 and Pam3CSK4 are synthetic diacylated and triacylated lipopeptide agonists of the TLR2/6 and 2/1 heterodimers, respectively.

sections from C57BL/6 mice were treated in culture with 10 ng/ml of Pam2CSK4 (a Tlr 2/6 heterodimer agonist), 100 ng/ml of Pam3CSK4 (a Tlr 2/1 heterodimer agonist) or 10 ng/ml of *E. coli* LPS. The results in figure 6 show significant *Defa* expression drops in untreated, cultured ileal explants with respect to the in vivo levels, which was predicted based on the proposed role of bacteria in *Defa* regulation. Expression increased 10–1,000-fold after treatment of the explants with TLR2 and TLR4 agonists. *Defa20* and *Defa23*, the genes most sensitive to TLR2 deficiency in vivo, were also the most sensitive to the ex vivo culture conditions (*Defa23* transcription became undetectable in untreated explants). In general, stimulation with Pam3CSK4 (the agonist of the TLR2/1 heterodimer) produced the highest increases in *Defa* expression.

Discussion

Paneth cell α -defensins are a major type of enteric antimicrobials. Reduced abundance or loss of function has been associated with Crohn's disease [22, 23], susceptibility to enteric pathogens [2, 3], changes in composition of microbiota and disruption of intestinal immune homeostasis [4]. Their production has been typically considered invariable and independent of bacterial stimulation [24]; however, we observed that transcript levels of several *Defa* genes are sensitive to antibiotic treatment and correlate with changes in the intestinal microbial composition.

Our results strongly associate *Lactobacillus* with maximal Paneth cell α -defensin expression and show that the commensal population plays a significant role in driving ileal *Defa* expression. Although it was not tested here, it is quite plausible that other Gram-positive bacteria are also able to induce expression. Observations in clinical specimens from patients with Crohn's disease support the role of commensal bacteria in regulating α -defensin expression since both, alterations in the intestinal microbial composition (including the depletion of lactobacilli) and reduction of α -defensin levels are observed in patients with Crohn's disease [25, 26].

Antibiotic treatments often lead to secondary clinical intestinal complications [18, 20]; it is possible that those arise due (at least in part) to alterations of α -defensin synthesis profile. There is evidence that Paneth cell α -defensins play a role in modulating the intestinal mucosal immune response directly [27, 28] and indirectly [4], and have been shown to inhibit the toxin B of *C. difficile* [29], a common opportunistic pathogen associated with antibiotic therapy [19]. Thus, reductions in *Defa* expression have the potential to impact the immunological tone of the gut in a significant way.

In contradiction with previous reports [12], we failed to observe any defect of *Defa* expression in NOD2-deficient mice. In contrast, the TLR-MyD88 pathway seems important for maximal *Defa* expression. Out of the TLRs tested, TLR2 was the most critical, being important for expression of most gene subgroups. This is in contrast with the work of Dessein et al. [30], who reported that

TLR2 and MyD88 deficiency had no impact on the expression of cryptdins. However, they studied *Defa1* and *Defa4*, two genes that are absent in C57BL/6 mice. In our study, most *Defa* genes displayed milder expression reductions in single *Tlr2*^{-/-} or *Tlr4*^{-/-} mice than in the double *Tlr2*^{-/-}/*Tlr4*^{-/-} or the *MyD88*^{-/-} strains, suggesting a compensatory action between these two TLRs. This is also supported by the responses of the intestinal explants to both TLR2 and TLR4 agonists.

Activation of the β -catenin pathway is so far the only mechanism known to regulate *Defa* gene expression [7, 8]. Our results demonstrate a role of MyD88 for cryptdins expression in the adult mouse intestine, as previously proposed [31] and provide clear evidence of the involvement of at least TLR2 and TLR4. This expands the known function of MyD88 in driving the expression of Paneth cell antimicrobials [32] and highlights the critical role of the TLR-MyD88 pathway in intestinal homeostasis. The normal expression of lysozyme in TLR- and MyD88-deficient mice suggests that the observed defects in *Defa* expression are not the result of abnormal Paneth cell development.

It is still unclear how the signaling pathway relevant for *Defa* expression proceeds downstream of MyD88. We observed that *Defa* expression was insensitive to NF κ B_{p50} null deficiency in vivo (not shown) but considering the functional compensation between the Rel family members [33], these results are not conclusive. It should be noted that all the knockout mice tested here are lacking their specified genes in their germ line and are not cell-specific deficient mice. Thus, the dramatic impact of MyD88 deficiency may not reflect the loss of MyD88 signaling within the Paneth cells, but it may instead reflect indirect actions, i.e. the involvement of MyD88 within other cell types, such as macrophages or myofibroblasts resulting in the release of factors that regulate defensin expression by Paneth cells.

In summary, we have presented here novel observations on ileal *Defa* gene expression in C57BL/6 mice. Future studies are necessary to establish how the observed differences in transcript levels translate into differences in luminal concentrations of active α -defensins and/or intestinal antibacterial activity. We have shown that intestinal commensals are directly linked with α -defensin expression through the TLR-MyD88 pathway. It would be interesting to assess whether the synthesis of a related group of antimicrobial peptides, the cryptdin-related sequences, is subject to similar regulation. As alterations of the intestinal microbial flora and homeostasis are associated with an increasing number of pathological conditions (e.g. inflammatory bowel disease, infection and cancer) our findings bear relevance to the understanding of the molecular mechanisms underlying such conditions.

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