INVESTIGATION INTO THE REGULATION AND FUNCTION OF ALKALINE PHOSPHATASE (ALP) IN HOST-MICROBE INTERACTIONS

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DISSERTATION ABSTRACT

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Title: Investigation into the Regulation and Function of Alkaline Phosphatase (ALP) in Host-Microbe Interactions

This dissertation describes our investigation into the regulation and function of the innate immune modulator alkaline phosphatase (ALP). Animal intestine harbors a vast and complex microbial community, the gut microbiota. While resident microbes perform vital functions and confer tremendous benefits on their hosts, they also provide antigens and toxins that provoke host immune responses, which, if uncontrolled, could be detrimental to the host. Our lab previously demonstrated a negative feedback loop mediated by intestinal ALP (ALPI) which promotes immune tolerance to the commensal microbiota in zebrafish. We continue to investigate regulation mechanisms of *ALP* genes and explore their roles in modulating host-microbe interactions in various models. We have characterized four zebrafish *alp* genes, and we engineered tools for functional studies of these genes. Phylogenic analyses involving zebrafish *alp* genes revealed distinct evolution histories of animal *ALP* genes and implied their diversified functions. We then tested whether the regulation mechanism and the roles of zebrafish *alpi* were conserved in mice. We found the *ALPI* gene *Akp3* was specifically upregulated by microbiota and played a role in immune education. We demonstrated the contribution of innate immune signaling to animal weight gain induced by high fat diet feeding. Finally,

we discovered the positive correlation between neonatal ALPI activity and gestational age, suggesting potential therapeutic value of ALP supplementation for preventing necrotizing enterocolitis development in preterm infants.

This dissertation includes previously published and unpublished co-authored material.

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CHAPTER I

INTRODUCTION

The Mutually Beneficial Relationship Forged between Host and the Gut Microbiota Requires Fine-Tuning of Host Immune Responses

Vertebrates live in symbiosis with a consortium of microorganisms, the microbiota. The most populous microbial community is present in the intestine. The human intestinal microbiota are comprised of as many as 1,000 distinct species and 100 trillion microorganisms, dominated by bacteria (Turnbaugh et al., 2007). A mutually beneficial relationship has been forged between the host and its associated gut microbiota. While the host intestine provides a hospitable and nutrient-rich environment for the microbes, the gut microbiota modulate host nutrient intake and metabolism (Backhed et al., 2005; Tremaroli and Backhed, 2012), promote maturation of the innate and adaptive immunity (Jarchum and Pamer, 2011), preserve the intestinal epithelial barrier function (Sharma et al., 2010), and prevent growth of pathogens and abnormal microbiota (Stecher and Hardt, 2011).

Despite these benefits, the intestinal microbiota are a continuous source of antigens and toxins that have the potential to provoke host inflammatory responses. One classic example is the activation of host innate immune responses by bacterial lipopolysaccharide (LPS). LPS, also referred to as endotoxin, is a constituent of the outer membrane of Gram-negative bacteria (Elin and Wolff, 1976) and abundantly present in the intestine. LPS is a classic microbial associated molecular pattern (MAMP) and potent inducer of innate immune signaling in both vertebrates and invertebrates (Beutler and

Rietschel, 2003). In mammals, LPS binds specifically to a complex consisting of Toll-like receptor 4 (TLR4) and MD-2 through two phosphate groups of its lipid A moiety (Kim et al., 2007; Park et al., 2009), and induces inflammatory responses by activating two distinct pathways, namely NF-κB (through MyD88-dependent and independent pathways) and IRF-3 (through TRIF/TRAM).

Uncontrolled inflammatory responses to the resident microbiota could damage the beneficial microbes and be detrimental to host. Regulatory mechanisms must exist to prevent unlimited immune activation. On the other hand, irresponsive immunity may fail to defend against invading pathogens. A delicate balance between the proinflammatory and immunosuppressive responses must therefore be actively maintained by both host and microbes to achieve a healthy steady state in which the gut microbiota are tolerated and simultaneously well restrained (Chow et al., 2010; Pamer, 2007; Round and Mazmanian, 2009; Sansonetti, 2004).

Alkaline Phosphatase (ALP) Mitigates Host Inflammatory Responses to Bacteria by Dephosphorylating LPS

Alkaline phosphatases (ALPs) are a superfamily of metalloenzymes that are widely found in organisms ranging from bacteria to human (McComb RB, 1979) and catalyze the hydrolytic removal of phosphate from a variety of molecules (Millan, 2006). Importantly, ALPs have been shown to remove the lipid A phosphates of LPS (Bentala et al., 2002; Riggle et al., 2013; Tuin et al., 2009; van Veen et al., 2006). By dephosphorylating LPS, ALPs decouple LPS and the TLR/MD-2 receptor complex, thereby ameliorate LPS-induced inflammation and reduce LPS endotoxic properties

(Lukas et al., 2010; Park et al., 2009; Poelstra et al., 1997a; Poelstra et al., 1997b; Rader et al., 2012; Riggle et al., 2013; Tuin et al., 2009). The LPS dephosphorylation activity could also explain the protective effects of ALPs against various inflammation-related injuries (Bates et al., 2007; Bol-Schoenmakers et al., 2010; Campbell et al., 2010; Heemskerk et al., 2009; Kats et al., 2009; Lukas et al., 2010; Martinez-Moya et al., 2012; Pickkers et al., 2012; Ramasamy et al., 2011; Rentea et al., 2013; Rentea et al., 2012a; Rentea et al., 2012b; Riggle et al., 2013; Tuin et al., 2009; van Veen et al., 2006; van Veen et al., 2005; Verweij et al., 2004; Whitehouse et al., 2010).

The mammalian ALP family consists of several isozymes that can be classified into tissue-nonspecific ALPs (liver-bone-kidney type) and tissue-specific ALPs (intestinal, placental, and germ cell type) (Yang et al., 2012). The intestinal ALP (ALPI) is abundantly present in the apical microvilli of the brush border of enterocytes (Hanna et al., 1979), and actively secreted into the intestinal lumen (McConnell et al., 2009; Shifrin et al., 2012). The physiological location of ALPI at the interface between the intestinal tissue and the gut microbiota suggests its involvement in shaping and sustaining the host-microbe mutualism in the intestine. Recent studies have shown that ALPI regulates neutral pH in the intestine (Mizumori et al., 2009), protects gut barrier function (Goldberg et al., 2008; Kaliannan et al., 2013; Martinez-Moya et al., 2012; Rentea et al., 2012b), preserves the normal homeostasis of the gut microbiota (Malo et al., 2010), and dephosphorylates proinflammatory molecules including nucleotide uridine diphosphate (Moss et al., 2013), flagellin (Chen et al., 2010), unmethylated cytosine-phosphate-guanine deoxyribonucleic acid (CpG DNA) (Chen et al., 2010), and of course, LPS.

The anti-inflammation role of ALPI is supported by many observations. Cell culture studies showed that in human intestinal epithelial cells (i.e., HT-29, T84 and IEC-6) overexpressing ALPI, LPS-activated NF-κB nuclear translocation was significantly inhibited (Goldberg et al., 2008). At the whole animal level, administration of bovine ALPI proved to reduce local/systemic inflammation and improve tissue morphology in the mouse polymicrobial sepsis model induced by cecal ligation and puncture (van Veen et al., 2005), in the rat liver ischemia–reperfusion model (van Veen et al., 2006), in the murine chronic colitis model induced by dextran sulfate sodium (DSS) (Campbell et al., 2010; Ramasamy et al., 2011; Tuin et al., 2009) or TNBS (Martinez-Moya et al., 2012), and in the neonatal necrotizing enterocolitis rat models (Rentea et al., 2013; Rentea et al., 2012a; Rentea et al., 2012b; Riggle et al., 2013; Whitehouse et al., 2010). In human clinical trials, exogenous ALPI exerted protective anti-inflammatory effects on patients after cardiopulmonary surgery (Kats et al., 2009), patients with moderate to severe ulcerative colitis (Lukas et al., 2010) and patients with severe sepsis or septic shock (Heemskerk et al., 2009; Pickkers et al., 2012). Collectively, these findings confirm that ALPI is an important innate immune regulator, both locally and systemically.

ALPI Contributes to Maintaining the Peaceful Relationship between Zebrafish and the Resident Microbiota

Our studies using zebrafish larvae have demonstrated an integral role of ALPI in maintaining peace between host immunity and the gut microbiota (Bates et al., 2007; Bates et al., 2006). We showed that intestinal colonization of Gram-negative bacteria upregulated zebrafish *ALPI* gene *alpi.1* through the LPS-induced innate immune

signaling, which requires the common TLR adaptor protein Myd88, and that ALPI functioned to dampen zebrafish inflammatory responses to the resident gut microbiota. The LPS-ALPI negative feedback loop therefore promotes zebrafish immune tolerance to the commensal Gram-negative bacteria.

Several interesting questions then arise. What are the roles of other *ALP* genes in zebrafish? Is the ALPI-mediated anti-inflammatory innate immune response conserved in mammals? What is the implication of ALPI in human necrotizing enterocolitis (NEC)? To answer these questions, we have explored the regulation and function of different *ALP* genes in various systems. Chapter II describes characterization of zebrafish *ALP* genes and includes previously published material that was co-authored with A. M. Wandler, J. H. Postlethwait and K. Guillemin. Chapter III covers the study of mouse *ALPI* gene *Akp3* in host-microbe interactions and includes material that will be published with co-authors J. Mecsas, J. Sonnenburg, J. L. Millán, and K. Guillemin. Chapter IV reports the finding of ALPI deficiency in preterm neonates who are prone to NEC and includes material that will be published with co-authors, R. Bent and K. Guillemin. And finally, Chapter V summarizes the conclusions.

CHAPTER II

CHARACTERIZATION OF ZEBRAFISH *ALP* GENES

This chapter includes work previously published in Volume 3 of Frontiers in Immunology in October 2012 with co-authors A. M. Wandler, J. H. Postlethwait and K. Guillemin. A. M. Wandler, K. Guillemin, and I designed the research. I characterized the four zebrafish ALP genes. J. H. Postlethwait performed phylogenic studies. K. Guillemin analyzed the microbiota profiles associated with different mammals. K. Guillemin, J. H. Postlethwait, and I wrote the paper. A. M. Wandler provided editorial assistance.

Introduction

The broad spectrum of substrates ALPs are able to dephosphorylate suggests versatile roles of ALPs. Distinct expression patterns of animal *ALP* genes further indicate their diversified functions. For example, while ALPI has been extensively studied for its anti-inflammatory role in health and disease, the tissue-nonspecific ALP (ALPL) is well recognized for its role in osteogenesis by promoting bone mineralization (Whyte, 2010). To dissect out how various *ALP* genes function in host-microbe interactions, we characterized *ALP* genes in zebrafish. The phylogeny study involving these genes revealed the dynamic evolution of vertebrate ALPs. I have also developed protocols and engineered genetic tools for functional analyses of individual zebrafish *ALP* genes.

Materials and Methods

Use of Vertebrate Animals

All zebrafish experiments were performed using protocols approved by the University of Oregon Institutional Care and Use Committee, and followed standard protocols (Westerfield, 2007).

Protein Sequence Alignment

Protein sequences used in the alignment are obtained from Ensembl (http://www.ensembl.org). ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align the following sequences: human ALPL (ENSP00000363973) and ALPI (ENSP00000295463); zebrafish Alpl (ENSDARP00000117214), Alpi.1 (ENSDARP00000016216), Alpi.2 (ENSDARP00000070354), and Alp3 (ENSDARP00000019098).

Sanger Sequencing of Zebrafish *ALP* Gene Coding Sequences

Fresh RNA was extracted from 7 dpf (days post fertilization) zebrafish larvae (illustra RNAspin mini kit, GE Healthcare). Total cDNA was synthesized using RNA as template (SuperScript III reverse transcription kit, Life technologies). The coding sequences of *ALP* genes were amplified from cDNA by PCR using Phusion DNA polymerase (Thermo Scientific). Primers used include: alplF: 5'-ATGTGGGAATGTGGATGCTTTCTTG-3', alplR: 5'-TCAGCAAAGCAGCCATTTGACC-3'; alpi.1F: 5'-ATGTGTTTGGTTTACGGTCGGGC-3', alpi.1R: 5'-

TCATCTCAAAACAAGCCAAAACACG-3'; alpi.2F: 5'-ATGGCCAAAACACAAGCCCTG-3', alpi.2R: 5'-CTAAATAAGAGCAGTAATGGAGGACATCAG-3'; alp3F: 5'-ATGTTTGCTGTCCGTGTGTCC-3', alp3R: 5'-TCAGTGCAGTAAAATCCTCATCAGTG-3'. PCR products were evaluated by DNA gel electrophoresis for purity and then extracted from gel. The purified PCR products were cloned into the pCR® - Blunt II TOPO® vector (ZeroBlunt® TOPO® PCR Cloning kit, Life Technologies). Clones carrying *ALP* gene coding sequences were sequenced (Sequetech). The complete coding sequences were assembled using the plasmid editor ApE (http://biologylabs.utah.edu/jorgensen/wayned/ape/), and submitted to GenBank.

PCR Detection of Zebrafish *ALP* Gene Transcription

Total cDNA was synthesized from fresh RNA prepared from 7 dpf whole fish, dissected intestines, or carcasses with guts removed. Gene-specific primers were used in PCR to test the presence of gene transcripts (alplF: 5'-TATTTCTTGGAGATGGGATGGGTG-3', alplR: 5'-TTCAAAGAGTTGTCTGGCGATGTC-3'; alpi.1F: 5'-GCACCGCGCCAAAGCACAAG-3', alpi.1R: 5'-CGGGCTTCGGAGGGCACATC-3'; alpi.2F: 5'-TGCGCTTTACGGAAACGGTCCA-3', alpi.2R: 5'-TGCGCCATCGGGCCTTTAGC-3'; alp3F: 5'-ATGTTTGCTGTCCGTGTGTCC-3', alp3R: 5'-ACGA GAAACCGCCTCATCCAG-3'). Equal amounts of cDNA (200 ng/µl, NanoDrop 1000) were added as template in PCR.

In Situ Hybridization

Zebrafish cDNA was obtained as describe above and used in PCR to amplify a unique fragment of *alpl* (alpl-F: TTCCAGAGCAAGAGAAGCGG; alpl-R: GTCTTAGAGAGGGCGACGTG), *alpi.1* (alpi.1-F: CGACCGGGCGATTCAGAGAG; alpi.1-R: TGGTGTACGGCTCAAGGCAC), *alpi.2* (alpi.2-F: TCACTAACGGGACTCGACCT; alpi.2-R: AGGCCATAGCGTGAGGAATG) and alp3 (alp3-F: CAGGGTCATCACTCCAGTCG; alp3-R: TCTGGACGCTTGTTGTTGGT). The purified PCR product was cloned into the pCR® - Blunt II TOPO® vector (ZeroBlunt® TOPO® PCR Cloning kit, Life Technologies) and validated by sequencing (Sequetech). The verified plasmid was used as template in PCR and T7 RNA polymerase binding sites were added to the gene fragment. Primers used include alpl-F: TTCCAGAGCAAGAGAAGCGGC, alplT7-R: GTAATACGACTCACTATAGGGGTCTTAGAGAGGG; ali.1p-F: CGACCGGGCGATTCAGAGAG, alpi.1T7-R: GTAATACGACTCACTATAGGGTGGTGTACGGCTCAA; alpi.2-F: TCACTAACGGGACTCGACCTGATGT, alpi.2T7-R: GTAATACGACTCACTATAGGGAGGCCATAGCGTGAG; and alp3-F: CAGGGTCATCACTCCAGTCGGGC, alp3T7-R: GTAATACGACTCACTATAGGGTCTGGACGCTTGTTG. The purified PCR product was used as template to synthesize the DIG labeled RNA probe (DIG RNA Labeling Mix (T7), Roche Applied Science).

7 dpf zebrafish larvae were fixed in 4% PFA overnight at 4°C and then washed in 1×PBST. Rinsed larvae were embedded in 1.5% agar and cryo-cut into 16 µm thick

sections. Sections were defrosted and air died overnight at room temperature. Diluted RNA probes were added on slides and incubated overnight at 70°C. After hybridization, slides were rinsed with the wash solution (1xSSC, 50% formamide, 0.1% Tween-20). Slides were then treated with the block solution (MABT, 2% blocking reagent, 20% heat inactivated sheep serum) for 3 hours. Following that, first antibody solution (AP conjugated anti-DIG) was applied to slides and incubated overnight at 4°C. Slides were then washed in AP staining buffer (5M NaCl, 1M $MgCl_2$, 1M Tris pH 9.5, 20% Tween-20) and incubated with NBT/BCIP color regents in the dark at 37°C. The color developing process was stopped by washing slides in PBST. Slides were then rinsed in ddH_2O, dehydrated in ethanol and eventually mounted in Permount. Images were taken under a Nikon TE2000 inverted microscope with a CoolSNAP camera (Photometrics).

Transgenic Zebrafish Lines

Transgenic zebrafish lines were constructed following the Tol2kit protocol (Kwan et al., 2007). Briefly, full length coding sequences of *alpi.1* and *alpi.2* were amplified by PCR using AB/Tuebingen zebrafish cDNA as template. Additional rounds of PCR were designed to add the viral 2A peptide sequence (Provost et al., 2007) before the start codon, and then add *att*B2 sites to the 5' end and attB3 sites to the 3' end. Purified PCR products, after verification by sequencing, were used in the BP recombination reaction (Invitrogen) to generate 3' entry clones. The *tdTomato* middle entry clone was obtained from Dr. Judith Eisen's lab at the University of Oregon. The 5' entry clone carrying the *bactin2* promoter was obtained from Dr. Chi-bin Chien's lab at the University of Utah, and the clone carrying the *ifabpb2* promoter was generated by Zac Stevens in our lab.

Finally, specific 5', middle and 3' entry clones were combined in the LR recombination reaction (Invitrogen) to create the Tol2 constructs, which, after verified by sequencing, were injected with the Tol2 transposase into zebrafish embryos at one cell stage. Embryos positive for tdTomato were raised up and crossed to AB/Tuebingen zebrafish. The progeny were selected for tdTomato signal and glowers were kept as stable transgenic lines.

Targeted Mutagenesis Using TALENs

The following paired TALE sequences were designed to specifically bind regions in exon 2 of *alpl*, *alpi.1*, *alpi.2*, or *alp3* using the online tool "Paired Target Finder" (https://tale-nt.cac.cornell.edu/node/add/talef-off-paired): alpl-TAL1RVD: HDNINNNGNGNNNGHDNGNNNNHDNIHDHDHDNGNGNN, alpl-TAL2RVD: NGNINGHDNGNINGHDNINNHDHDNGNNHDNNNGNGNG; alpi.1-TAL1RVD: NNNININGNNNNNNNINNNININNNNNINGHDHDHDNN, alpi.1-TAL2RVD: HDNGNNHDNINNHDNNNGHDHDNNNGHDNGNNNNHDHD; alpi.2-TAL1RVD: NNNNNGNINGNNNGNININININNNHDNGNININININNI, alpi.2-TAL2RVD: NNNGNGNNNININGNNNGHDNINGNGNNNINNNINNHD; alp3-TAL1RVD: NGHDNGNNNNNINNNNNNININNNGHDNNNNHDNGHDNI, alp3-TAL2RVD: NNNINGNGNGNGHDHDNGNNHDNGHDNINNNINNHDHD. These TALEs were assembled and cloned into the TALEN backbone plasmid following the previously published protocol (Sanjana et al., 2012). The resulting plasmids, after verified by sequencing, were used as templates to make TALEN transcripts using the Ambion mMessage mMachine T7 Ultra Kit (Life Technologies). After purification, paired

TALEN transcripts targeting individual *ALP* genes were injected to zebrafish embryos at one cell stage.

E. tarda and *V. cholerae* Infections

The *Edwardsiella tarda* strain isolated from catfish was kindly provided by Dr. Carol H. Kim (University of Maine, Orono, ME) and the *Vibrio cholerae* strain was isolated from zebrafish water at the University of Oregon. Liquid cultures were grown overnight at 28 °C with shaking. Rifampicin-resistant colonies were selected by plating bacterial cultures on TSA plates containing 50 μg/ml rifampicin, and stocked in 15% glycerol at -80 °C. Liquid cultures of rifampicin-resistant *E. tarda* or *V. cholerae* were grown overnight at 28 °C with shaking, diluted 1:10 early next morning, grown for another 3 hours at 28 °C with shaking, and centrifuged in 50 ml tubes at 5,000 rpm for 10 min to harvest bacteria. Bacteria were resuspended in zebrafish embryo medium (EM) (0.29 g/l NaCl, 0.013 g/l KCl, 0.048 g/l CaCl2·2H2O, 0.082 g/l MgCl2·6H2O, pH 7.2). Bacterial concentration was calculated based on absorbance at OD600 (1 OD600 = 10^9 cfu/ml). Zebrafish embryos were collected from natural crossings between AB/Tuebingen parent fish. At 3 dpf, 25 embryos were put into a 250 ml flask containing 50 ml EM, and infection was done in triplicate flasks by inoculating EM with *E. tarda* or *V. cholera* to a final concentration of 5×10^7 cfu/ml. After inoculation, zebrafish mortality was monitored daily till 8 dpf and the results were analyzed using the Prism software (GraphPad).

Results

The Four Zebrafish *ALP* Genes Are Distributed among Three Vertebrate *ALP* Gene Clades

We had previously characterized two zebrafish *alp* genes, which we called *alp* (accession number NM_201007.1) on chromosome 11 and *alpi* (accession number NM_001014353.1) on chromosome 22, and had shown that the former gene is ubiquitously expressed and the latter is intestinal specific (Bates et al., 2007). BLAST searches against the subsequent refinement of the zebrafish genome sequence revealed two additional *alp*-related genes: *zgc:110409* (accession number NM_001025188.1) on chromosome 22, and the most recently described *alp*-related gene (accession number XM_003201677.1) on unassembled Scaffold Zv9 NA903 that Ensembl annotated as "*alpl*". All four genes likely encode enzymatically active ALPs based on their conserved ALP motifs. Protein sequence alignment against human ALPL and ALPI showed that active site residues and proposed metal-binding residues are invariant in the proteins encoded by the two human genes and four zebrafish genes (Figure 1, see Appendix A for all figures).

To better understand the identity and origin of the four zebrafish *alp*-related genes, we investigated their evolutionary history. Phylogenetic analysis of vertebrate ALP protein sequences rooted on non-vertebrate chordate sequences revealed three distinct clades of *ALP* genes, the tissue-nonspecific ALPL clade, the intestinal ALPI clade, and the fish-exclusive ALP3 clade (Figure 2). Analysis of the four zebrafish *alp* genes within this phylogeny revealed that i) the chromosome 11 "*alp*" is a genuine ortholog of the human tissue non-specific gene *ALPL*; ii) the chromosome 22 "*alpi*" and

the neighboring "*zgc:110409*" are tandem duplicates derived from the ancestral *alpi* gene and represent co-orthologs of human *ALPI*; and iii) the new Scaffold Zv9 NA903 *alp* annotated in Ensembl as "*alpl*" belongs to the Alp3 clade maintained in teleosts but lost in tetrapods.

Based on these findings, we developed a new nomenclature for the four genes, i.e., the current "*alp*" is renamed *alpl*, "*alpi*" is *alpi.1*, "*zgc:110409*" becomes *alpi.2*, and "*alpl*" is *alp3*. We cloned and sequenced the complete coding DNA sequences of the four zebrafish *alp* genes and submitted to Genbank: *alpl* (accession number JX847415), *alpi.1* (accession number JX847416), *alpi.2* (accession number JX847417), and *alp3* (accession number JX847418).

Three of the Zebrafish *ALP* Genes Show Enriched Expression in the Intestine

We further explored the four zebrafish *ALP* genes by investigating their tissue expression patterns. We used semi-quantitative reverse transcription PCR to estimate the abundance of the transcripts in intestinal tissue ("I") versus the rest of the body (referred to as carcass, "C"). Transcript levels of *alpl* were abundant in the carcass as well as the intestine (Figure 3A). In contrast, transcripts of the other three genes were enriched (*alpi.1* and *alpi.2*) or exclusively expressed (*alp3*) in intestinal tissue (Figure 3A). We next performed *in situ* hybridization with gene-specific RNA probes to further examine the expression patterns of the four *alp* transcripts. Consistent with our previous analysis (Bates et al., 2007), we observed that *alpl* was diffusely expressed in many tissues (Figure 3B). Also, as we showed previously (Bates et al., 2007), a*lpi.1* was highly expressed in the intestine (Figure 3C). We also observed high levels of intestinal-specific

expression of *alpi.2* (Figure 3C & D), indicating that the tissue-specific expression of this *alpi* co-ortholog has been maintained. Finally, we observed intestinal-specific expression of the teleost-specific *alp3* gene (Figure 3C & E), suggesting that intestinal-specific expression of *alp* genes is an ancestral trait possessed by the single *ALP* gene found in non-vertebrate chordates (Yang et al., 2012).

Development of Genetic Tools for Functional Study of Zebrafish *ALP* Genes

To facilitate functional analyses of individual zebrafish *ALP* genes, we have made a collection of valuable reagents. We have successfully constructed three transgenic zebrafish lines overexpressing *ALP* genes: the *bactin2p:tdtomato:2Aalpi.1* line, which have ectopic expression of *alpi.1* in whole fish; the *ifabb2p:tdtomato:2Aalpi.1* line, which have ectopic expression of *alpi.1* in the intestine; and the *ifabb2p:tdtomato:2Aalpi.2* line, which have ectopic expression of *alpi.2* in the intestine. On the other hand, we have made efforts to mutagenize zebrafish *alp* genes using designed TALENs (transcription activator-like effector nucleases) (Sanjana et al., 2012). Given the high efficacy of our designed TALENs for targeted genome editing in zebrafish, as demonstrated by our preliminary study, we hope to get loss-of-function fish lines of each *ALP* genes in the near future.

These genetically modified zebrafish will be examined for interactions with commensal microbiota, as well as responses to Gram-negative pathogens.

Development of an Infection Model for Functional Study of Zebrafish *ALP* Genes

To study roles of zebrafish ALPs in regulating animal responses to Gram-negative pathogens, we established a standard zebrafish infection model. Zebrafish larvae hatch and open mouths around 3 dpf. We thus inoculated 3 dpf zebrafish larvae by adding Gram-negative pathogens into zebrafish embryo medium so that bacteria entered and colonized fish guts through the orally route. Fish mortality was monitored daily till 8 dpf. We tested the Gram-negative pathogen *E. tarda*, which was associated with freshwater that causes a systemic infection in *fish* (Pressley et al., 2005), and the opportunistic Gram-negative pathogen *V. cholerae*, which was isolated from fish water at the University of Oregon. We found that inoculation with rifampicin-resistant *E. tarda* or *V. cholerae* produced reproducible survival curves (Figure 4).

Discussion

We have characterized four zebrafish *ALP* genes, one tissue-nonspecific (*alpl*) and three enriched in the intestine (*alpi.1*, *alpi.2*, and *alp3*). We developed a new nomenclature for the four genes based on their phylogenic traits. We found that the vertebrate *ALP* genes arose from a single ancestral *ALP* gene but diversified into three major clades in vertebrates: the *ALPL* clade which includes zebrafish *alpl*, the *ALPI* clade which includes zebrafish *alpi.1* and *alpi.2*, and the *ALP3* clade which includes zebrafish *alp3* and is lost in tetrapods. While *ALPL* genes remained relatively stable across vertebrates, *ALPI* genes have undergone rapid evolution, especially in mammals.

We speculate the basis for the dramatically different evolutionary histories of the tissue non-specific *ALPL* genes and the intestinal *ALPI* genes, which encode enzymes

with well-conserved catalytic activities, lies in their different patterns of tissue expression (Yang et al., 2012). The ubiquitously expressed ALPL plays an important role in bone mineralization in mammals and modest changes in ALP function at sites of bone mineralization could lead to deleterious phenotypes, possibly restricting the evolution of copy number variation of this gene once it became dedicated to this function. On the contrary, intestinal *ALP* genes that regulate metabolism and host-microbiota interactions are under strong evolutionary pressure derived from the dramatic changes in animal dietary, gut morphologies, associated microbial communities, and pathogens.

To facilitate future functional studies of the diversified zebrafish *ALP* genes, we have engineered specific gene gain-of-function and loss-of-function fish. And a standard infection model was established to test requirements for individual zebrafish *ALP* genes in defending against infections and evaluate their potential protective effects.

CHAPTER III

REGULATION AND FUNCTION OF MOUSE *ALPI* GENE *AKP3*

This chapter includes work that will be published with co-authors, J. Mecsas, J. Sonnenburg, J. L. Millán, and K. Guillemin. K. Guillemin and I designed the research. I performed the experiments and analyzed the data. J. Mecsas provided the pathogen strain and gave instructions on the infection protocol. J. Sonnenburg provided conventional and germ-free mouse intestine tissue samples. J. L. Millán provided *Akp3*-KO mice. The writing is mine. K. Guillemin provided editorial assistance.

Introduction

The mouse genome contains four *ALP* genes: the tissue-nonspecific type *Alpl*, the embryonic *Alppl2*, the intestinal *Alpi*, and the duodenal *Akp3* (Millan, 2006; Yang et al., 2012). *Alppl2*, *Alpi* and *Akp3* all contribute to the ALP activity in the mouse intestine, but exhibit distinct expression patterns (Narisawa et al., 2007). *Alppl2* and *Alpi* are expressed immediately after birth throughout the intestine but enriched in the duodenum. In comparison, *Akp3* expression starts around postnatal day 13-15 and is restricted in the duodenum. The concordant timing of *Akp3* expression with weaning and the establishment of the adult gut microbiota in mouse led us to test the possibility that *Akp3* is induced by microbiota.

The three murine *Alpi* genes, *Alppl2*, *Alpi* and *Akp3*, arose from recent gene duplication in the rodent lineage and are not direct orthologues of the human *ALPI* genes or the *alpi* genes in zebrafish (Yang et al., 2012). It is therefore likely that *Akp3* has

acquired novel functions in evolution. Since the generation of *Akp3*$^{-/-}$ mice (Narisawa et al., 2003), several studies have probed the role of *Akp3* in animal health and disease. Like other ALPs, Akp3 has LPS dephosphorylation activity, and *Akp3*$^{-/-}$ mice had significantly lower ALP enzyme and LPS dephosphorylating activities in stools and duodenal mucosa compared with wild type littermates (Goldberg et al., 2008). *Akp3*$^{-/-}$ mice had increased gut permeability, which caused more LPS translocation from the intestinal lumen into the blood (Kaliannan et al., 2013). Consequently, *Akp3*$^{-/-}$ mice displayed higher hepatic expression of MHC class II molecules (Chen et al., 2011) and signs of metabolic endotoxemia (Kaliannan et al., 2013). *Akp3*$^{-/-}$ mice were also found to contain dramatically fewer and also different types of aerobic and anaerobic microbes in stools compared with wild type mice (Malo et al., 2010). Together, these observations from routinely maintained animals indicate that *Akp3* plays a role in maintaining the normal gut barrier function and regulating the gut microbial composition. The role of Akp3 as a gut mucosal defense factor is more evident when mice were challenged. Compared to wild type mice, *Akp3*$^{-/-}$ mice suffered from more severe gut mucosal disruption and higher bacterial translocation after ischemia/reperfusion injuries (Goldberg et al., 2008), and were more vulnerable in dextran sulfate sodium (DDS)-induced colitis (Ramasamy et al., 2011). Interestingly, *Akp3*$^{-/-}$ mice showed immune tolerance to intraperitoneal LPS injection and resistance to *Salmonella typhimurium* infection (Chen et al., 2011).

It is worthwhile to note that all the aforementioned results were obtained from full-grown mice but little is known about the biological relevance of *Akp3* in developing animals. We therefore compared intestinal inflammatory responses of wild type and *Akp3*$^{-/-}$ mice at different ages to resident microbiota, to orally administered LPS, and to

Gram-negative pathogen *Yersinia pseudotuberculosis*. Based on our previous observations in zebrafish (Bates et al., 2007), we speculated that the partial reduction in ALPI activity due to disruption of one *ALPI* gene could give rise to subtle inflammatory reactions to the microbiota in *Akp3*$^{-/-}$ mice during development, which in the long run might alter their immune sensitivity to future challenges like LPS. And to test whether the reported resistance of *Akp3*$^{-/-}$ mice to *S. typhimurium* infection is pathogen-specific, we tested the requirement of *Akp3* for defending against infection by *Y. pseudotuberculosis*.

Although *Akp3*$^{-/-}$ mice appear normal under normal laboratory conditions, they had accelerated fat absorption and weight gain when maintained long term on a high fat diet (HFD) (Kaliannan et al., 2013; Narisawa et al., 2003). However, the underlying mechanism is not well understood. We proposed that the LPS-mediated innate immune signaling could provide a link between HFD feeding and the faster weight gain. Long-term HFD feeding was shown to increase plasma LPS (Amar et al., 2008; Cani et al., 2007; Erridge et al., 2007; Ghanim et al., 2009), and increasing plasma LPS alone by continuous infusion of LPS could induce mouse weight gain by a similar extent to HFD feeding (Cani et al., 2007). More direct evidence for implication of LPS-sensing in HFD-induced weight gain come from the observation that loss-of-function mutation of Tlr4 or CD14 (a co-receptor required for LPS detection in mammals) protects mice against HFD-induced obesity (Cani et al., 2007; Davis et al., 2008; Radin et al., 2008; Tsukumo et al., 2007). We examined whether the dysregulated LPS/TLR4 signaling due to loss of Akp3 contributed to the faster weight gain phenotype observed in *Akp3*$^{-/-}$ mice receiving HFD.

Materials and Methods

Mice

Animals were maintained and all procedures were performed with approval of the University of Oregon Institutional Animal Care and Use Committee. Pairs of *Akp3*$^{+/-}$ C57BL/6 mice were obtained from Dr. José Luis Millán's group (the Sanford-Burnham Medical Research Institute, La Jolla, CA). Generation of the *Akp3*$^{-/-}$ mice was described previously and the genotyping protocol was established (Narisawa et al., 2003). Pairs of *MyD88*$^{+/-}$ and *CD14*$^{+/-}$ C57BL/6 mice were obtained from the Jackson Laboratory and genotyping was done following instructions described in JAX® Mice database. Heterozygous mice were bred at the University of Oregon animal facility to acquire homozygous mutants and wild type littermates. For HFD feeding, a female *Akp3*$^{-/-}$ mouse was crossed to a male *CD14*$^{-/-}$ mouse to generate *Akp3*$^{+/-}$;*CD14*$^{+/-}$ mice, which were subsequently mated to produce progeny of desired genotypes. Age-matched isogenic germ-free and conventional mouse intestinal tissue samples were provided by Dr. Justin Sonnenburg's lab (Stanford University, Stanford, CA).

ALP Activity Assay

1 cm segments of the proximal duodenum from mice were collected and cut open longitudinally. The lumen side was gently washed with PBS. Cells on the lumen side were harvested using cell scrapers, homogenized in 1 ml lysis buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100), and centrifuged at 14,000 rpm for 15 min at 4°C. The cell-free supernatant was diluted in double distilled H_2O (ddH_2O) and protein concentration was measured using the Bio-Rad protein assay

kit (Bio-Rad Laboratories Inc.). Mouse stools were collected, weighed and homogenized in 1 ml ddH_2O and centrifuged at 14,000 rpm for 15 min at 4°C to collect the supernatant. Diluted supernatants processed from duodenal samples and stools were assayed for ALP activity using the phosphate substrate kit (Thermo Fisher Scientific Inc.). ALP activities were normalized to protein concentrations for duodenal samples, and to mass for stools. Data were analyzed using the Prism software (GraphPad).

LPS Treatment

LPS (purified from *Salmonella enterica* serotype *typhimurium*, Sigma-Aldrich) was dissolved in ddH_2O by 2-min sonication. Age-matched female wild type and *Akp3*$^{+/-}$ mice were deprived of food for 3 hours, and then weighed to calculate volumes of the LPS solution for oral administration. Gavage was done using sterilized 20 G 1 1/2" feeding needles (curved, ball-tipped, Popper & Sons) in 8-week and 10-week-old mice, and sterilized 14 G 1" needles (straight, ball-tipped, Roboz Surgical Instrument) in 4-week-old mice. Water gavage was administered to the control group. 3 hours after gavage mice were euthanized. 1 cm segments of the proximal duodenum from mice were collected for Q-PCR examination of ALPI levels. 2 cm segments of the ileum were collected for Q-PCR examination of inflammatory markers. Mice were treated and tissue samples were collected at the same time.

Y. pseudotuberculosis Infection

The wild type *Y. pseudotuberculosis* strain (YPIII) was provided by Dr. Joan Mecsas (Tufts University, Medford, MA). The infection was performed following the

protocol described previously (Logsdon and Mecsas, 2003). Briefly, female wild type and *Akp3*$^{+/-}$ mice (10 weeks old) were fasted overnight. Infection was performed by gavage at 2×10^9 cfu/animal. Uninfected animals were used as control. 3~5 mice of each group were saved for the weight loss study, and weighed daily for 5 days. To quantify bacterial colonization, infected mice were euthanized 24 hours after the oral inoculation, and Peyer's patches along the small intestine were collected and homogenized in sterile PBS and plated on TSA plates containing 2 μg/ml irgasan to determine cfu/gram of tissue. Data were analyzed using the Prism software (GraphPad). 2 cm segments of the ileum tissue from mice were collected for Q-PCR examination of inflammatory markers. Mice were treated and tissue samples were collected at the same time.

Quantitative PCR (Q-PCR)

Total RNA was isolated from collected mouse intestinal tissues using the PureLink RNA mini kit (Life Technologies Co.) and cDNA was synthesized using the SuperScript III first-strand synthesis system (Life Technologies Co.). Quantitative PCR was performed on the StepOnePlus real-time PCR system (Life Technologies Co.) using the KAPA SYBR fast qPCR kit (Kapa Biosystems). Endogenous reference genes were tested, and *36B4*, *Gapdh* and *Rpl13a* were selected for Q-PCR normalization based on the GeNorm and the Normfinder algorithms using the GenEx software (Multid Analyses AB). Primers used in Q-PCR were as follows: 36B4fwd, 5'-TCCAGGCTTTGGGCATCA-3'; 36B4rev, 5'-CTTTATCAGCTGCACATCACTCAGA-3'; Gapdhfwd, 5'-GCACCACCAACTGCTTAGC-3'; Gapdhrev, 5'-GGCATGGACTGTGGTCATGAG-

3’; Rpl13afwd, 5’-TTCTGGTATTGGATGGCCGAG-3’; Rpl13arev, 5’-ATGTTGATGCCTTCACAGCG-3’; Akp3fwd, 5’-ACATTGCTACACAACTCATCTCC-3’; Akp3rev, 5’-TCCTGCCATCCAATCTGGTTC-3’; Alppl2fwd, 5’-AATGCCTGCCTCAGCGCTACAGGA-3’; Alppl2rev, 5’-TGGTCTGGTGTCCCCTTGGGAA-3’; Alpifwd, 5’-GGCTACACACTTAGGGGGACCTCCA-3’; Alpirev, 5’-AGCTTCGGTGACATTGGGCCGGTT-3’; Tnfαfwd, 5’-AGGCTGCCCCGACTACGT-3’; Tnfαrev, 5’-GACTTTCTCCTGGTATGAGATAGCAAA-3’;Il1βfwd, 5’-GAAATGCCACCTTTTGACAGTG-3’; Il1βrev, 5’-TGGATGCTCTCATCAGGACAG-3’; Il6fwd, 5’-TGGTACTCCAGAAGACCAGAGG-3’; Il6rev, 5’-AACGATGATGCACTTGCAGA-3’; Lcn2fwd, 5’-TGGCCCTGAGTGTCATGTG-3’; and Lcn2rev, 5’-CTCTTGTAGCTCATAGATGGTGC-3’. Primer amplification efficiency was determined using the LinRegPCR software and incorporated to normalize the relative levels of gene expression to endogenous references using the ΔΔCt analysis. Data were analyzed using the Prism software (GraphPad).

High Fat Diet (HFD) Feeding

Male mice of indicated genotypes (at least 10 in each group) from the same generation were fed a 36.0% fat diet (Bio-Serv, F3282/S3282) after weanling. HFD feeding continued for about 6 months and mouse body weights were recorded weekly. Data were analyzed using the Prism software (GraphPad).

Results

Akp3 Is Upregulated by Microbiota

If *Akp3* is upregulated by microbiota, we would expect lower levels of Akp3 in mice raised in the absence of microbes (germ-free mice) as compared with those colonized normally (conventional mice). We thus measured transcription levels of *Akp3* in the duodenum of age-matched isogenic conventional mice and germ-free mice using quantitative real-time PCR (Figure 5A). As expected, we found that germ-free mice had significantly lower levels of *Akp3* transcription comparing to conventional mice. In comparison, no significant differences in *Alppl2* or *Alpi* transcription were observed between germ-free and conventional mice. The results were consistent in two independent experiments (one shown in Figure 5A), suggesting that the microbiota specifically upregulate *Akp3*.

We next investigated whether LPS exposure would induce *Akp3*, similar to the regulation of zebrafish *alpi.1*.We treated wild type mice with water or LPS by oral gavage and compared *Akp3* transcription levels. Interestingly, oral administration of LPS did not stimulate *Akp3* in the mouse duodenum (Figure 5B). Furthermore, we found that *Myd88*$^{-/-}$ mice had similar levels of *Akp3* transcription to their wild type littermates (Figure 5C). Loss of Myd88 has no effects on *Akp3* expression, again in contrast to the regulation of zebrafish *alpi.1*. We conclude that whereas the microbiota upregulate *Akp3* in the mouse intestine, the mechanism is different from that in zebrafish.

Basal Inflammatory Responses in *Akp3*$^{-/-}$ Mice Are Comparable to Those in Wild Type Mice

We used the *Akp3* loss-of-function mice to study the role of ALPI in modulating mouse intestinal inflammatory responses to resident microbiota (basal inflammatory responses) during development. We first confirmed that ALPI activities were significantly reduced in *Akp3*$^{-/-}$ mice. The *in vitro* ALP activity assay showed that as compared to the wild type control, *Akp3*$^{-/-}$ mice had significantly lower ALP activity in duodenal epithelia (Figure 6A) and in stools (Figure 6B). The Duodenal ALP deficiency is consistent with disruption of *Akp3*, and the fecal ALP deficiency suggests reduced ALP activity throughout the intestine of mutant mice. We detected the ALPI deficiency in *Akp3*$^{-/-}$ mice as early as 2 weeks of age, and the deficit sustained into adulthood.

Our hypothesis that mouse ALPI ameliorates host inflammatory responses to the gut microbiota predicts stronger basal inflammatory responses in *Akp3*$^{-/-}$ mouse due to their low ALPI activity. The duodenum, the proximal portion of the small intestine, is sparsely colonized by bacteria (Uhlig and Powrie, 2003) and consequently has the lowest levels of inflammatory responses along the length of the intestine (data not shown). Low colonization also implies that the immunoregulatory role of *Akp3* may be less important in the duodenum. In comparison, the ileum, the terminal portion of the small intestine, harbors a large population of bacteria and *Akp3* could be essential. Since ALPI is secreted into the lumen and functions throughout the intestine, we examined basal inflammatory responses in the mouse ileum. We measured transcription levels of proinflammatory cytokines (Tnf-α, Il-1β and Il-6) and the innate immune protein Lcn2 using quantitative real-time PCR, and compared wild type mice to *Akp3*$^{-/-}$ mice. We detected comparable

levels of the four inflammatory markers in ileal samples from wild type and *Akp3*$^{-/-}$ mice of different ages (Figure 7). Loss of Akp3 does not significantly affect basal inflammatory responses, suggesting that *Akp3* is not required for host immune tolerance to commensal microbes.

Akp3 Is Not Required in Defense against *Yersinia pseudotuberculosis* Infection

Although disruption of *Akp3* has no obvious effect on basal intestinal inflammatory responses in healthy animals, we reasoned that the functional requirement for *Akp3* might be more pronounced when the equilibrium between host and the gut microbiota is severely disturbed, as in the case of infection. We speculated that *Akp3*$^{-/-}$ mice would be more susceptible to Gram-negative pathogen infection due to their lack of ability to dephosphorylate and detoxify LPS.

Yersinia pseudotuberculosis is a well-studied Gram-negative pathogen that colonizes mouse small intestine (Autenrieth and Firsching, 1996; Logsdon and Mecsas, 2003). We infected 10-week-old wild type and *Akp3*$^{-/-}$ mice with *Y. pseudotuberculosis* by oral gavage, and monitored animal weight loss over 5 days. We observed similar weight loss in wild type and *Akp3*$^{-/-}$ mice (Figure 8A), suggesting their equal susceptibility to infection. To look into the infection dynamics, we examined dissemination of *Y. pseudotuberculosis* into Peyer's patches along the mouse small intestine 24 hours after the initial inoculation. We found that *Y. pseudotuberculosis* colonized at comparable levels in Peyer's patches of wild type and *Akp3*$^{-/-}$ mice (Figure 8B), indicating *Akp3* does not prevent translocation of the pathogen. We then assessed ileal inflammatory responses of wild type and *Akp3*$^{-/-}$ mice at this time point, when they

were equally colonized. We found that Tnf-α and Il-1β levels were significantly elevated in wild type and *Akp3*$^{-/-}$ mice; Il-6 also seemed to be induced although not significantly from the basal level in wild type and *Akp3*$^{-/-}$ mice; and interestingly, Lcn2 appeared significantly upregulated only in *Akp3*$^{-/-}$ mice, which might suggest increased sensitivity of *Akp3*$^{-/-}$ mice to *Y. pseudotuberculosis* infection in this specific innate immune response (Figure 8C). Taken together, our data demonstrated no immediate requirement of *Akp3* in defense against *Y. pseudotuberculosis* infection.

Akp3$^{-/-}$ Mice Develop Immune Tolerance to Low Doses of LPS in the Small Intestine

We speculated that the large influx of LPS introduced by oral administration of *Y. pseudotuberculosis* might resemble the severe septic shock situation (Morrison and Ryan, 1987) and that the amount of LPS greatly surpassed the dephosphorylation capacity of the mouse intestine, thus masking possible functional requirements for Akp3 to detoxify LPS in a less extreme situation. To experimentally test this, we challenged mice with high or low doses of LPS.

First we administered a high dose of LPS (*Salmonella typhimurium*, 200 mg/kg body weight) by oral gavage. We expected this would mimic the *Y. pseudotuberculosis* infection procedure and result in similar inflammatory responses in wild type and *Akp3*$^{-/-}$ mice. Indeed, we saw comparable induction of the inflammatory markers Tnf-α, Il-1β and Il-6 and Lcn2 by the large dose of LPS in wild type and *Akp3*$^{-/-}$ mice (Figure 9).

We next challenged mice with a low dose of LPS (*S. typhimurium*, 100 mg/kg body weight), which we reasoned might be effectively dephosphorylated only in wild type mice and result in stronger inflammatory responses in *Akp3*$^{-/-}$ mutants. On the

contrary, we observed that after the low dose LPS gavage all four inflammatory markers examined were significantly upregulated in wild type mice but not in *Akp3*$^{-/-}$ mice (Figure 10). Whereas the basal levels of these inflammatory markers were similar in the control groups of both genotypes, after LPS gavage wild type mice showed significantly higher levels of Tnf-α, Il-1β and Lcn2 in the small intestine than *Akp3*$^{-/-}$ mice.

The hyporesponsiveness of the intestinal innate immune system after the low dose LPS gavage in adult *Akp3*$^{-/-}$ mice (10 weeks old) reminded us of a well-recognized phenomenon, endotoxin tolerance. Endotoxin/LPS tolerance describes a situation where animals or innate immune cells become refractory to endotoxin challenge after a prior exposure to small amounts of LPS (Fan and Cook, 2004; Ziegler-Heitbrock, 1995). We reasoned that the reduced LPS dephosphorylation activity of *Akp3*$^{-/-}$ mouse intestine resulted in higher concentrations of phosphorylated microbiota-derived LPS, and this continuous exposure to more immunostimulatory LPS during development trained mouse intestinal innate immune cells to be less responsive to further LPS challenge. To test this theory, we examined intestinal inflammatory responses to small doses of LPS in younger mice. If there was a process of training, we would not see LPS tolerance at early stages of development. As expected, we found that low dose LPS gavage caused similar inflammatory responses in the small intestine of wild type and *Akp3*$^{-/-}$ mice at 19 days and 4 weeks old (Figure 11 & 12). Interestingly, 8-week-old *Akp3*$^{-/-}$ mice exhibited "partial" tolerance to the low dose LPS challenge (Figure 13): whereas Tnf-α and Il-1β were induced to a similar degree as seen in the wild type mice, Il-6 and Lcn2 levels remained low, in contrast to the significant upregulation in the wild type mice. Together, our data demonstrated that *Akp3*$^{-/-}$ mice acquired immune tolerance to low doses of LPS

through a long period of development, suggesting a role of *Akp3* in educating the intestinal immune system.

LPS-Induced Innate Immune Signaling Is Required for the Accelerated Weight Gain of *Akp3*$^{-/-}$ Mice Maintained on HFD

We proposed that the slightly more active LPS-induced innate immune signaling in *Akp3*$^{-/-}$ mice, stimulated by accumulated intact LPS due to the ALPI deficiency, led to faster weight gain when animals were kept on HFD. To test this hypothesis, we asked whether the HFD-induced accelerated weight gain in *Akp3*$^{-/-}$ mice was dependent on their ability to sense LPS. We reasoned that if we blocked LPS-sensing in *Akp3*$^{-/-}$ mice by mutating the CD14 co-receptor, they should no longer be affected by the slightly elevated phosphorylated LPS levels and should be resistant to the HFD-induced faster weight gain. We thus fed HFD to wild type, *Akp3*$^{-/-}$, *CD14*$^{-/-}$, and *Akp3*$^{-/-}$;*CD14*$^{-/-}$ mice, and compared their weight gains over time (Figure 14). As reported, *Akp3*$^{-/-}$ mice showed faster weight gain as compared to wild type mice. But we found that the weight gain curve of *Akp3*$^{-/-}$;*CD14*$^{-/-}$ mice was statistically indistinguishable from that of wild type or *CD14*$^{-/-}$ mice. The results demonstrate that the LPS-sensing machinery is required for the HFD-induced accelerated weight gain in *Akp3*$^{-/-}$ mice.

Discussion

Microbiota regulate a great number of genes in animal intestines (Larsson et al., 2012; Mutch et al., 2004). Here we show that the mouse microbiota specifically upregulate *Akp3*, which could explain its unique expression patterns among mouse *ALPI*

genes. But in contrast to zebrafish where *alpi.1* is induced by LPS-mediated innate immune signaling (Bates et al., 2007), mouse *Akp3* is upregulated by microbiota independent of innate immunity. The discrepancy is not surprising considering the evolution history of the two *ALPI* genes (Yang et al., 2012). *Akp3* arises from a recent duplication of mouse gene *Alpi*, long after the divergence of zebrafish *alpi.1* and mouse *Alpi*. The newly-acquired *Akp3* is very likely regulated by different mechanisms from *alpi.1*. In addition, mammals and teleosts have evolved different strategies for immune recognition of bacteria (Sullivan et al., 2009), indicating that LPS or the microbiota may not act in a conserved way to modulate *ALPI* genes.

Microbiota have profound influences on host gut development. Intestinal epithelial cell proliferation is reduced in germ-free mice (Uribe et al., 1997). However, the reduced *Akp3* expression in germ-free mice was unlikely due to their developmental defects because otherwise *Alppl2* or *Alpi* would also be downregulated in germ-free mice. Instead, we observed that *Alppl2* and *Alpi* were not affected by microbiota colonization. Many dietary components like fat (Eliakim et al., 1991; Kaur et al., 2007; Vazquez et al., 2000), and micronutrients like calcium (Brun et al., 2012), have been reported to modulate ALPI expression or activity. We propose that the mouse microbiota upregulate *Akp3* through metabolic interactions with the host. The gut microbiota perform essential metabolic functions that are not encoded in the host genome, including the processing of complex proteins and indigestible dietary fibers, and synthesis of vitamins like vitamin K (Resta, 2009). Interestingly, vitamin K was reported to induce ALPI activity in rodents (Haraikawa et al., 2011; Sogabe et al., 2007). And specifically, oral administration of vitamin K boosted *ALPI* gene expression in the mouse intestine, with a more dramatic

effect on *Akp3* than *Alpi* (Haraikawa et al., 2011). We speculate that production of vitamin K, which requires the gut microbiota but is independent of host immune responses, may be one mechanism by which microbiota upregulate *Akp3*. And being more a potent inducer of *Akp3* than *Alpi*, vitamin K when synthesized by the gut microbiota at the physiological levels may only be adequate to significantly elevate *Akp3* expression but not *Alpi*, which could explain the specific upregulation of *Akp3* by microbiota.

Akp3 is not required for establishing immune tolerance to resident microbiota in the mouse intestine, as suggested by the similar basal inflammatory responses in wild type and *Akp3*$^{-/-}$ mice. We reasoned that the considerable level of ALP activity retained in *Akp3*$^{-/-}$ mouse intestine, presumably from *Alppl2* and *Alpi*, was adequate to effectively prevent excessive inflammatory responses to the gut microbiota. *Alppl2* and *Alpi* are reported to be upregulated in *Akp3*$^{-/-}$ mice (Narisawa et al., 2007). Thus, the *ALPI* gene redundancy and the supplementary mechanism for loss of one gene may combine to keep the inflammatory responses in check in *Akp3*$^{-/-}$ mice. The unaffected basal inflammatory responses in the small intestine after disruption of *Akp3* also imply, from another perspective, the robustness of the equilibrium between host immunity and the gut microbiota.

Exogenous ALPI treatment has been reported to preserve the normal gut barrier function (Kaliannan et al., 2013; Ramasamy et al., 2011; Rentea et al., 2012b) and prevent gut bacterial translocation after experimental injury in rodent models (Martinez-Moya et al., 2012). Consistent with the role of ALPI in promoting the gut barrier function, disruption of *Akp3* caused increased gut permeability (Kaliannan et al., 2013)

and more gut bacterial translocation into mesenteric tissues after intestinal ischemia/reperfusion injury (Goldberg et al., 2008). Here we observed that *Akp3* provided no protection against *Y. pseudotuberculosis* invasion into Peyer's patches. We think that these results are not contradictory but could be representative of the fundamental differences between commensal bacteria and virulent pathogens. In the injury models, ALPI elicited protective effects by promoting the intestinal epithelial integrity, which was competent to block the passive flow-through of the commensal bacteria. However, pathogens like *Y. pseudotuberculosis* have developed sophisticated apparatus to breach the gut barrier, making the role of ALPI trivial during infection.

With respect to intestinal inflammatory responses to *Y. pseudotuberculosis* infection, our results again showed that *Akp3* had no beneficial effects. This further demonstrated that *Akp3* plays little role in defense against the pathogen infection. We speculated that the large amount of LPS introduced by *Y. pseudotuberculosis* inoculation exceeded the dephosphorylation and detoxification capacity of the mouse intestine and experimentally tested this by administering a high dose of LPS to mice. We showed that disruption of *Akp3* did not affect mouse intestinal inflammatory responses to the high dose LPS, confirming a limited immunoregulatory role of the endogenous ALPI. This could explain why exogenous supplementation of ALPI is usually necessary to elicit beneficial effects in various injury and disease models.

Although *Akp3* is not required at the host-microbiota equilibrium or in defense against *Y. pseudotuberculosis* infection, we demonstrated an important role of *Akp3* in educating the mouse immune system. By characterizing intestinal inflammatory responses to low doses of LPS in developing animals, we showed that *Akp3*$^{-/-}$ mice

acquired immune tolerance to intestinal LPS through a long period of development and that LPS desensitization did not occur until about 8 – 10 weeks of age. We reason that the ALPI deficiency in *Akp3*$^{-/-}$ mice causes increased levels of phosphorylated LPS in the gut lumen, which induces a slightly more active innate immune response and in the long term trains the intestinal immune system to be less responsive to additional LPS challenge.

LPS desensitization could benefit *Akp3*$^{-/-}$ mice by saving energy when mice confront commensal gut bacteria, but may also profoundly influence animal responses to infections. Mice with induced endotoxin tolerance were reported to be more resistant to *S. typhimurium* and *Cryptococcus* infections (Lehner et al., 2001; Rayhane et al., 2000), yet more susceptible to *Escherichia coli* (Lu et al., 2008). Correspondingly, *S. typhimurium* was seen to caused more dramatic weight loss and bacterial translocation in wild type mice as compared with *Akp3*$^{-/-}$ mice (Chen et al., 2011). The resistance of *Akp3*$^{-/-}$ mice to *S. typhimurium* infection, in contrast to their similar susceptibility to *Y. pseudotuberculosis* as compared to wild type mice, suggests that these results are pathogen-specific. And considering only adult *Akp3*$^{-/-}$ mice exhibit immune tolerance to LPS, their resistance to *S. typhimurium* infection could also depend on the developmental stage. We speculate that adult *Akp3*$^{-/-}$ mice could be more vulnerable to *E. coli* infection, and *Akp3*$^{-/-}$ pups that haven't developed LPS tolerance might not be resistant to *S. typhimurium* infection.

ALPI activities are inversely related to the obesity proneness in rats receiving HFD (de La Serre et al., 2010; Sefcikova et al., 2008). Analogously, mice lacking Akp3 exhibited accelerated weight gain when maintained on HFD as compared to wild type mice (Narisawa et al., 2003). A direct role of mouse *ALPI* gene *Alpi* in promoting fatty

acid intake was proposed and upregulation of *Alpi* in *Akp3*$^{-/-}$ mice could contribute to the HFD-induced faster weight gain (Hansen et al., 2007; Lynes et al., 2011; Narisawa et al., 2007). However, it is not clear whether the elevation of Akp6 itself is sufficient to cause the HFD-induced phenotype in *Akp3*$^{-/-}$ mice, and other factors may kick in. Here we tested the contribution of LPS-induced innate immune signaling and we showed that by blocking LPS-sensing we abrogated the faster weight gain of *Akp3*$^{-/-}$ mice receiving HFD. This provides direct evidence that LPS-induced innate immune signaling is required for the HFD-induced phenotype of *Akp3*$^{-/-}$ mice, and implies that a subtle impairment in LPS dephosphorylation due to ALPI deficiency can manifest, in the long term, in significant physiological differences among individuals. The elevated LPS signaling might also underlie the inverse relationship between ALPI activities and the obesity proneness of rats receiving HFD and it would be interesting to evaluate contributions of LPS sensing in these models.

In conclusion, we have discovered that the mouse *ALPI* gene *Akp3* is specifically upregulated by microbiota, but the mechanisms are different from those observed in zebrafish. We have showed that disruption of *Akp3* leads to immune desensitization to luminal LPS in adult mice, suggesting that *Akp3* plays a role in immune education. Lastly, we have demonstrated elevated LPS/TLR signaling leads to the HFD-induced accelerated weight gain in *Akp3*$^{-/-}$ mice.

CHAPTER IV

NEONATAL ALPI AND NECROTIZING ENTEROCOLITIS

This chapter includes work that will be published with co-authors, R. Bent and K. Guillemin. K. Guillemin designed the research. I performed the experimental work. R. Bent organized meconium sample collection. The writing is mine. K. Guillemin provided editorial assistance.

Introduction

Necrotizing enterocolitis (NEC) is a multifactorial disease that predominately affects premature infants (Beeby and Jeffery, 1992) and is a leading cause of late mortality and morbidity in very preterm infants (Caplan, 2008; Christensen et al., 2010; Guthrie et al., 2003; Morgan et al., 2011). Despite advances in neonatal care and medical research, the incidence of NEC has increased (Petrosyan et al., 2009) and the mortality rate has not significantly changed (Blakely et al., 2005; Holman et al., 1997; Holman et al., 2006). The most important risk factor NEC is prematurity (Henry and Moss, 2009) and the incidence of NEC is inversely related to an infant's gestational age (Gregory et al., 2011; Lin and Stoll, 2006; Llanos et al., 2002).

Although the etiology of NEC is not clearly defined (Dominguez and Moss, 2012; Gephart et al., 2012), NEC is believed to represent a severe inflammatory disorder in the intestine (Ballance et al., 1990; Zhang et al., 2011). Excessive inflammatory responses to environmental insults in the immature intestine are a hallmark of NEC (Chan et al., 2009). Specifically, increased levels of LPS/TLR4 signaling have been suggested to

contribute to the pathogenesis of NEC (Chan et al., 2009; Fusunyan et al., 2001; Leaphart et al., 2007; Nanthakumar et al., 2011). And inhibition of LPS/TLR4 signaling attenuates intestinal inflammation and mitigates NEC pathology in animal models (Chan et al., 2009; Gribar et al., 2009).

ALPs been shown by many studies to inhibit LPS-induced TLR4 activation by dephosphorylating LPS (Lalles, 2010). Interestingly, a dynamic transition of ALPI isozyme forms is associated with the maturation of fetal intestine (Mulivor et al., 1978; Sugiura et al., 1981), implying ALPI activity could change with fetal development. We hypothesized that ALPI activities in neonates increased with gestational age, *i.e.*, preterm neonates have lower ALPI activities than full-term infants. We reasoned that the ALPI deficiency in preterm neonates could lead to overactive LPS/TLR4 signaling due to decreased LPS-detoxifying activity in the premature intestine colonized by microbes. We tested the hypothesis by comparing meconium ALP activities from infants of different gestational ages. Meconium, the first stool of a newborn infant, is primarily composed of water, intestinal epithelial cells and mucus, and thus provides an accurate measurement of the ALP activity in the neonatal intestine.

Materials and Methods

The use of meconium samples for this study was reviewed by the University of Oregon Institutional Review Board and Research Compliance Services and determined to qualify for an exemption as per Title 45 CFR Part 46.101 (b)(4). A total of 122 meconium samples from infants of gestational ages ranging from 24 to 41 weeks were collected from the Peacehealth Neonatal Intensive Care Unit and the Peacehealth Nurse

Midwifery Birth Center (Springfield, OR). Samples were frozen upon collection and subsequently analyzed as follows. The samples were homogenized in double distilled H_2O and centrifuged at 14,000 rpm for 15 min at 4°C to collect the supernatants. The supernatants were then diluted and assayed for protein concentrations using the Bio-Rad protein assay kit (Bio-Rad Laboratories Inc.) and ALP activities using the PNPP substrate kit (Thermo Fisher Scientific Inc.). ALP activities were compared to standard shrimp alkaline phosphatase (Thermo Fisher Scientific Inc.) and normalized to meconium protein concentrations. Data were grouped by weeks of completed gestation at birth, and plotted using the Prism software (GraphPad software). Correlation between meconium ALP activities and gestational age was analyzed using one-way ANOVA (post test for linear trend). Meconium ALP deficiency in premature newborns (gestational age ≤ 35 weeks) was determined using one-way ANOVA (followed by Tukey's multiple comparison test) and one-tailed *t*-test.

Results and Discussion

Figure 15 shows the average meconium ALP activities, normalized to total protein concentrations, of meconium samples grouped by weeks of gestation. There is a strong positive correlation between meconium ALP activity and gestational age ($p < 0.0001$; one-way ANOVA and post test for linear trend). When comparing ALP activities spanning the gestational age from 24 weeks to 41 weeks, we found the samples were statistically separated into two groups, the preterm group (gestational age ≤ 35 weeks) and the full-term group (gestational age ≥ 36 weeks) (one-way ANOVA and Tukey's multiple comparison test). A comparison of all meconium samples from full-term infants

(gestational age ≥ 36 weeks, n=34), with all samples from premature infants (gestational age ≤ 35 weeks, n=88) reveals the latter group to have significantly lower ALP activities ($p < 0.0001$; one-tailed *t*-test).

Our data indicate that premature infants have reduced ALPI activity as compared to full-term infants. We speculate that the reduced capacity of preterm infant intestines to dephosphorylate proinflammatory LPS may increase the risk of excessive inflammatory responses to bacterial colonization and NEC development. Based on these findings, we propose that prophylactic ALPI supplementation to premature infants may warrant further study as a strategy for decreasing the risk of NEC. Indeed, lab studies using rat models have demonstrated the protective effects of ALPI administration against intestinal inflammation and injury in experimental NEC (Rentea et al., 2013; Rentea et al., 2012a; Rentea et al., 2012b; Riggle et al., 2013; Whitehouse et al., 2010). Future prospective studies that record meconium ALPI activities of infants who go on to develop NEC or remain healthy may provide additional insights into the possible utility of ALPI supplementation for premature neonates.

CHAPTER V

CONCLUSION

We characterized four *ALP* genes in zebrafish, *alpl*, *alpi.1*, *alpi.2* and *alp3*. We found the four genes were distributed among three vertebrate *ALP* gene clades. The ALPL clade, which includes zebrafish *alpl*, remains relatively stable among species. The ALPI clade, which includes zebrafish *alpi.1* and *alpi.2*, has undergone rapid evolution. And the ALP3 clade, which includes zebrafish *alp3*, is maintained in teleosts but lost in tetrapods. We propose that the distinct evolution histories of the three clades are derived from different functional requirements for ALPL versus ALPI. The dramatic changes of the ALPI clade in vertebrates imply diversified roles of individual *ALPI* genes, which we have developed reagents and protocols to explore in zebrafish. In mice, we found that the *ALPI* gene *Akp3* is specifically induced by microbiota, a process independent of host immune recognition of microbiota. We propose that microbiota-mediated metabolic functions stimulate *Akp3* expression. We show that loss of Akp3 has little effect on intestinal inflammatory responses in animals maintained routinely or infected by *Y. psedotuberclosis*, but the ALPI deficiency due to disruption of one *ALPI* gene causes subtle elevation of LPS-mediated innate immune signaling, which in the long term results in LPS tolerance and weight gain. And finally, we report a positive correlation between meconium ALP activity and gestational age, suggesting preterm neonates have reduced ALPI activity, which could predispose them to inflammation and NEC.

APPENDIX

FIGURES

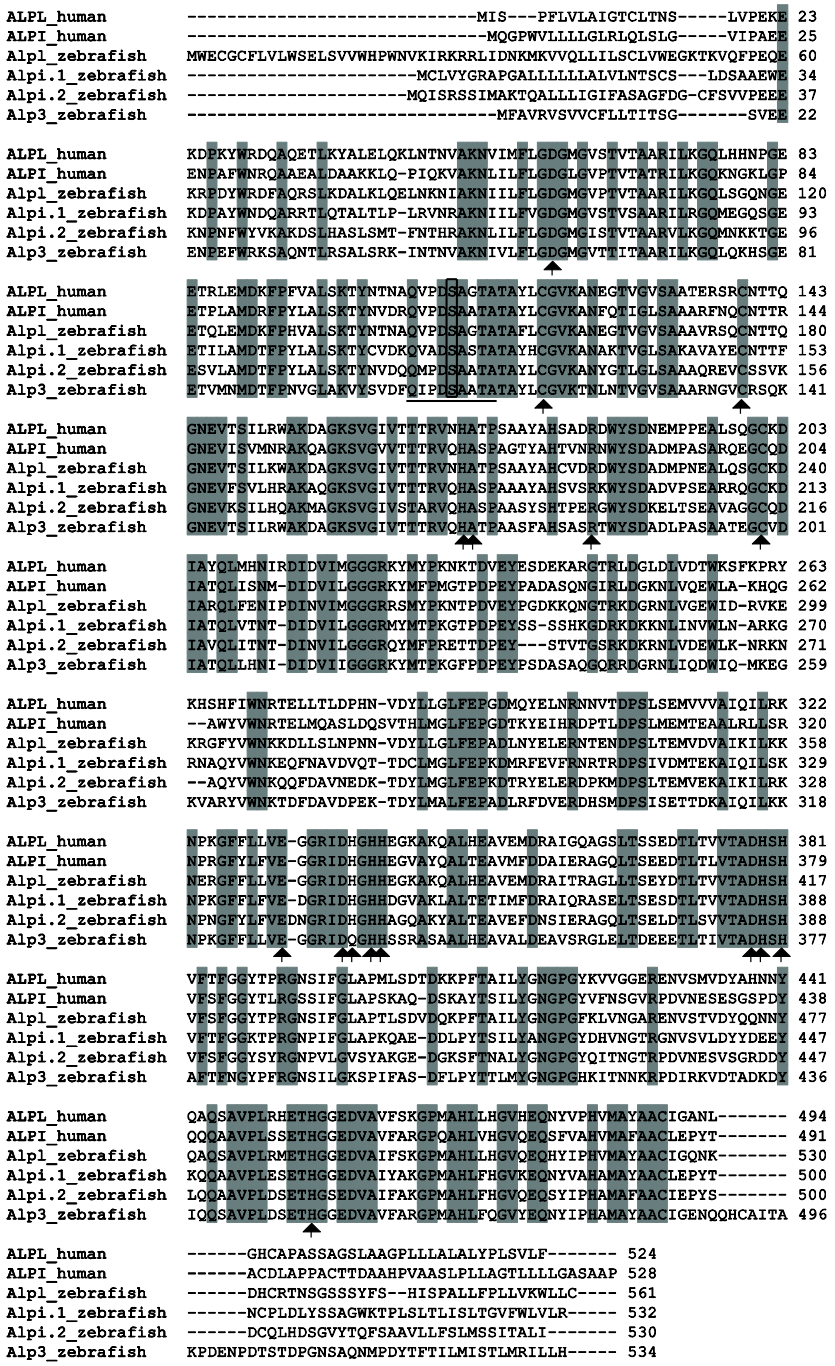

Figure 1. Sequence alignment of human and zebrafish ALPs. Identical amino acids are shaded gray, putative metal-binding sites are indicated by arrows, active sites are underlined, and the conserved serine required for enzyme activity is boxed.

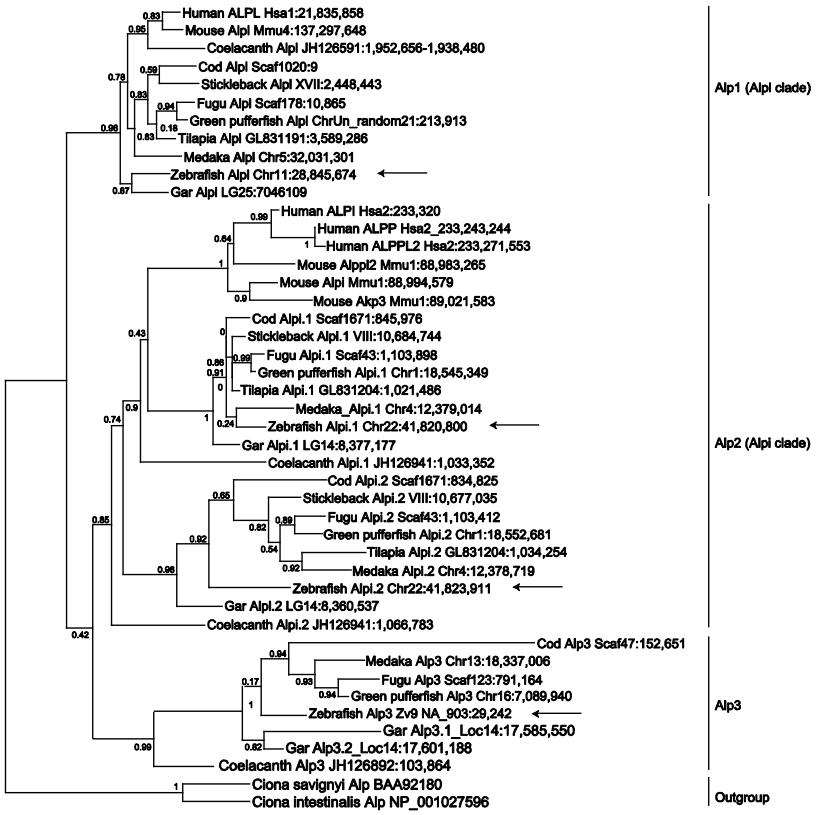

Figure 2. Diversification of vertebrate ALP protein sequences. Sequences are indicated on this maximum likelihood tree with the species name, gene name, and genomic location. For example, the taxon 'Human ALPL Hsa1:21,835,858' indicates the start of the human *ALPL* gene encoding this protein on human chromosome 1 at nucleotide position 21,835,858 according to Ensembl Release 68 (July 2012) and Pre Ensembl Release 66 (April 2012). The tree has three major clades (Alp1 (Alpl clade), Alp2 (Alpi clade), and Alp3). Arrows point to the four zebrafish *ALP* genes.

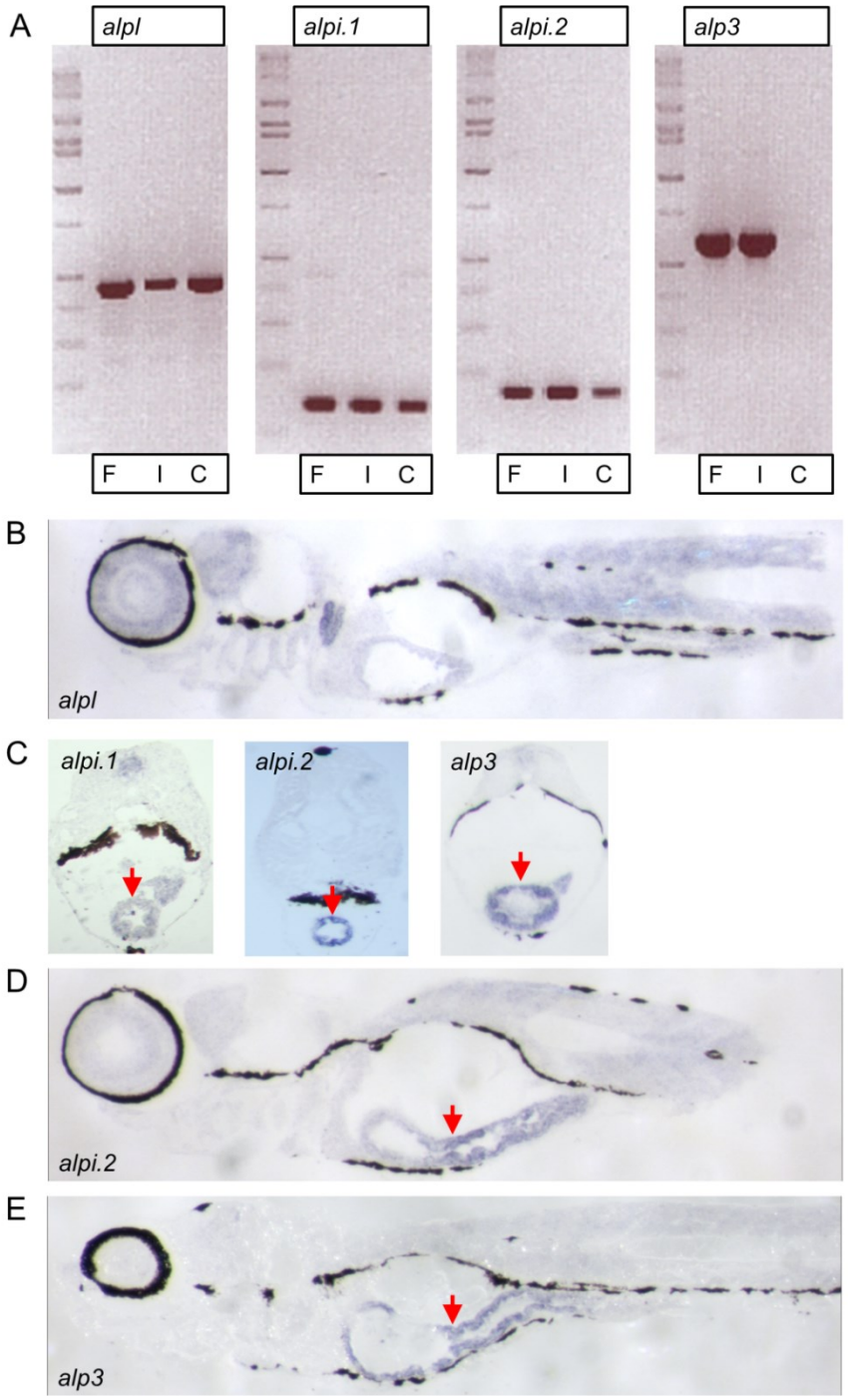

Figure 3. Expression of zebrafish *ALP* genes. (A) Semi-quantitative reverse transcription PCR analysis of *alp* gene transcript levels at 7 days post fertilization (dpf) in whole fish (F), dissected intestines (I), and carcasses with intestine removed (C). *In situ* hybridization of 7 dpf larval sagittal **(B, D, E)** and transverse **(C)** sections with probes to *alpl*, *alpi.1*, *alpi.2* and *alp3* as indicated. The hybridization is visible in blue. Arrows point to the intestinal epithelium.

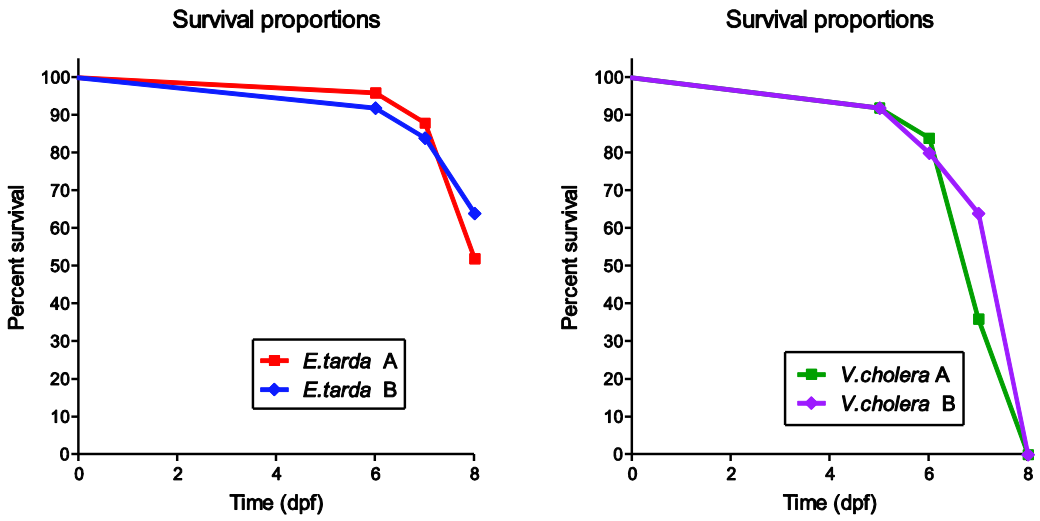

Figure 4. Zebrafish survival curves in infection. Inoculation of rifampicin-resistant E. tarda (left) and V. cholera (right) gave rise to reproducible survival curves. Zebrafish embryos were inoculated at 3 dpf and mortality was monitored till 8 dpf. Survival curves shown were obtained from two independent experiments (A & B).

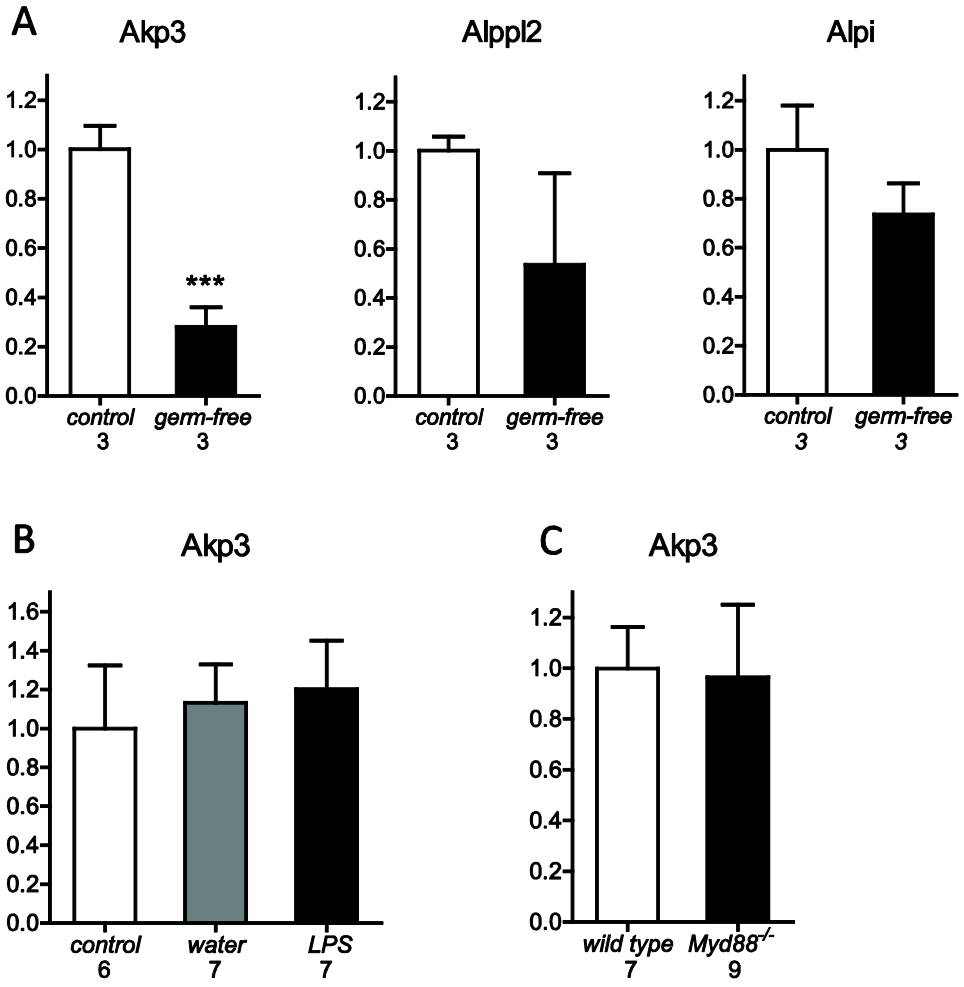

Figure 5. Duodenal transcription levels of mouse *ALPI* genes. (**A**) Comparison of *Akp3*, *Alppl2* and *Alpi* levels between 4-week-old control and germ-free mice. (**B**) Comparison of *Akp3* transcription levels between 8-week-old control, water-treated, and LPS-treated wild type mice. (**C**) Comparison of *Akp3* transcription levels between 4-week-old wild type and *Myd88*$^{-/-}$ mice. Error bars represent standard deviations. The number below the graph indicates the sample size. Asterisks mark the significant difference (***, $p < 0.001$; student's *t*-test).

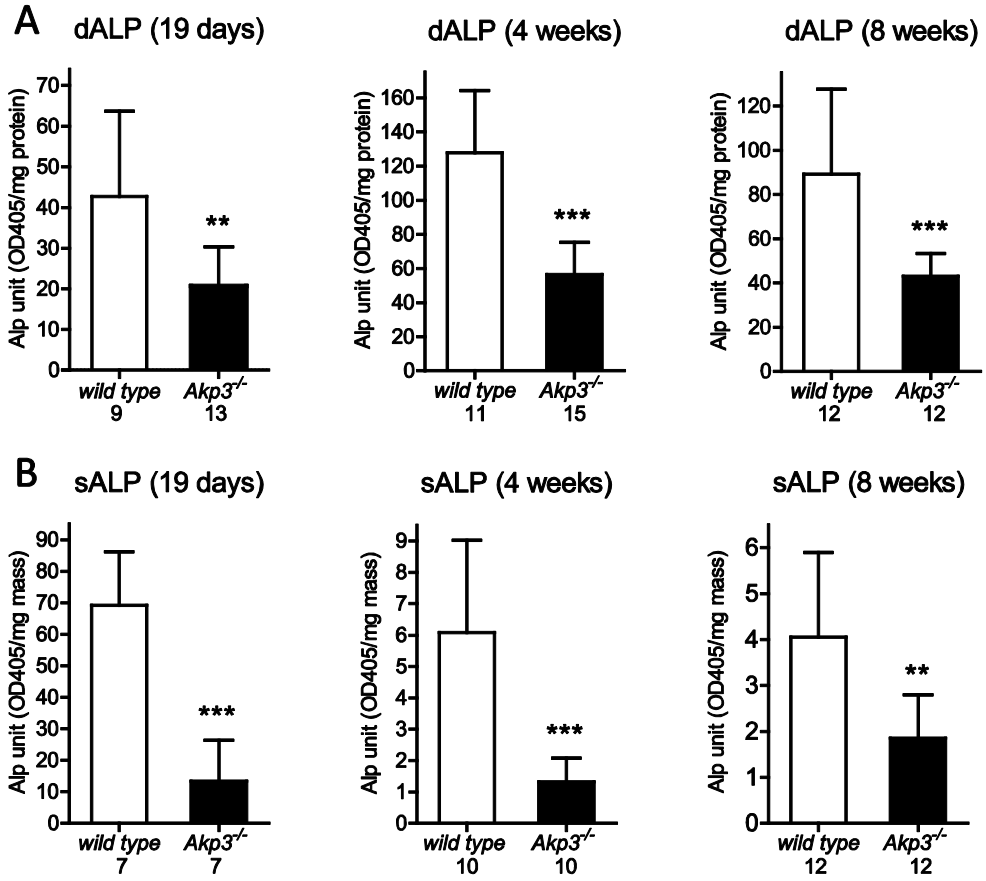

Figure 6. Mouse ALPI activities. Comparison of ALP activities between wild type and *Akp3*$^{-/-}$ mice in **(A)** duodenal epithelia (dALP) and **(B)** stools (sALP), at different ages as indicated at the top of each bar graph. Error bars represent standard deviations. The number below the graph indicates the sample size. Asterisks mark the significant difference (**, $p < 0.01$; ***, $p < 0.001$; student's *t*-test).

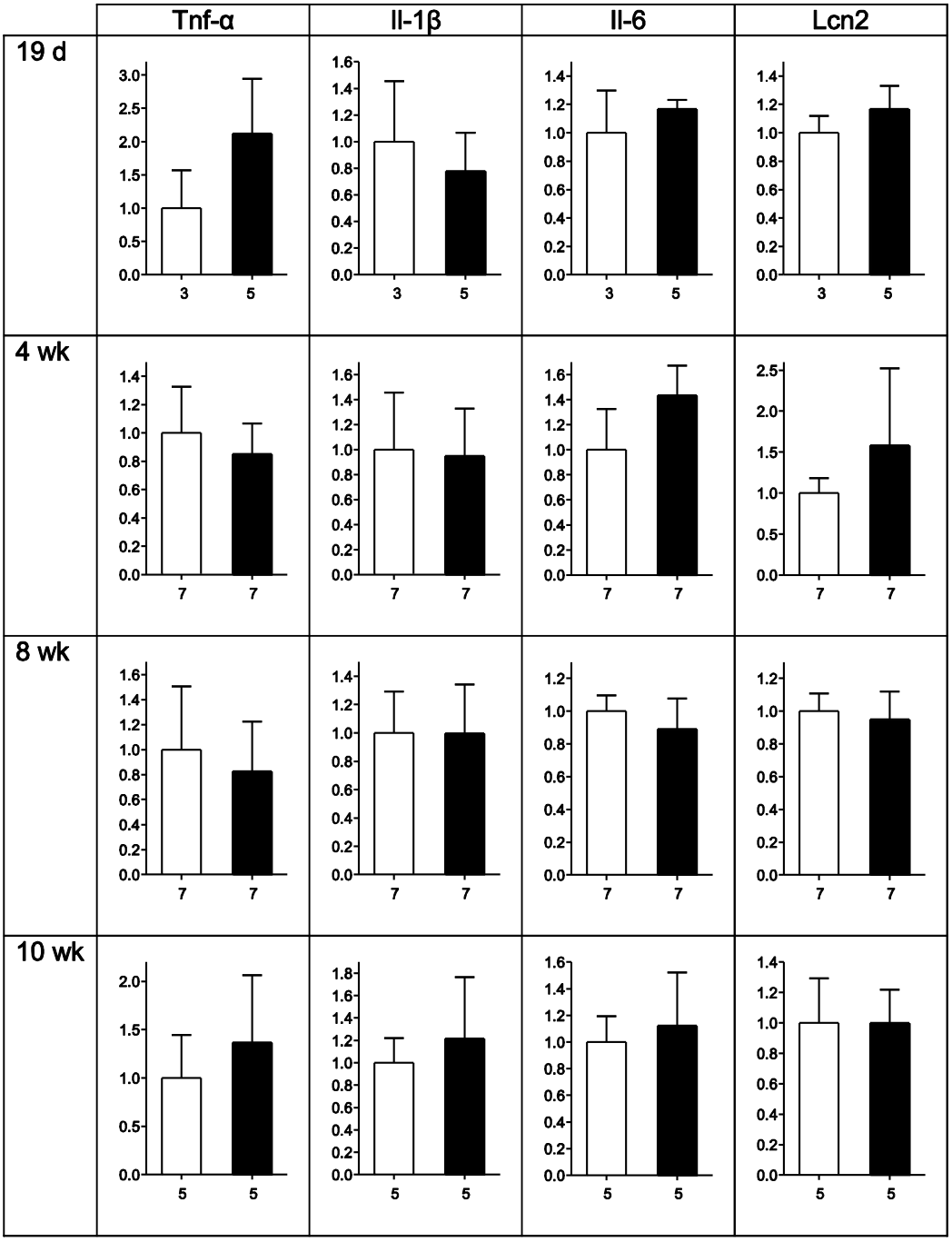

Figure 7. Basal intestinal inflammatory responses. Comparison of ileal transcriptional levels of Tnf-α, Il-1β, Il-6 and Lcn2 between wild type (white column) and *Akp3*$^{-/-}$ mice (black column) at different ages. Error bars represent standard deviations. The number below the graph indicates the sample size.

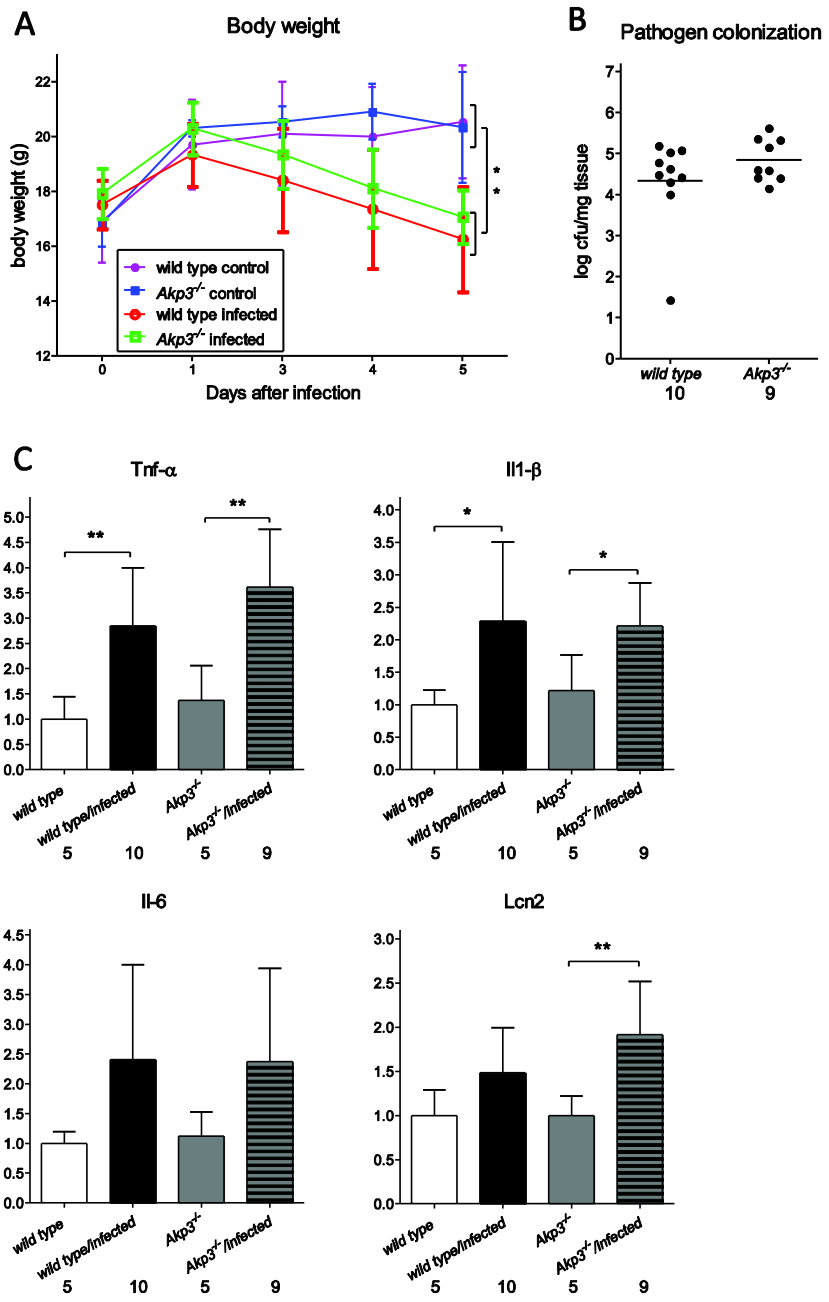

Figure 8. ***Yersinia pseudotuberculosis*** **infection. (A)** Body weight curves of control or infected wild type and *Akp3*$^{-/-}$ mice. Error bars represent standard deviations. **(B)** Colonization of *Yersinia pseudotuberculosis* in Peyer's patches. Each dot represents a mouse. **(C)** Real-time PCR quantification of transcription levels of Tnf-α, Il-1β, Il-6 and Lcn2 in the small intestine of control or infected wild type and *Akp3*$^{-/-}$ mice (10 weeks old). Error bars represent standard deviations. The number below the graph indicates the sample size. Asterisks mark the significant difference (*, $p < 0.05$; **, $p < 0.01$; one-way ANOVA followed by Bonferroni test).

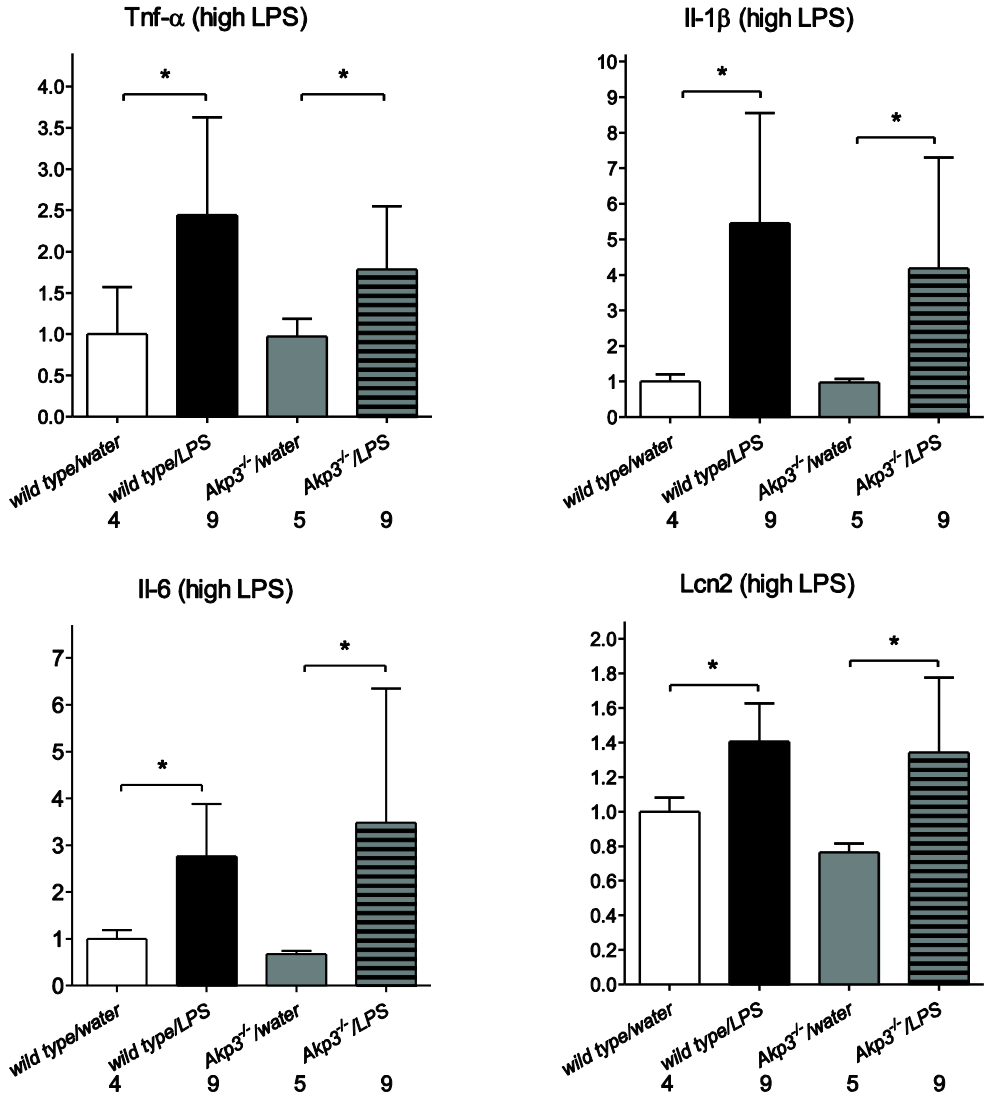

Figure 9. High dose LPS treatment in 10-week-old mice. Real-time PCR quantification of transcription levels of Tnf-α, Il-1β, Il-6 and Lcn2 in the small intestine of water- or high dose LPS-treated wild type and *Akp3*$^{-/-}$ mice (10 weeks old). Error bars represent standard deviations. The number below the graph indicates the sample size. Asterisks mark the significant difference (*, $p < 0.05$; one-way ANOVA followed by Bonferroni test).

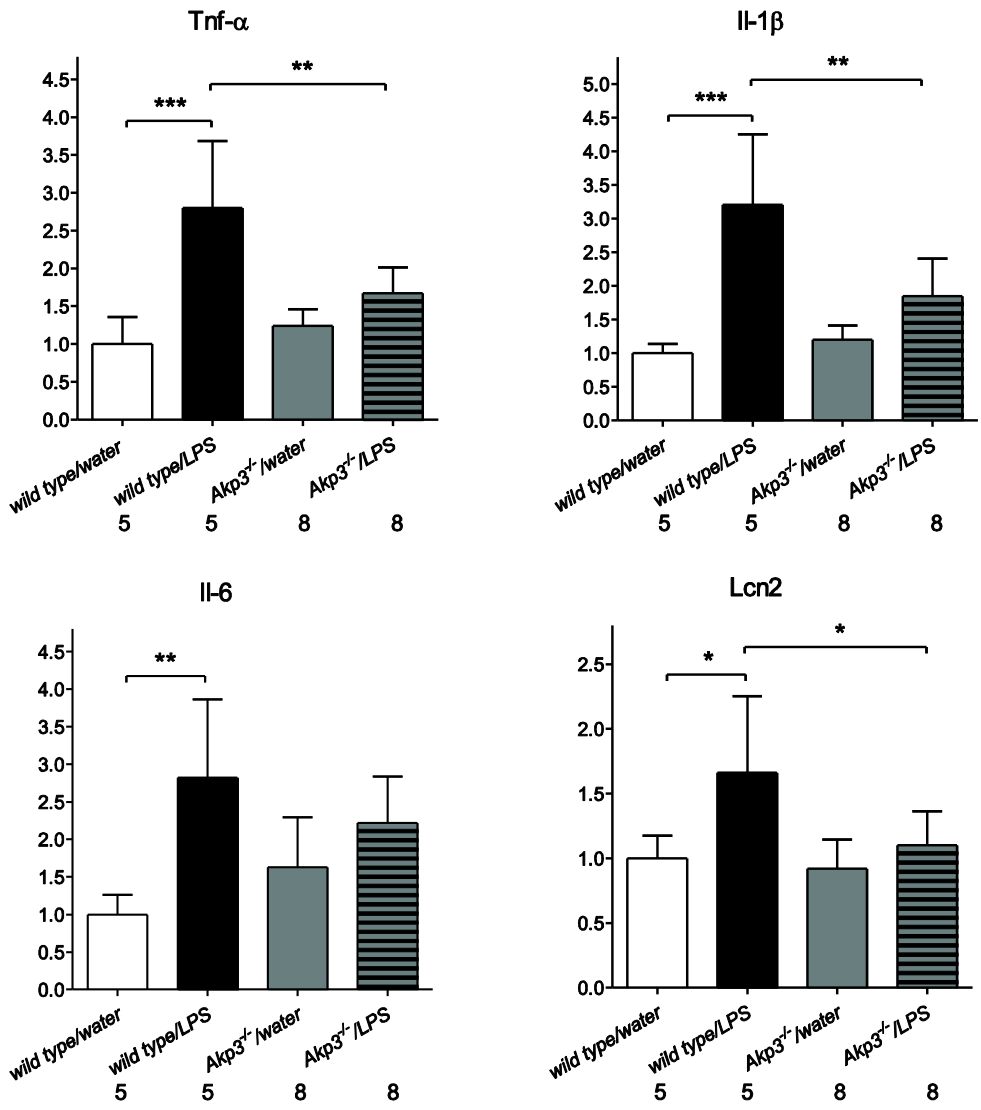

Figure 10. Low dose LPS treatment in 10-week-old mice. Real-time PCR quantification of transcription levels of Tnf-α, Il-1β, Il-6 and Lcn2 in the small intestine of water- or low dose LPS-treated wild type and *Akp3*$^{-/-}$ mice (10 weeks old). Error bars represent standard deviations. The number below the graph indicates the sample size. Asterisks mark the significant difference (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; one-way ANOVA followed by Bonferroni test).

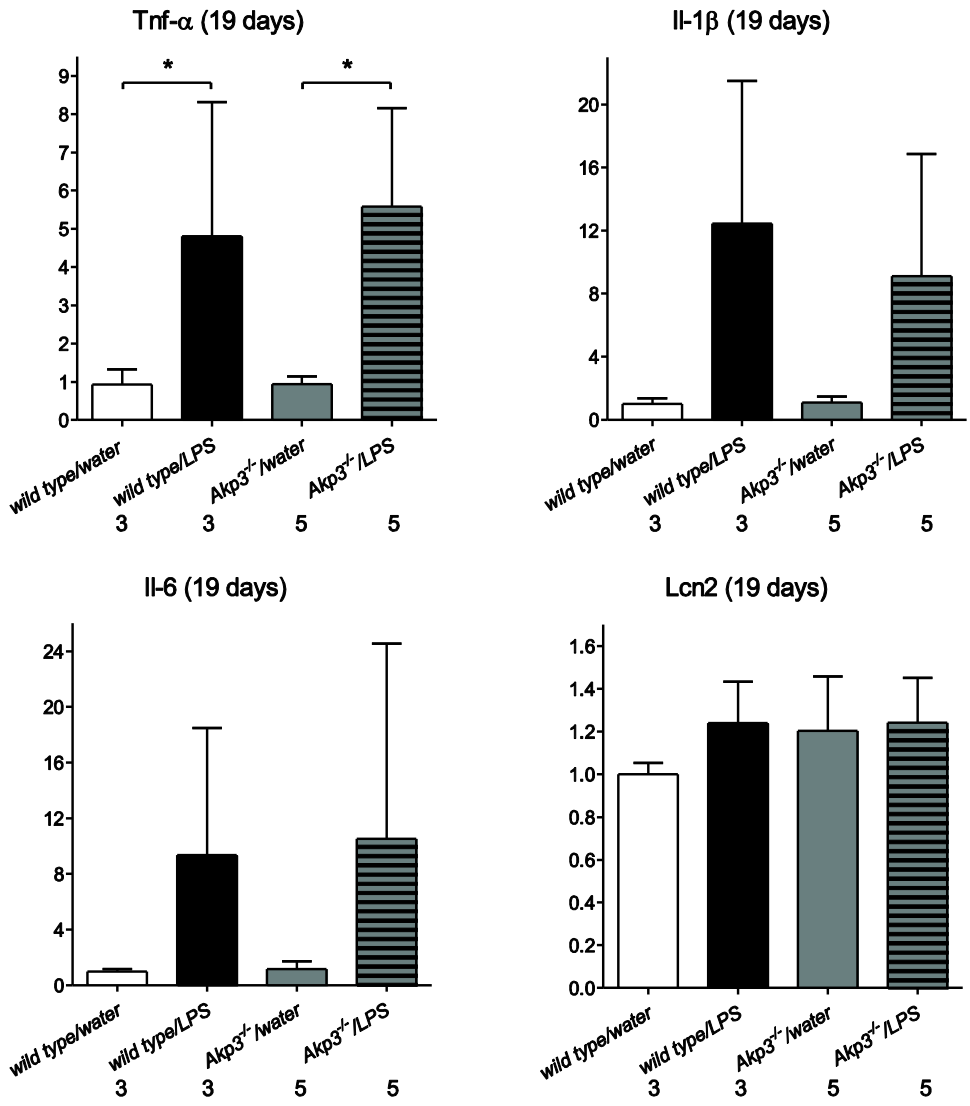

Figure 11. Low dose LPS treatment in 19-day-old mice. Real-time PCR quantification of transcription levels of Tnf-α, Il-1β, I-l6 and Lcn2 in the small intestine of water- or low dose LPS-treated wild type and *Akp3*$^{-/-}$ mice at 19 days old. Error bars represent standard deviations. The number below the graph indicates the sample size. Asterisks mark the significant difference (*, $p < 0.05$; one-way ANOVA followed by Bonferroni test).

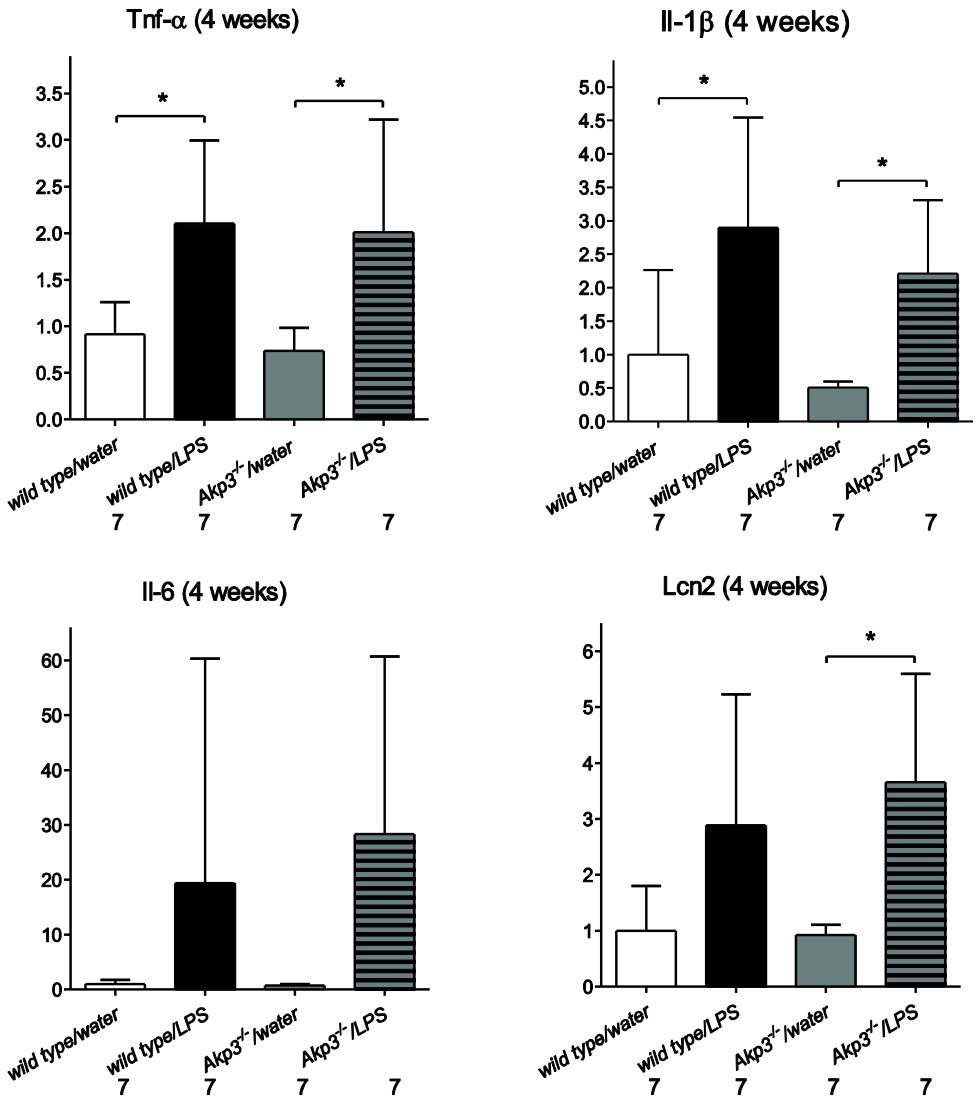

Figure 12. Low dose LPS treatment in 4-week-old mice. Real-time PCR quantification of transcription levels of Tnf-α, Il-1β, I-l6 and Lcn2 in the small intestine of water- or low dose LPS-treated wild type and *Akp3*$^{-/-}$ mice at 4 weeks old. Error bars represent standard deviations. The number below the graph indicates the sample size. Asterisks mark the significant difference (*, $p < 0.05$; one-way ANOVA followed by Bonferroni test).

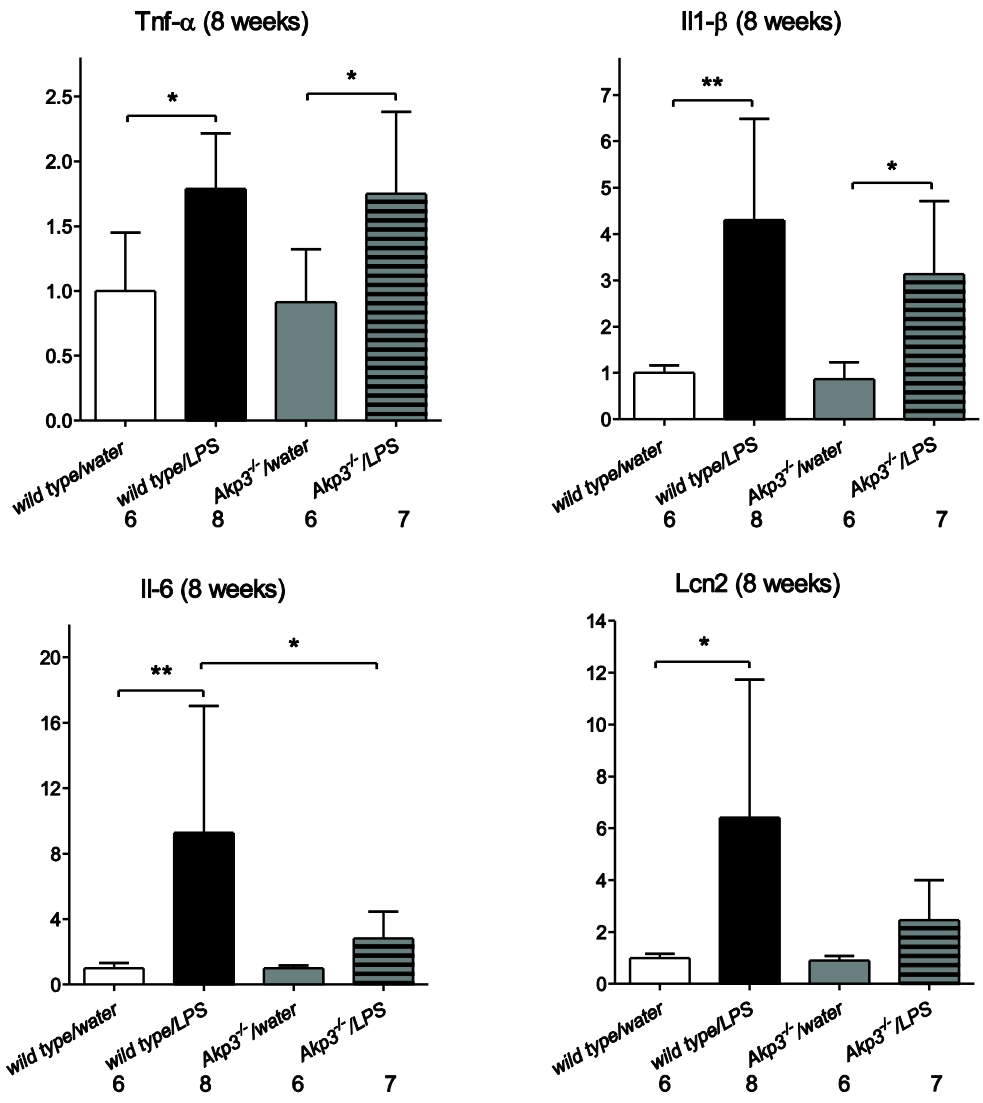

Figure 13. Low dose LPS treatment in 8-week-old mice. Real-time PCR quantification of transcription levels of Tnf-α, Il-1β, I-l6 and Lcn2 in the small intestine of water- or low dose LPS-treated wild type and *Akp3*$^{-/-}$ mice at 8 weeks old. Error bars represent standard deviations. The number below the graph indicates the sample size. Asterisks mark the significant difference (*, $p < 0.05$; **, $p < 0.01$; one-way ANOVA followed by Bonferroni test).

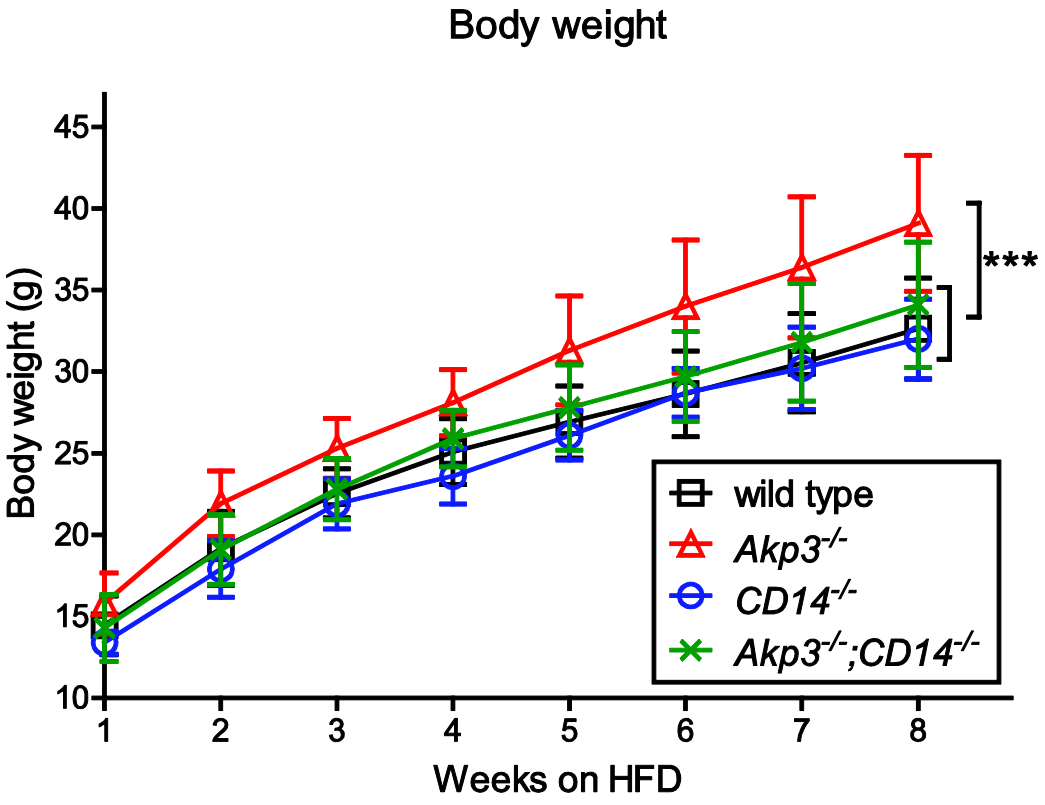

Figure 14. Weight gains of mice receiving HFD . Weight gains of wild type, *Akp3*$^{-/-}$, *CD14*$^{-/-}$, and *Akp3*$^{-/-}$;*CD14*$^{-/-}$ mice on HFD. Error bars represent standard deviations. Asterisks mark the significant difference (***, $p < 0.001$; two-way ANOVA).

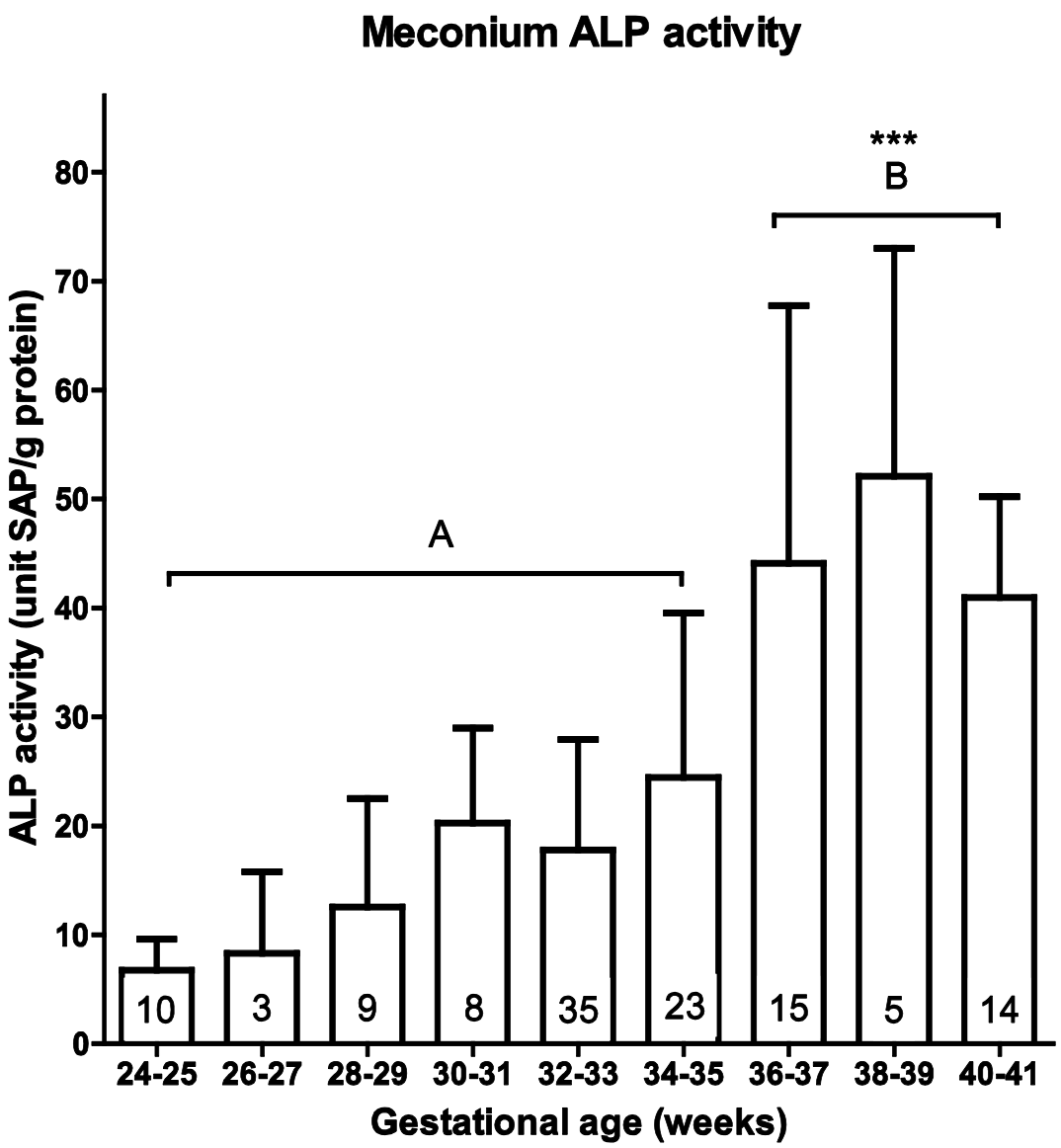

Figure 15. Meconium ALP activity is positively related to gestational age. Meconium ALP activities increased with gestational age ($p < 0.0001$; one-way ANOVA and post test for linear trend). Samples were statistically separated into two different groups, A and B (one-way ANOVA and Tukey's multiple comparison test). Preterm infants (group A, gestational age ≤ 35 weeks) had significantly lower meconium ALP activities than full-term infants (group B, gestational age ≥ 36 weeks) (***, $p < 0.0001$; one-tailed *t*-test). Error bars represent standard deviations. Numbers in columns indicate sample sizes.

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