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Supplementary materials

Nutrient-Sensing Ghrelin Receptor in Macrophages Modulates Bisphenol A-Induced Intestinal Inflammation in Mice

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Abstract: Bisphenols are environmental toxins with endocrine disruptor activity, yet bisphenol A (BPA) and its analogs are still widely used in manufacturing plastic products. There is evidence showing that BPA elicits inflammation in humans and animals, but the target cell types of BPA are not well understood. In this study, we sought to determine the BPA's direct effect on macrophages and the BPA immunotoxicity in mouse intestine. Ghrelin is an important nutrient-sensing hormone, acting through its receptor growth hormone secretagogue receptor (GHSR) to regulate metabolism and inflammation. We found that BPA promotes intestinal inflammation, showing increased infiltrating immune cells in colons and enhanced expression of *Ghsr* and pro-inflammatory cytokine and chemokine, such as *Il6* and *Ccl2*, in colonic mucosa. Moreover, we found that both long- and short-term BPA exposure elevated pro-inflammatory monocytes and macrophages in mouse peripheral blood mononuclear cells (PBMC) and peritoneal macrophages (PM), respectively. To determine the role of GHSR in BPA-mediated inflammation, we generated *Ghsr* deletion mutation in murine macrophage RAW264.7 using CRISPR gene editing. In wild-type RAW264.7 cells, the BPA exposure promotes macrophage pro-inflammatory polarization and increased *Ghsr* and cytokine/chemokine *Il6* and *Ccl2* expression. Interestingly, *Ghsr* deletion mutants showed a marked reduction of pro-inflammatory cytokine/chemokine expression in response to BPA, suggesting that GHSR is required for the BPA-induced pro-inflammatory response. Further understanding how nutrient-sensing GHSR signaling regulates BPA intestinal immunotoxicity will help design new strategies to mitigate BPA immunotoxicity and provide policy guidance for BPA biosafety.

Keywords: ghrelin; GHSR; bisphenol A; macrophage; inflammation

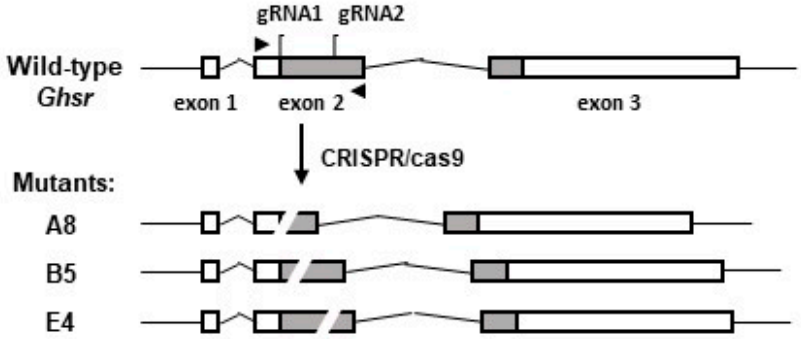
Table S1. Antibodies used in analysis of mouse PBMC and PM

Panel	Ab Name	Fluorophore	Catalog
PBMC panel			
	anti-CD45	eFluor450	Thermo 48-0451-82
	anti-F4/80	PE-Cy7	Thermo 25-4801-82
	anti-CD11b	APC-Cy7	BD 561039
	anti-CD38	BV750	BD 747103
	anti-Ly6G	PerCP	Biolegend 127654
	anti-CD206	BV650	Biolegend 141723
	anti-iNOS	AF488	Thermo 53-5920-82
PM panel			
	anti-CD45	BV510	Biolegend 103138
	anti CD11b	PE	Biolegend 101208
	anti Ly6C	eFluor-450	Thermo 48-5932-82
	anti-Ly6G	BV785	Biolegend 127645
	anti-CD115	APC	Biolegend 135509
	anti-CX3CR1	BV650	Biolegend 149033
	anti-CCR2	FITC	Biolegend 150607

Table S2. Oligonucleotides used in mouse gene editing, genotyping and qPCR

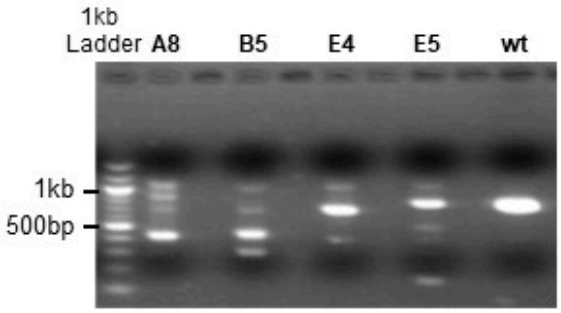
Oligo Type	Name	Sequence (5′ --> 3′)	Note
Guide RNAs			
	gRNA1	GUGGAACGCGACGCCCAGCG	Target *Ghsr* at coding aa 7-8
	gRNA2	CGGCACUCGUUGGUGUCCCG	Target *Ghsr* at coding aa 192-193
Genotyping primers			
	Ghsr-F	CTCCTCAGGGGACCAGATTT	740 bp (wt) or 150-700 bp (CRISPR del)
	Ghsr-R	GAGCACAGTGAGGCAGAAGA	
Gene expression primers			
	Ghsr-F1123	AAGATGCTTGCTGTGGTGGT	Priming *Ghsr* exons 2 - 3 (wt)
	Ghsr-R1284	AGCGCTGAGGTAGAAGAGGA	
	Ghsr A8/B5-F112	CTCAGGGGACCAGATTTCCG	Priming a Ghsr A8/B5 deletion region
	Ghsr A8/B5-R261	GCAGCAGTTCGTCAGAGAGT	
	Ghsr E4-F684	CCCATCTTCGTGCTGGTG	Priming a Ghsr E4 deletion region
	Ghsr E4-R941	ACCACCACAGCAAGCATCT	
	Ghrl-F248	AGCTGGAGATCAGGTTCAATGC	Priming *Ghrl* exons 2 - 3
	Ghrl-R453	GCTGAGGCGGATGTGAGTTC	
	Gper1-F372	ACACTCACACACTCTGGGTGC	
	Gper1-R607	TTCCCTCGGCAGTTTTCAGG	
	Il1b-F149	TGTTCTTTGAAGTTGACGGACCC	
	Il1b-F476	TCATCTCGGAGCCTGTAGTGC	
	Il6-F162	ACAAGTCCGGAGAGGAGACT	
	Il6-R299	GAATTGCCATTGCACAACTCT	
	Ccl2-F177	CACTCACCTGCTGCTACTCA	
	Ccl2-R293	GCTTGGTGACAAAAACTACAGC	
	Ccl20-F87	TCCTTGCTTTGGCATGGGTA	
	Ccl20-R156	CAGTCGTAGTTGCTTGCTGCTTC	
	Actb-F30	ACTGTCGAGTCGCGTCCA	
	Actb-R117	TCATCCATGGCGAACTGGTG	
	Ppia-F351	GCTGGACCAAACACAAACGG	
	Ppia-R422	ATGCTTGCCATCCAGCCATT	

A



B

Genotyping Primers:	
F/R = 740 bp (wt) + 150-700 bp (del)	
Ghsr F	CTCCTCAGGGGACCAGATTT
Ghsr R	GAGCACAGTGAGGCAGAAGA



C

Clone name	PCR results	Sequencing results
A8	dominant mutation band around 400bp	deletion aa1-119: GTGGAACGCGACGCCCAGCGAGGAGCCGGAGCCTAACGTCACGCTGGACCTGGA CTGGGACGCTTCTCCCGGCAACGACTCACTCTCTGACGAACTGCTGCCACTGTT CCCCGCGCCGCTGCTGGCGGGCGTCACTGCCACCTGCGTGGCGCTCTTCGTGGT GGGCATCTCGGGCAACCTGCTCACCATGCTGGTGGTGTCCCGCTTCCGCGAGCT GCGCACCACCACCAACCTCTACCTATCCAGCATGGCCTTCTCCGATCTGCTCAT CTTCCTGTGCATGCCGCTGGACCTCGTCCGCCTCTGGCAGTATCGGCCCTGGAA CTTCGGCGACCTGCTCTGCAAACTCTTCCAG
B5	dominant mutation band around 400bp, also weaker 300 bp	deletion aa4-110: CGACGCCCAGCGAGGAGCCGGAGCCTAACGTCACGCTGGACCTGGACTGGGACG CTTCTCCCGGCAACGACTCACTCTCTGACGAACTGCTGCCACTGTTCCCCGCGC CGCTGCTGGCGGGCGTCACTGCCACCTGCGTGGCGCTCTTCGTGGTGGGCATCT CGGGCAACCTGCTCACCATGCTGGTGGTGTCCCGCTTCCGCGAGCTGCGCACCA CCACCAACCTCTACCTATCCAGCATGGCCTTCTCCGATCTGCTCATCTTCCTGT GCATGCCGCTGGACCTCGTCCGCCTCTGGCAGTATCGGCCCTGGAACTTC
E4	dominant mutation band around 700bp	deletion aa181-197 GTGGGCGTGGAGCACGAGAACGGCACAGATCCCCGGGACACCAACGAGTGCCGC GCCACCGAGTTCGCTG
E5	wt band 740bp and weak mutant 150 bp band	no mutations found
wt RAW264.7	wt band 740bp	

Figure S1. Generation of RAW264.7 *Ghsr* deletion mutants by CRISPR gene editing. (**A**) Two gRNAs targeted to Ghsr exon 2 for cas9-mediated deletion. (**B**) The isolated RAW264.7 cell clones were firstly checked by genotyping PCR, which primers were flanked the gRNAs targeting region in exon 2. (**C**) The genotyping identified clones were further confirmed by DNA sequencing.

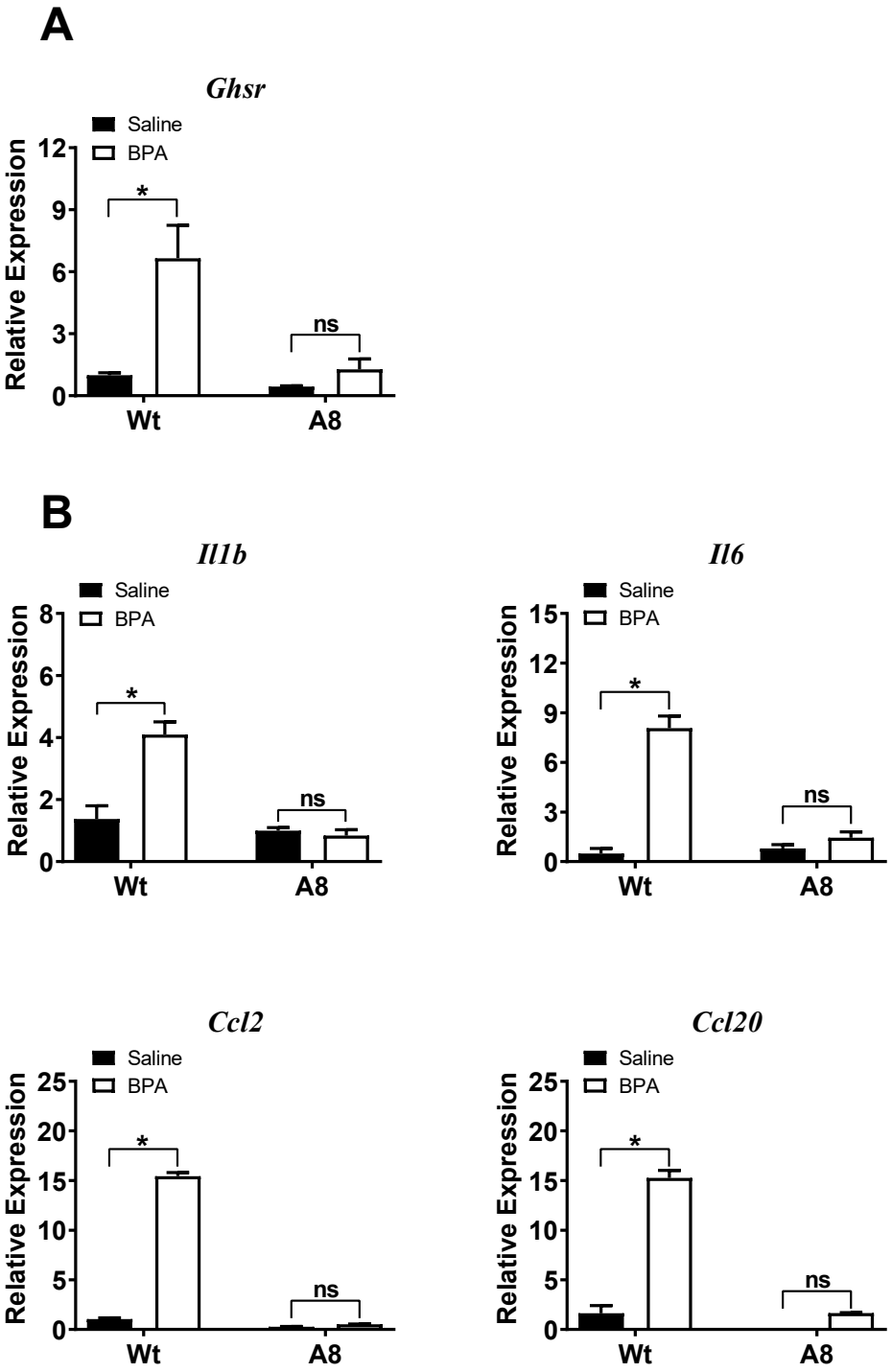


Figure S2. BPA-induced macrophages activation was suppressed in *Ghsr* mutant clone A8. (**A**) Gene expression analysis showed that BPA induced *Ghsr* gene expression in RAW264.7 parental cells (Wt), while the induction of *Ghsr* expression was suppressed in mutant A8. (**B**) The BPA-induced expressions of pro-inflammatory cytokines *Il1b* and *Il6* and chemokine *Ccl2* and *Ccl20* were blunted by *Ghsr* mutation in mutant A8 cells. Data are reported as mean ± SEM. *: $p < 0.05$; ns: not significant

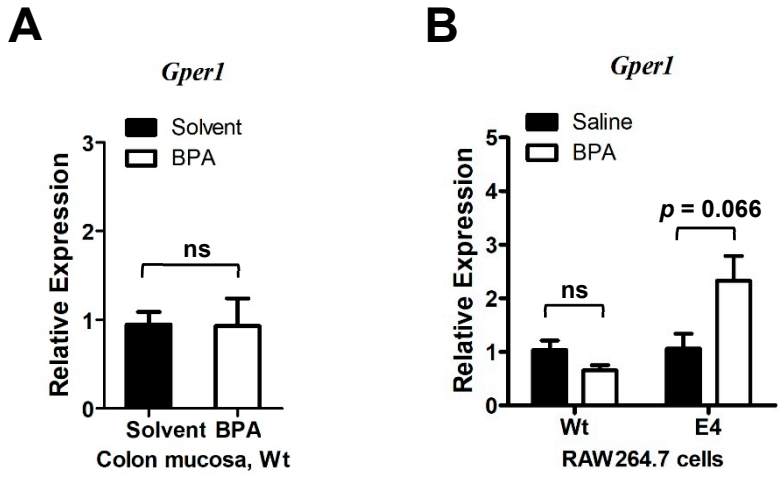


Figure S3. Expression of *Gper1* in mouse colonic mucosa and RAW264.7 cells. (**A**) The expression of *Gper1* in mouse colonic mucosa was analyzed by qPCR assay, showing little changes in *Gper1* expression influenced by BPA stimulation. (**B**) RAW264.7 cells also express *Gper1*, which is not significantly affected by *Ghsr* mutation. Mouse group n = 4 in colon mucosa samples. Data are reported as mean ± SEM. ns: not significant.