

High Beta-Palmitate Fat Controls the Intestinal Inflammatory Response and Limits Intestinal Damage in Mucin $Muc2$ Deficient Mice

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Abstract

Background: Palmitic-acid esterified to the sn-1,3 positions of the glycerol backbone (alpha, alpha'-palmitate), the predominant palmitate conformation in regular infant formula fat, is poorly absorbed and might cause abdominal discomfort. In contrast, palmitic-acid esterified to the sn-2 position (beta-palmitate), the main palmitate conformation in human milk fat, is well absorbed. The aim of the present study was to examine the influence of high alpha, alpha'-palmitate fat (HAPF) diet and high beta-palmitate fat (HBPF) diet on colitis development in $Muc2$ deficient ($Muc2^{-/-}$) mice, a well-described animal model for spontaneous enterocolitis due to the lack of a protective mucus layer.

Methods: $Muc2^{-/-}$ mice received AIN-93G reference diet, HAPF diet or HBPF diet for 5 weeks after weaning. Clinical symptoms, intestinal morphology and inflammation in the distal colon were analyzed.

Results: Both HBPF diet and AIN-93G diet limited the extent of intestinal erosions and morphological damage in $Muc2^{-/-}$ mice compared with HAPF diet. In addition, the immunosuppressive regulatory T (Treg) cell response as demonstrated by the up-regulation of *Foxp3*, *Tgfb1* and *Ebi3* gene expression levels was enhanced by HBPF diet compared with AIN-93G and HAPF diets. HBPF diet also increased the gene expression of *Pparg* and enzymatic antioxidants (*Sod1*, *Sod3* and *Gpx1*), genes all reported to be involved in promoting an immunosuppressive Treg cell response and to protect against colitis.

Conclusions: This study shows for the first time that HBPF diet limits the intestinal mucosal damage and controls the inflammatory response in $Muc2^{-/-}$ mice by inducing an immunosuppressive Treg cell response.

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Introduction

Human milk provides the best nourishment for full-term infants, by which approximately half of the dietary calories are supplied as fat [1]. More than 98% of milk fat is in the form of triglycerides containing fatty acids esterified to glycerol, with palmitic acid (C16:0) representing about 20–25% of total milk fatty acid. In human milk, palmitic acid is predominantly esterified to the sn-2 position of the triglyceride (beta-palmitate) [2]. However, in vegetable oils, the main constituents of infant formula fat, the palmitic acid is predominantly esterified to the sn-1,3 positions of the triglycerides (alpha, alpha'-palmitate) [3]. Structured triglycerides (synthetic beta-palmitate) are synthesized through enzymatic processing, whereby a large amount of alpha, alpha'-palmitate fat is converted to beta-palmitate fat [4,5]. It has been reported that high beta-palmitate fat (HBPF) rather than high alpha, alpha'-palmitate fat (HAPF) enables easy digestion and absorption of fatty acid [6,7,8,9] and calcium [10]. Moreover, HBPF was shown to

enhance the proliferation of spleen lymphocytes [11]. However, the influence of HBPF on intestinal damage remains unknown.

Mucins are the principal components of the intestinal mucus layer [12], which forms a physical barrier protecting the underlying epithelium against luminal substances and microbes [13,14,15]. Deficiency of $Muc2$ affects the protective capacities of the mucus layer [16], and as a consequence, bacteria are in direct contact with the intestinal epithelial cells [17]. This in its turn leads to the development of spontaneous colitis in $Muc2^{-/-}$ mice, a well-described animal model for enterocolitis [18,19,20]. In this model, colonic mucosal damage is not observed before weaning when immunosuppressive regulatory T (Treg) cells dominate the immune response. Interestingly, after weaning the Treg cell response declines and mucosal damage appears [21]. These data suggest that components of human milk might be able to limit intestinal inflammation and beta-palmitate could be such a component. Furthermore, increasing evidence has shown a

protective role of Treg cells in inflammatory diseases [22], and patients with inflammatory bowel diseases (IBD) have reduced Treg cell numbers compared with patients with non-IBD inflammatory diseases [23].

The present study was designed to investigate the influence of alpha-palmitate fat and beta-palmitate fat on colitis development in *Muc2*^{-/-} mice. We hypothesized that HBPF diet, which mimics the fat composition and properties of human milk fat, limits the intestinal mucosal damage and controls the inflammatory response in *Muc2*^{-/-} mice. Therefore, *Muc2*^{-/-} mice were fed AIN-93G reference diet [24], HAPF diet or HBPF diet for 5 weeks after weaning, and clinical symptoms and intestinal damage and inflammation were analyzed.

Methods

Animals

The 129Sv-*Muc2*^{-/-} mice were generated from *Muc2* heterozygous mice as previously described [19]. All mice were housed in the same specific pathogen-free environment with free access to acidified tap water in a 12-hour light/dark cycle. All animal experiments were reviewed by and performed with approval of the Erasmus MC Animal Ethics Committee (approval number: EMC 2087), Rotterdam, the Netherlands. All mice were tested negative for *Helicobacter hepaticus* and norovirus infection.

Experimental Setup

Muc2^{-/-} mice were divided into three diet groups which only differed in fat compositions: standard AIN-93G diet as a reference group, HAPF diet and HBPF (InFatTM, Advanced Lipids AB) diet containing 11.1%, 16.7% and 16.8% total palmitic acid, with 6.3%, 11.0% and 50.4% of the palmitic acid esterified to the sn-2 position, respectively (see Table 1 for more detailed information on fatty acid composition). Three male and 3 female mice were included in each group. All three diets were prepared by Research Diet Services, Wijk bij Duurstede, the Netherlands. Animals were weaned from mother's milk at the age of 23 or 24 days, housed separately, and received one of the above described diets *Ad libitum* for 5 weeks.

The food intake and body weight were recorded each week. Animals were sacrificed at the end of experiment, and colonic tissue samples were excised immediately and either fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline or stored in RNAlater (Qiagen, Venlo, the Netherlands) at -20°C.

Histology and Immunohistochemistry

Paraformaldehyde fixed colonic tissues were embedded in paraffin, and 4-µm-thick sections were stained with hematoxylin and eosin to study histologic changes as described previously [25]. The erosion score was assessed as follows: 0, no erosions; 1, 0–25% of the epithelium is erosive; 2, 25–50% of the epithelium is erosive; 3, 50–75% of the epithelium is erosive; 4, 75–100% of the epithelium is erosive; 5, 100% erosions (i.e. no epithelium present). To detect differences in mucosal and epithelial thickness in the colon, 5 to 10 well-oriented crypts were chosen per intestinal segment and measured using calibrated Leica Image Manager 500 software (Leica Microsystems, Rijswijk, the Netherlands). All data were obtained in a blinded fashion by 2 independent investigators.

Immunohistochemistry was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, United States) and 3,3'-diaminobenzidine as staining reagent as previously described [19,20]. Antigen unmasking was carried out by heating

Table 1. Fatty acid composition in the diets.

	AIN-93G	HAPF	HBPF
Fatty acid composition (%)			
C8	nd	nd	nd
C10	nd	nd	nd
C12	nd	nd	0.2
C14	nd	0.3	0.2
C15	nd	nd	nd
C16	11.1	16.7	16.8
C16:1	0.1	0.2	0.1
C17	nd	nd	nd
C18	4.5	3.4	3.8
C18:1	24.7	36	42.2
C18:2 (n-6)	51.6	40.3	34
C18:3 (n-3)	6.6	1.8	1.5
C20	0.4	0.3	0.3
C20:1	0.2	0.3	0.3
C22	0.4	0.4	0.4
C22:1	0.3	0.1	0.1
C24	0.1	0.2	0.1
C16 sn-2	2.1	5.5	25.4
C16 sn-2/total C16 (%)	6.3	11.0	50.4
Total fat content/diet (%)	7.2	7.2	7.1

nd: not detected.

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the sections for 20 min in 0.01 M sodium citrate (pH 6.0) at 100°C. CD3e-positive and S100a8-positive cells were detected using antibodies against CD3e (Dako, Glostrup, Denmark) and S100a8 (R&D Systems, Abingdon, United Kingdom), respectively.

RNA Isolation and Quantitative PCR (qPCR)

Total RNA was isolated using the QIAamp RNA midi-kit (Qiagen, Venlo, the Netherlands) following the manufacturer's protocol. Complementary DNA was synthesized from 1.5 mg RNA using M-MLV reverse transcriptase (Promega, Leiden, the Netherlands). The qPCR analysis was performed based on the intercalation of SYBR Green on an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, United States) as previously described [19]. The relative mRNA expression levels were normalized against β -Actin (*Actb*) expression levels of each mouse. The sequences of all primers used for qPCR are given in Table 2. The oligonucleotide sequences were designed using OLIGO 6.22 software based on the gene sequences and purchased from Invitrogen. All primers had a melting temperature between 65°C and 66.5°C. The specificity and efficiency of all primer sets were tested, and all PCRs performed with comparable efficiencies of 95% or higher.

Statistical Analysis

The data are expressed as median or mean \pm SEM. The data from HBPF and HAPF groups were compared using Chi-square test or Mann-Whitney test. AIN-93G group served as a reference group; therefore all parameters were also compared with this group. The data were considered statistically significant at $p < 0.05$.

Table 2. Primer sequences used for qPCR in this study.

Target gene	Forward Primer (5'–3')	Reverse Primer (5'–3')
<i>Actb</i>	GGGACCTGACGGACTAC	TGCCACAGGATTCCATAC
<i>Cd45</i>	TTTGGGAACATTACTGTGAA	TGGAGCACATGAGTCATTAG
<i>Cd3e</i>	CCAGCTCAAATAAAAACA	TTGGCCTTCCTATTCTTG
<i>Tnf</i>	TGGCCTCCCTCTCATC	GGCTGGCACCAGTAGTT
<i>Foxp3</i>	ACACCCAGGAAAGACAG	GGCAGTGCTTGAGAAAC
<i>Tgfb1</i>	AACCAAAGACATCTCACACA	GCCAGGAATTGTTGCTAT
<i>Ebi3</i>	CCCGGACATCTTCTCTCT	GAGGCTCCAGTCACTTG
<i>Ii12a</i>	GCCTTGGTAGCATCTATGAG	TCGGCATTATGATTAGAGAGA
<i>Pparg</i>	CAGTTTCGATCCGTAGAAGC	CCATAAAGTCACCAAAGGGC
<i>Sod1</i>	GATCGTGTGATCTCACTCTC	TTGTTTCTCATGGACCAC
<i>Sod3</i>	GAAGTTCACAGAGGGAA	GACATGGTGACAGAGCC
<i>Gpx1</i>	CCCGTGCAATCAGTTC	TTCGCACTTCTCAAACAA

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Results

Clinical Symptoms

The fats did not affect total food intake as no significant differences among the HAPF, HBPF and AIN-93G groups was observed during the study period (Figure 1A). Weight loss or growth retardation is considered as one of the major clinical symptoms of colitis, and therefore, we next compared body weights. The mean value of body weight of mice fed HAPF diet was slightly lower than that of mice fed AIN-93G or HBPF diets, but the differences were not statistically significant at any time point investigated (Figure 1B). Another clinical symptom of colitis is rectal bleeding. At the age of 8 weeks, 2 of 6 mice (33%) from the AIN-93G diet group, 3 of 6 mice (50%) from the HAPF diet group and only 1 of 6 mice (16%) from the HBPF diet group showed rectal bleeding.

HAPF, but not HBPF, Increases Intestinal Mucosal Damage

Ruffled, flattened and erosive epithelia were observed in the distal colon of all *Muc2*^{-/-} mice. However, the extent of morphological changes within the surface epithelium varied among the groups (Figure 2A). The remaining surface epithelium in the AIN-93G group consisted of approximately 20–30% normal, polarized, columnar epithelial cells, and 30–70% of the epithelial cells were ruffled and flattened. However, the remaining surface epithelium of the animals in the HAPF group showed extensive damage, and normal epithelial cells were not observed in these animals. The HBPF group exhibited similar results to those observed in the AIN-93G reference group. In line with this, the erosion score was significantly increased in the HAPF group compared with HBPF and AIN-93G groups (Figure 2B). We previously identified crypt lengthening as a site-specific marker for colitis severity in *Muc2*^{-/-} mice [19,20]. Therefore we analyzed crypt lengths in *Muc2*^{-/-} mice fed the different diets. No significant differences were observed among the three diet groups (Figure 2C).

HAPF and HBPF do not Alter the Mucosal T cell Influx in the Distal Colon

Following the relatively normal surface epithelial morphology in HBPF group compared with HAPF group, we next determined whether HBPF suppressed the inflammatory response in *Muc2*^{-/-} mice. We used the influx of CD3e-positive T cells as a marker for intestinal inflammation. In the distal colon, the amount of CD3e-positive T cells did not differ among the three diet groups (Figure 3A). We also performed immunohistochemistry for S100a8, an inflammation related protein which is mainly produced by neutrophils. Similarly, the abundance of S100a8-positive cells did not differ among the three diet groups (Figure 3B).

In addition to the immunohistochemical analysis, the mRNA expression levels of *Cd45* and *Cd3e* were also quantified. No differences were seen in the mRNA expression levels of *Cd45* and *Cd3e* among the three diet groups (Figure 3C & 3D), indicating that the total numbers of hematopoietic cells and T cells were not altered by the type of diet studied.

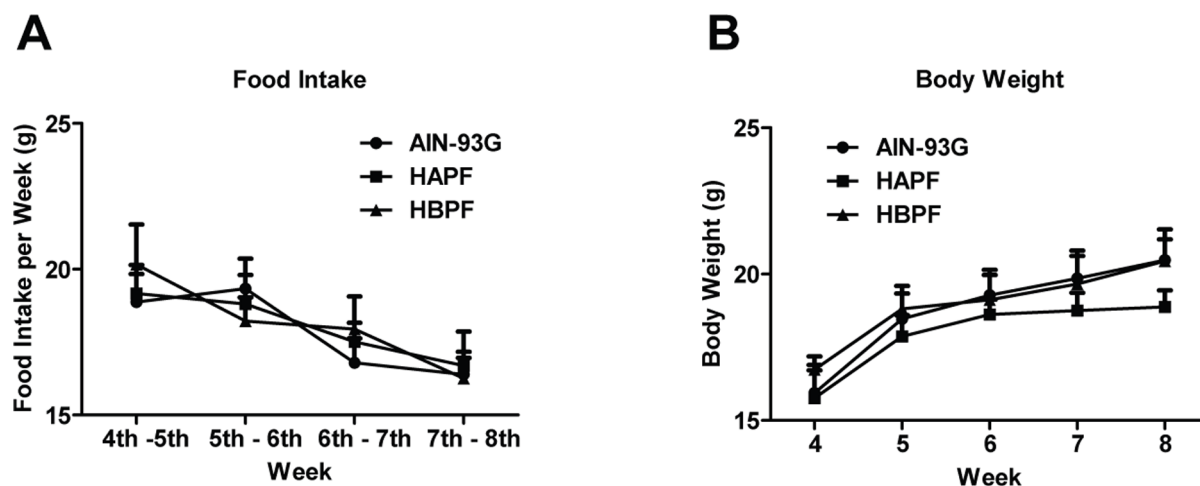
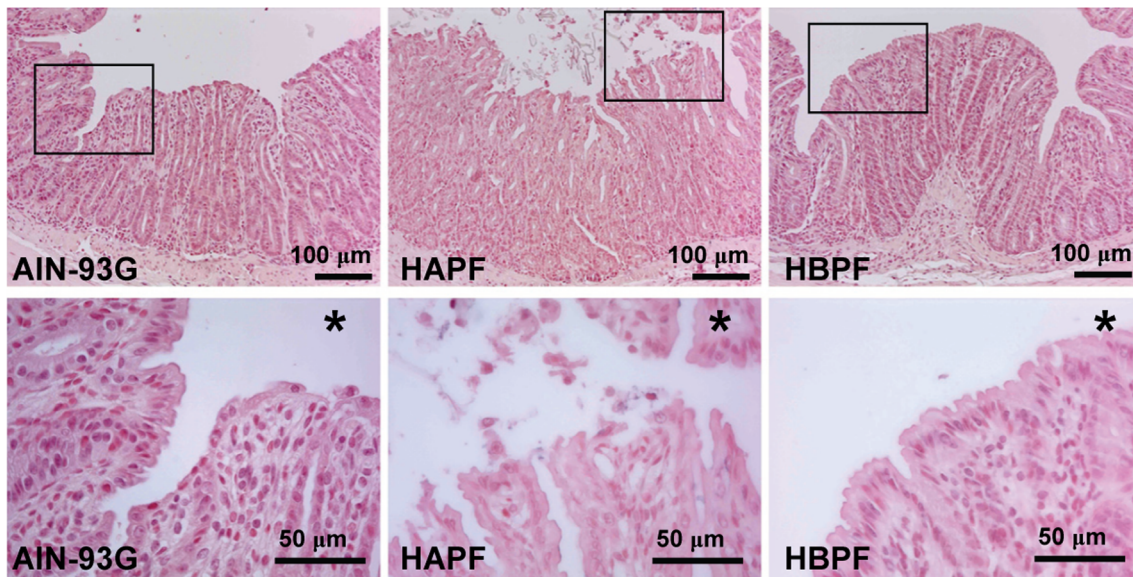
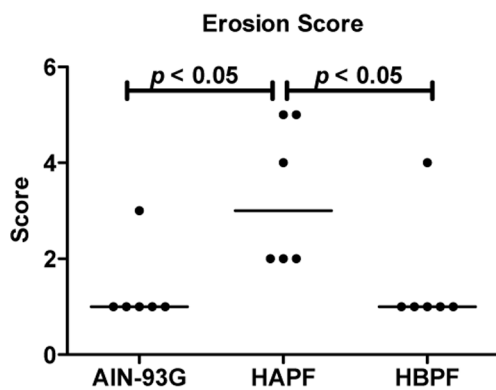


Figure 1. Clinical Symptoms of *Muc2*^{-/-} mice fed AIN-93G, HAPF or HBPF diet. Food intake (A) and body weights (B) of mice fed AIN-93G, HAPF or HBPF diet were recorded from the age of 4 weeks until the end of the study. Each group represents 6 animals in total; 3 males and 3 females. doi:10.1371/journal.pone.0065878.g001

A



B



C

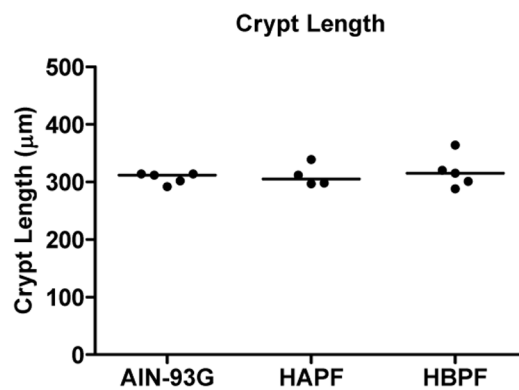


Figure 2. Morphology of the distal colon of *Muc2*^{-/-} mice fed AIN-93G, HAPF or HBPF diet. Distal colonic sections of mice fed with different diets were stained with haematoxylin and eosin, and representative sections of each diet group are shown (A). Panels with an asterisk represent a higher magnification of the surface epithelium of the distal colon. Erosion scores (B) and crypt lengths (C) within in each diet group are shown. Images are representative for all the mice in each diet group. doi:10.1371/journal.pone.0065878.g002

We next quantified the expression of the pro-inflammatory cytokine tumor necrosis factor- α (*Tnf*). There was no significant difference in *Tnf* expression levels between mice fed HBPF and HAPF diets (Figure 3E), and there were also no significant differences in the mRNA expression levels of other inflammatory markers such as inducible nitric oxide synthase 2 (*iNos2*), interleukin 1 beta (*Il1b*), interleukin 4 (*Il4*), and the Th17 signature cytokines interleukin 17 (*Il17*) and interleukin 22 (*Il22*) (data not shown).

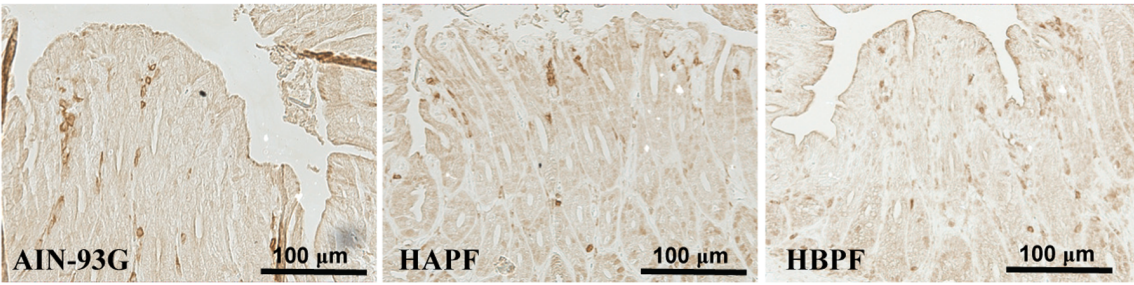
HBPF Enhances Regulatory T cell Response

Treg cells are shown to have immunosuppressive capacities [22], and therefore, we next determined the Treg response in the three diet groups. The *Foxp3* mRNA expression did not differ between HAPF and AIN-93G groups, but it was significantly up-

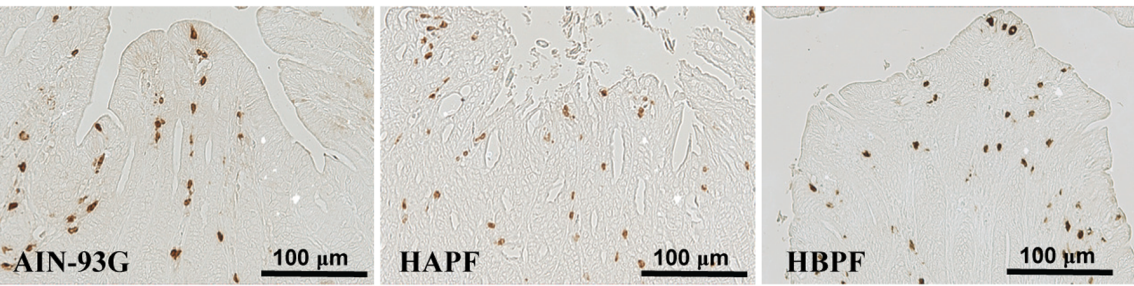
regulated in HBPF group compared with HAPF and AIN-93G groups (Figure 4A), suggesting that HBPF induced an immunosuppressive Treg cell response.

Treg cells suppress the pro-inflammatory response by inhibiting effector T cells primarily through the production of cytokines, such as transforming growth factor beta 1 (*Tgfb1*) and interleukin 35 (*Il35*) [26,27]. HBPF diet significantly up-regulated *Tgfb1* mRNA expression levels in the distal colon compared with HAPF diet (Figure 4B). *Il35* is a heterodimeric cytokine composed of an interleukin 12A (*Il12a*) subunit and an Epstein-Barr virus induced 3 (*Ebi3*) subunit. Interestingly, *Ebi3* mRNA expression levels were up-regulated in the HBPF group compared with the HAPF group and the AIN-93G (Figure 4C), and *Il12a* mRNA levels trended higher in the HBPF group (Figure 4D). There were no differences

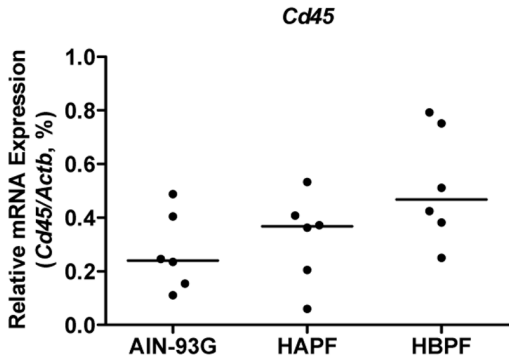
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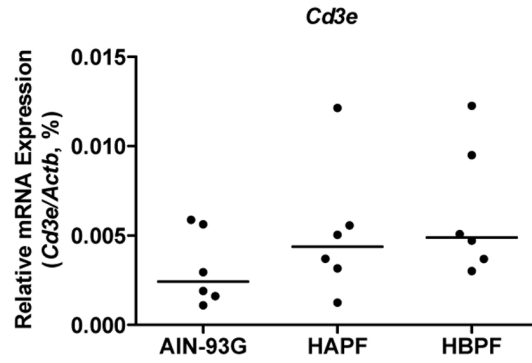
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D



E

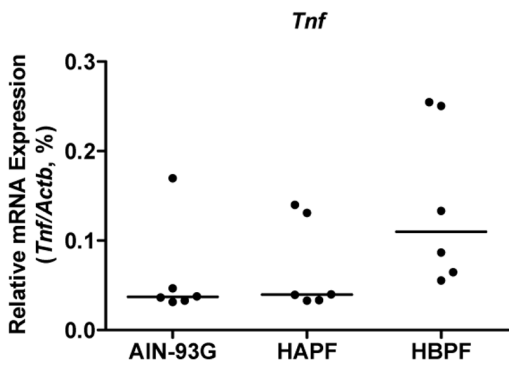


Figure 3. Inflammatory response in the distal colon of *Muc2*^{-/-} mice fed AIN-93G, HAPF or HBPF diet. Representative stainings of CD3ε-positive T cells (A) and S100a8-positive cells (B) in the distal colon of each diet group are shown. Transcription of *Cd45* (C), *Cd3e* (D) and *Tnf* (E) in the distal colon of mice was analyzed using qPCR. No significant difference was observed. Each group represents 6 animals in total; 3 males and 3 females.
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in the mRNA expression levels of these Treg signature cytokines between HAPF and AIN-93G groups (Figure 4B to 4D).

HBPF Up-regulates Proliferator-activated Receptor Gamma (PPAR-gamma) and Enzymatic Antioxidants

PPAR-gamma is shown to prevent gut inflammation by favouring the recruitment of Treg to the mucosal inductive sites [28]. HAPF diet did not change the *Pparg* mRNA expression levels compared with AIN-93G diet. However, *Pparg* mRNA expression was significantly up-regulated in the HBPF group compared with the HAPF group, and there was a trend towards increasing *Pparg* mRNA expression levels in the HBPF group compared with the AIN-93G group (Figure 5A).

Finally, as antioxidants modulate the inflammation in IBD [29], we quantified the mRNA expression levels of enzymatic antioxidants cytoplasm superoxide dismutase 1 (*Sod1*), extracellular superoxide dismutase 3 (*Sod3*) and glutathione peroxidase 1 (*Gpx1*). The mRNA expression levels of these three antioxidant enzymes

did not differ between HAPF and AIN-93G groups, but they were all up-regulated in the HBPF group (Figure 5B to 5D).

Discussion

The HAPF and HBPF diets used in this study contain a similar amount of total palmitic acid. However, HBPF diet contains high levels of palmitic acid esterified to the sn-2 position of the triglycerides and mimics the fat composition and properties of human milk fat, while HAPF diet contains high levels of palmitic acid esterified to the sn-1,3 positions. In the current study, HAPF diet increased the incidence of erosion and mucosal damage in the distal colon of *Muc2*^{-/-} mice compared with the AIN-93G reference diet, but HBPF diet did not. Moreover, HBPF diet induced an immunosuppressive Treg response.

The different fat blends did not affect the total food intake in the three diet groups. However, compared with mice fed AIN-93G reference diet, which contains soybean oil as fat source, mice fed

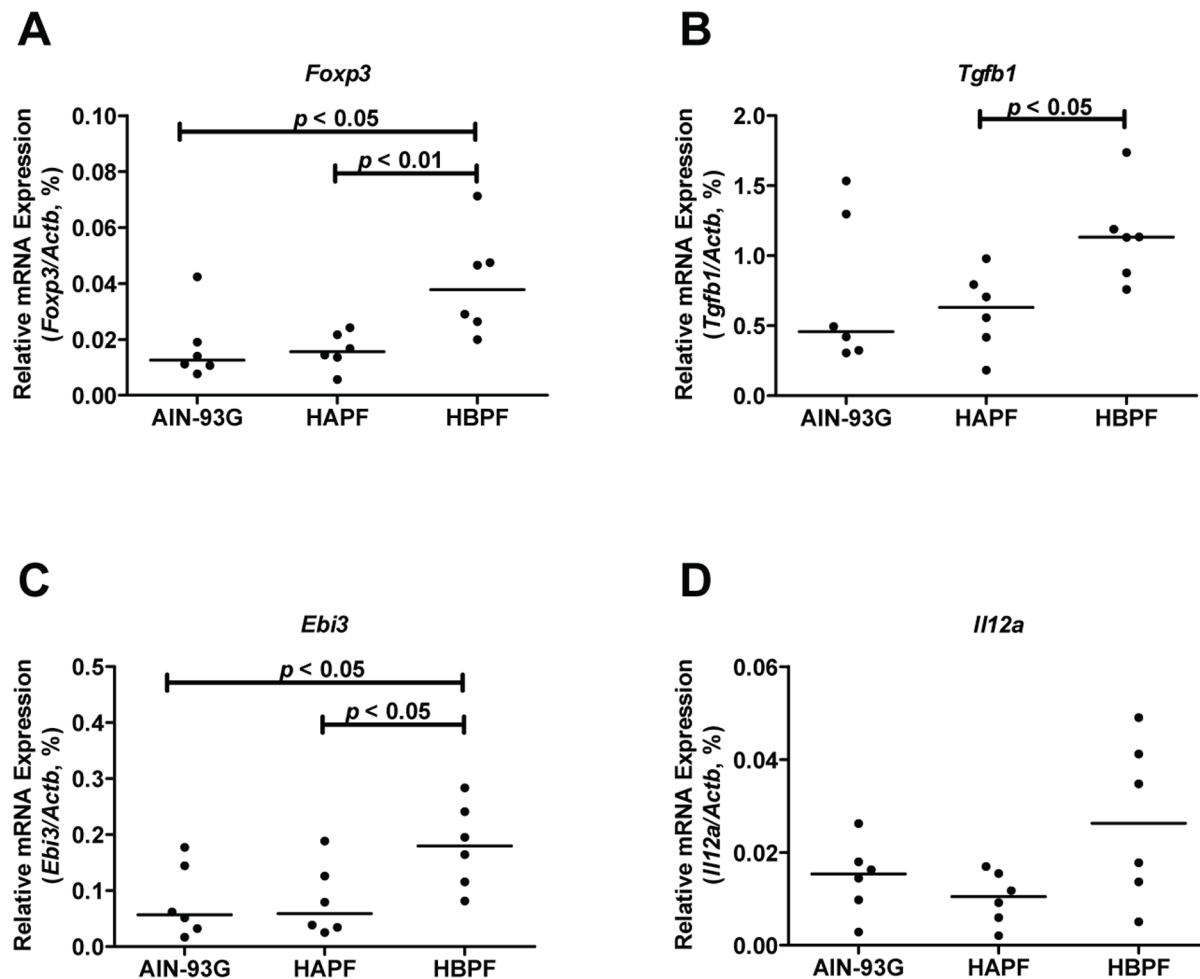


Figure 4. Enhanced regulatory T cell response in the distal colon of *Muc2*^{-/-} mice fed HBPF diet. The transcription levels of *Foxp3* (A), *Tgfb1* (B), *Ebi3* (C) and *Il12a* (D) in the distal colon of mice were analyzed using qPCR. Each group represents 6 animals in total; 3 males and 3 females.
doi:10.1371/journal.pone.0065878.g004

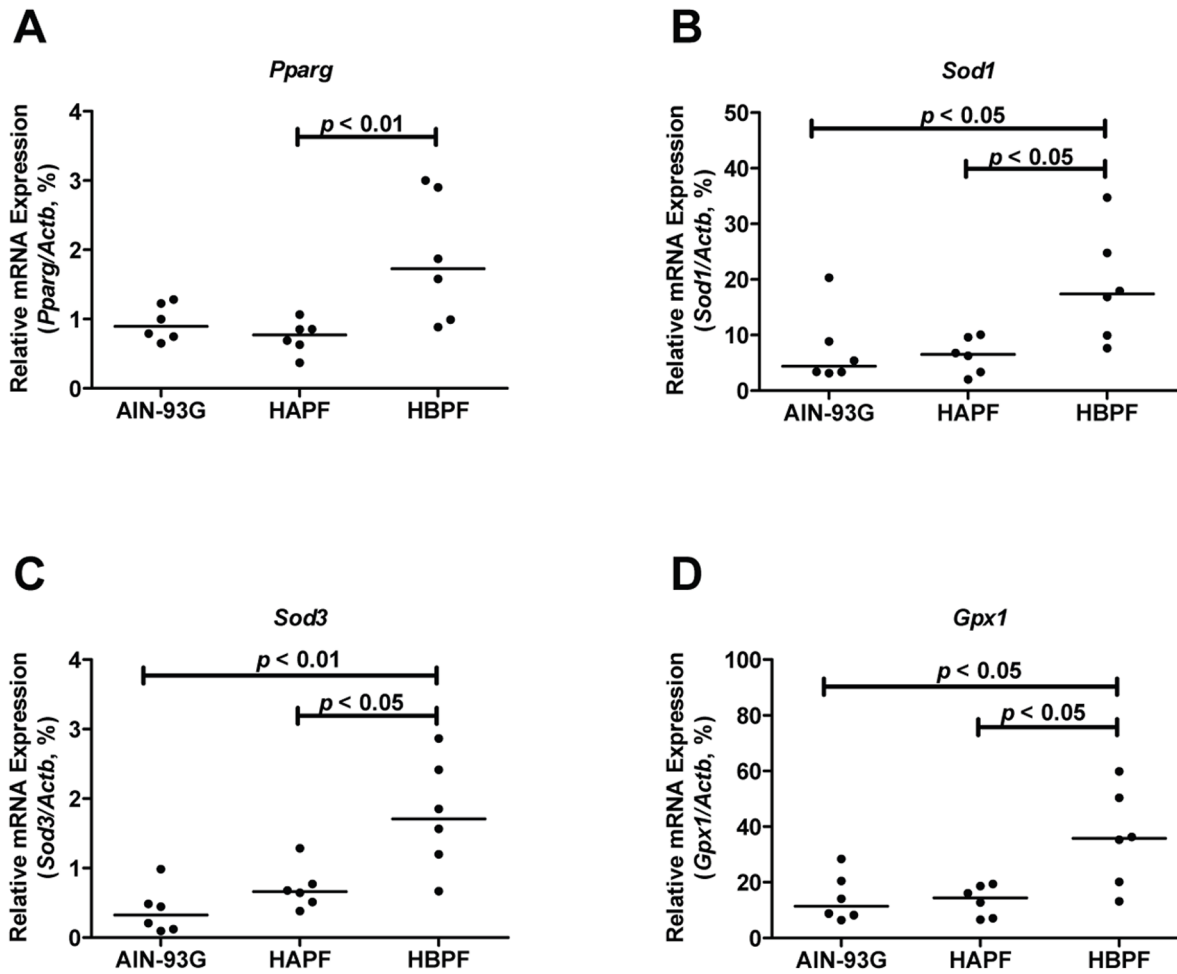


Figure 5. Increased *Pparg* and enzymatic antioxidants in the distal colon of *Muc2*^{-/-} mice fed HBPF diet. The transcription of *Pparg* (A), *Sod1* (B), *Sod3* (C) and *Gpx1* (D) in the distal colon of mice was analyzed using qPCR. Each group represents 6 animals in total; 3 males and 3 females. doi:10.1371/journal.pone.0065878.g005

HAPF diet, which contains palm oil with high levels of alpha, alpha'-palmitate and low levels of beta-palmitate, showed significantly higher erosion scores and more intestinal mucosal damage. These data imply that HAPF diet increased the colonic mucosal damage in *Muc2*^{-/-} mice compared with the AIN-93G reference diet. In contrast, HBPF diet, which contains palm oil with low levels of alpha, alpha'-palmitate and high levels of beta-palmitate, significantly reduced the occurrence of epithelial erosions and improved the intestinal morphology in the distal colon compared with HAPF diet. Additionally, less mice fed HBPF diet showed rectal bleeding compared with mice fed HAPF diet (1 of 6 versus 3 of 6). There were no differences regarding the erosion score, intestinal morphology, body weight and rectal bleeding between HBPF and AIN-93G groups.

Intestinal inflammation was extensively investigated using multiple inflammation markers. The abundance of CD3e-positive T cells and S100a8-positive neutrophils was not altered among the three diet groups, and the expression of inflammation markers (e.g. *Tnf*, *iNos2*, *Il1b*, *Il4*, *Il17* and *Il22*) remained unaltered among the three diet groups. In sharp contrast, morphological signs of inflammation such as flattening of surface epithelium, detachment of surface epithelium from the basement membrane, and erosions were limited in mice fed HBPF diet compared with HAPF diet. Based on these data, one could hypothesize that in the HBPF

group the pro-inflammatory response is counterbalanced by an immunosuppressive response, thereby limiting colonic damage. In line with this hypothesis are the increased levels of *Foxp3* and *Tgfb1* mRNA levels in the HBPF group, but not in HAPF and AIN-93G groups. Our data also show that IL35, a heterodimeric cytokine composed of IL12a and Ebi3 which is produced by Treg cells [27], was up-regulated by the HBPF diet group. Interestingly, we previously showed an up-regulated local immunosuppressive Treg response and no histological damage in the distal colon of *Muc2*^{-/-} mice before weaning [21]. However, after weaning the immunosuppressive Treg response in the distal colon had declined and colonic mucosal damage appeared in these mice. Based on this we suggest that, in our current study, the HBPF-induced immunosuppressive Treg response in *Muc2*^{-/-} mice limits mucosal damage and thereby colitis severity.

In line with the increased hallmarks of an immunosuppressive Treg response in HBPF group compared with HAPF and AIN-93G groups, HBPF diet also up-regulated the expression levels of *Pparg* and enzymatic antioxidants. Interestingly, PPAR-gamma has important immunoregulatory functions in the intestinal homeostasis and inflammation [30,31], and it is an important factor controlling the accumulation and phenotype of Treg cells residing in the intestine [32]. For example, endogenous PPAR-gamma activation down-regulates effector T cell function and prevents

colitis [33]. Moreover, PPAR-gamma agonist-induced Treg cells maintain a high level of Foxp3 expression [34], and deficiency of PPAR-gamma in T cells accelerates the onset of colitis and decreases the number of Treg cells [28]. It has also been documented that dietary antioxidants reduce Treg apoptosis and increase the yield of Treg cells, and therefore decrease the inflammation [35,36]. Enzymatic antioxidants, such as superoxide dismutases, were shown to ameliorate colitis in animal models [37] and improve ulcerative colitis in patients [38]. Further, Gpx1 activity is associated with enhanced Treg cell activity in mice [39], and mice deficient in both glutathione peroxidases, Gpx1 and Gpx2, develop spontaneous colitis [40]. Therefore, we suggest that in our study the up-regulation of *Pparg* and antioxidant enzymes (*Sod1*, *Sod3* and *Gpx1*) by HBPF might contribute to the induction of an immunosuppressive Treg response, thereby controlling the inflammatory response and limiting the incidence of erosions in *Muc2*^{-/-} mice.

Overall, based on these data it is tempting to speculate that HAPF, a fat constituent used in regular infant formula [3], might be involved in the onset of intestinal damage in infants prone to develop intestinal inflammation such as preterm infants. Replace-

ment of HAPF with HBPF (i.e., fat containing high levels of palmitic acid esterified to the sn-2 position), could enhance the immunosuppressive Treg response in the intestine and thereby prevent or limit intestinal inflammation. However, this is all highly speculative and should be investigated further.

In summary, our data show for the first time that HAPF diet increases the incidence of intestinal erosions and mucosal damage, while HBPF diet controls the damage by inducing an immunosuppressive Treg response in *Muc2*^{-/-} mice. Additionally, HBPF diet stimulates expression of *Pparg* and antioxidant enzymes (*Sod1*, *Sod3* and *Gpx*), which might be linked to the protective Treg response. Together, these data imply a crucial role for beta-palmitic acid in limiting intestinal inflammation.

Author Contributions

Conceived and designed the experiments: PL FBY LL YL JBvG IBR. Performed the experiments: PL JWb ACJMB AMKM. Analyzed the data: PL IBR. Contributed reagents/materials/analysis tools: FBY LL YL. Wrote the paper: PL IBR.

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