Received: 28 October 2020 | Revised: 16 February 2021 | Accepted: 16 February 2021

DOI: 10.1096/fj.202002340RR

RESEARCH ARTICLE

THE FASEB JOURNAL

Omega-3 fatty acids protect from colitis via an Alox15-derived eicosanoid

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Funding information
Deutsche Forschungsgemeinschaft (DFG), Grant/Award Number: KU961/11-1; Deutsche Forschungsgemeinschaft (DFG), Grant/Award Number: KU961/13-1; Deutsche Forschungsgemeinschaft (DFG), Grant/Award Number: WE2908/11-1

Abstract

An increased omega-3 polyunsaturated fatty acid (n-3 PUFA) tissue status can lead to a significant formation of anti-inflammatory lipid mediators and effective reduction in inflammation and tissue injury in murine colitis. Arachidonic acid lipoxygenases (ALOX) have been implicated in the pathogenesis of inflammatory bowel disease as well as in the formation of pro- and anti-inflammatory lipid mediators. To explore the role of Alox15 in the protective response found in *fat1* transgenic mice with endogenously increased n-3 PUFA tissue status *fat1* transgenic mice were crossed with *Alox15*-deficient animals and challenged in the dextran sulfate sodium (DSS)- and the 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced colitis model. Transgenic *fat1* mice rich in endogenous n-3 PUFAs were protected from colitis. However, additional systemic inactivation of the *Alox15* gene counteracted this protective effect. To explore the molecular basis for this effect Alox15 lipid metabolites derived from n-3 PUFA were analyzed in the different mice. *Alox15* deficiency suppressed the formation of n-3 PUFA-derived 15-hydroxy eicosapentaenoic acid

Abbreviations: AA, arachidonic acid; ALOX, arachidonic acid lipoxygenase; DAI, disease activity index; DHA, docosahexaenoic acid; DSS, dextran sulfate sodium; EPA, eicosapentaenoic acid; IBD, inflammatory bowel disease; LLOQ, lower limit of quantification; Lx, lipoxin; n-3/6, omega-3/6; PUFA, polyunsaturated fatty acid; Rv, resolvin; SPM, specialized pro-resolving mediator; TNBS, 2,4,6-trinitrobenzene sulphonic acid; 5-HETE, 5-hydroxy eicosatetraenoic acid; 12-HETE, 12-hydroxy eicosatetraenoic acid; 15-HETE, 15-hydroxy eicosatetraenoic acid; 15-HEPE, 15 hydroxy eicosapentaenoic acid; 17-HDHA, 17-hydroxy docosahexaenoic acid; 10-HDHA, 10-hydroxy docosahexaenoic acid; 7-HDHA, 7-hydroxy-docosahexaenoic acid.

(15-HEPE). In contrast, treating mice with intraperitoneal injections of 15S-HEPE protected wild-type mice from DSS- and TNBS-induced colitis. These data suggest that the anti-colitis effect of increased n-3 PUFA in the transgenic *fat1* mouse model is mediated in part via Alox15-derived 15-HEPE formation.

KEYWORDS

lipid peroxidation, lipoxygenases, eicosanoids, inflammatory bowel disease

1 | INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic gastrointestinal disorders with a high prevalence in the western world.[1] The pathogenesis of IBDs is diverse and genetic predispositions, environmental factors, nutrition, alterations in the intestinal microbiome, and the immune status of the patients have been implicated.[2,3] A number of studies suggested an attenuation of the risk for IBD by diets rich in omega-3 polyunsaturated fatty acids (n-3 PUFAs).[4,5] Although clinical evidence for beneficial effects of n-3 PUFAs in humans is controversial,[6,7] n-3 PUFA enriched diets markedly reduce the severity of inflammatory symptoms in animal colitis models.[8] Transgenic mice overexpressing the *C elegans fat1* gene, which encodes for an omega-6 fatty acid desaturase, have increased levels of circulating n-3 PUFA and tissue concentrations[9,10] and develop less severe inflammatory symptoms than wild-type animals in the DSS colitis model.[11] Biosynthesis of specialized pro-resolving mediators (SPMs) may be involved in this effect.[12] SPMs are n-3 PUFA-derived anti-inflammatory and pro-resolving mediators, which are formed *via* lipoxygenases.[13] In addition to different ALOX-isoforms (ALOX5, ALOX15, ALOX15B, ALOX12) aspirin-treated COX2 may also contribute to the biosynthesis of these compounds.[14]

The human genome involves six functional ALOX genes (*ALOX15, ALOX15B, ALOX12, ALOX12B, ALOXE3, ALOX5*), which encode for six catalytically active ALOX-paralogs.[15] In mice, a single enzyme ortholog exists for each human ALOX-paralog (Alox15, Alox15b, Alox12, Alox12b, Aloxe3, Alox5) but in addition an Aloxe12 is expressed in the skin.[15] Of the six human ALOX-paralogs ALOX5, ALOX15, and ALOX15B have been implicated in the pathogenesis of IBD.[16-18] *Alox5*-deficient mice were protected from chemically induced colitis, and animals treated with the ALOX5 inhibitor zileuton developed less severe inflammatory symptoms.[19] In Crohn's disease colonic 15-HETE levels are reduced.[17] This metabolite, which is formed in humans by ALOX15 and ALOX15B, upregulates expression of tight junction proteins in the healthy human intestine and thus, the two enzymes may prevent the loss of epithelial barrier function during intestinal inflammation.[17] However, the biological role of ALOX15 in inflammation is controversial since pro- and anti-inflammatory activities have been reported.[20] We recently observed that female *Alox15*-deficient mice developed less severe inflammatory symptoms in the DSS colitis model when they received a normal chow diet.[21]

To characterize the role of *Alox15* deficiency in *fat1* mice rich in n-3 PUFA, we crossed the two mouse strains and bred a colony of double genetically modified mice. These mice were subsequently challenged in two experimental colitis models (DSS and TNBS colitis).

2 | MATERIALS AND METHODS

2.1 | Mice and genotyping

Alox15-deficient mice (*Alox15*$^{-/-}$)[22] were back-crossed eight times with C57BL/6 animals to achieve genetic homogeneity. Then transgenic *fat1* mice[23] (C57BL/6) were crossed with homozygous *Alox15*$^{-/-}$ mice to produce founder individuals carrying the *fat1* transgene and one *Alox15* knockout allele (*fat1* + *Alox15*$^{+/-}$). From these founders, *fat1* + *Alox15*$^{+/+}$ as well as *fat1* + *Alox15*$^{-/-}$ were outbred and these mice were employed for the induction of experimental colitis. All mice were genotyped by PCR for the presence of the *fat1* transgene and for the *Alox15* gene locus and the employed primer combinations are given below. Mice were maintained under standard conditions in a specific pathogen-free environment at 12 hours day-night cycles according to the FELASA recommendation with food and water ad libitum. Starting 4 weeks before induction and during the experimental colitis, all mice were fed a modified AIN-76A diet containing 10% safflower oil (ssniff Spezialdiäten GmbH), which is high in n-6 and low in n-3 PUFAs. The experiments were approved by the state animal care committee (Landesamt für Gesundheit und Soziales) and were performed according to the guidelines for the care and use of laboratory animals adopted by the US National Institutes of Health, and the ARRIVE guidelines. Genotyping of the *Alox15* gene locus and the *fat1* transgene was carried out by PCR with genomic DNA from ear tissue biopsies using the following primer combinations. Wild-type *Alox15* allele: reverse: 5′-CCA TAG ACG AGA CCA GCA CA-3′, forward: 5′-GGC

TGC CTG AAG AGG TAC AG-3′. Mutant *Alox15* allele: reverse: 5′-GGG AGG ATT GGG AAG ACA AT-3′, forward: 5′-GGC TGC CTG AAG AGG TAC AG-3′. The wild-type band for the *Alox15* gene was 200 bp and the knockout band was 417 bp. The primers for the *fat1* transgene were: 5′-CTG CAC CAC GCC TTC ACC AAC C-3′ and reverse: 5′-CAC AGC AGC AGA TTC CAG AGA TT-3′.[24] The band for the *fat1* transgene was 251 bp.

2.2 | TNBS colitis model

Wild-type mice (n = 6), *fat1* + *Alox15*$^{+/+}$ mice (n = 5), *Alox15*$^{-/-}$ mice (n = 6) and *fat1* + *Alox15*$^{-/-}$ mice (n = 6) were kept at standard conditions and 8 to 10-weeks-old females with similar body weights were selected. 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis was performed in littermate, sex-matched, and cohoused mice sharing identical conditions independent of their genotype. To induce TNBS colitis, mice were first sensitized by the application of 100 µL 2.5% TNBS (Sigma-Aldrich) in 50% (*v/v*) ethanol to the dorsal skin. The control group was treated with presensitization solution without TNBS. Prior to intrarectal TNBS administration, food (but not water) was withdrawn overnight for 12 hours (day 0). Seven days after presensitization, colitis was induced by the intrarectal administration of 100 µl of 1.9% TNBS in 50% (*v/v)* ethanol (day 1). After TNBS instillation, mice were held in an upright position for 60 seconds to avoid reflux. Control animals were instilled with the same volume of 50% ethanol without TNBS. Mice were sacrificed 3 days after intrarectal TNBS injection by isoflurane anesthesia and cervical dislocation. The colon was prepared and inspected for macroscopic signs of inflammation. Colon length was determined and small tissue samples were formalin-fixed. The remaining colon was cut in pieces of 1 cm, the samples were shock-frozen in liquid nitrogen and stored at −80°C.

2.3 | DSS colitis model

Wild-type mice (n = 9), *fat1* + *Alox15*$^{+/+}$ mice (n = 9), *Alox15*$^{-/-}$ mice (n = 11) and *fat1* + *Alox15*$^{-/--}$ mice (n = 9) were kept at standard conditions and 10 to 12-weeks-old males were selected. Dextran sulfate sodium (DSS) colitis was performed in littermate, sex-matched, and cohoused mice sharing identical conditions independent of their genotype. For the induction of colitis, drinking water containing 2.5% (*w/v*) DSS (molecular weight = 36.000-50.000, ICN Biomedicals, Irvine, CA, USA) was provided ad libitum for 7 days. Then DSS water was replaced by normal drinking water. On the 8th day, mice were anesthetized with isoflurane and sacrificed by cervical dislocation. To quantify colitis severity, body weight, stool consistency, and the presence of blood in the stool were determined and a scoring system was applied.[25] Sample collection was carried out as described above for TNBS colitis.

2.4 | 15S-HEPE application

Wild-type mice received daily intraperitoneal (i.p.) injections of 50 µg/kg body weight 15S-HEPE (Cayman Chemical) or PBS as vehicle control, starting one day before the induction of colitis until the end of the experiment.

2.5 | Histological evaluation of colitis severity

Microscopic cross-sections (10 µm) of frozen colon segments were stained with the standard hematoxylin-eosin method and histopathological assessment was carried out in a blinded manner. Four different histological categories were evaluated: (a) Degree of histological inflammation (0 = no inflammation, 1 = mild, 2 = moderate, 3 = severe), (b) severity of intestinal damage (0 = no damage, 1 = mucosa damage, 2 = mucosa plus submucosa damage, 3 = transmural damage), (c) character of epithelial erosion (0 = intact epithelium, 1 = disruption of architectural structure, 2 = erosion, 3 = ulceration) and (d) extent of intestinal lesions (0 = no lesions, 1 = punctuate, 2 = multifocal, 3 = diffuse).

2.6 | Analysis of endogenous eicosanoid levels

Free eicosanoids from colon tissue were extracted and analyzed by LC-ESI-MS/MS[26] and a more detailed description is given in the supplementary material.

2.7 | Polyenoic fatty acid analysis of plasma lipids

The polyenoic fatty acid content of the plasma lipid hydrolysates was analyzed by RP-HPLC[27] and a more detailed description of the analyses procedure is given in the supplement.

2.8 | Statistical analysis

Statistical evaluation of data was performed using the Prism software package (GraphPad). Unless indicated otherwise,

all data were representative of at least two independent experiments and expressed as mean ± SEM. Since most results were not normally distributed, we employed the non-parametrical Mann-Whitney U Test. $P < .05$ were considered statistically significant.

3 | RESULTS

3.1 | Functional characterization of genetically modified animals

Previous experiments have shown that overexpression of the *fat1* gene protected mice from chemically induced colitis but the underlying mechanisms remained largely unclear.[11,28] The aim of the study was to investigate the potential role of *Alox15* in this protective effect. For this purpose, we crossed transgenic *fat1* mice with *Alox15*-deficient animals. Before using the animals in two different colitis models, we explored the functional alterations induced by the two genetic manipulations.

Fat1 mice carry the *C elegans fat1* transgene encoding for a n-6 PUFA desaturase. Acting on arachidonic acid (AA) this enzyme produces eicosapentaenoic acid (EPA). Docosapentaenoic (DPA) acid is converted to docosahexaenoic acid (DHA) and linoleic acid (LA) to alpha-linolenic acid (ALA). To test the impact of *fat1* transgene expression on the polyenoic fatty acid profile, we analyzed the PUFA composition of the plasma lipids by HPLC. From Figure 1A it can be seen that LA, AA, and DHA are the dominant polyenoic fatty acids found in wild-type mice. Smaller amounts of EPA, ALA, and n-3 DPA were also detected. A similar PUFA pattern was analyzed for *Alox15*$^{-/-}$ mice (Figure 1B) and there was no significant difference between these two genotypes. In contrast, for *fat1* mice (*fat1* + *Alox15*$^{+/+}$) the relative shares of DHA and EPA were significantly increased (Figure 1C). When we compared the molar EPA/AA- and the molar DHA/DPA(n-6) ratios (Table 1) we observed highly significant ($P < .001$) differences between wild-type and *fat1* mice. Interestingly, we did not find significant alterations between wild-type and *fat1* mice when the ALA/LA-ratios were compared (Table 1). A similar situation was observed when *Alox15*$^{-/-}$ mice were compared with *fat1* + *Alox15*$^{-/-}$ mice (Figure 1B,D). Here again (Table 1), a significant ($P < .001$) increase in the EPA/AA- and in the DHA/DPA(n-6) ratios was observed. These data indicate the in vivo functionality of the *fat1* transgene.

To quantify the functionality of the *Alox15* gene in the different genotypes, we carried out ex vivo Alox15 activity assays. For this purpose, we prepared peritoneal lavage cells, incubated them in PBS containing 100 μmol/L AA, and analyzed the formation of Alox15 products. As expected from previous studies,[22] 12-hydroxy eicosatetraenoic acid (12-HETE) was formed in large amounts when wild-type peritoneal lavage cells were used (Figure 1E). In contrast, peritoneal lavage cells of *Alox15*$^{-/-}$ mice did not form any 12-HETE. Here, the major oxygenation product was 5-HETE originating from the ALOX5 pathway (Figure 1F). Comparing the oxygenation products formed by peritoneal lavage cells prepared from *fat1* + *Alox15*$^{+/+}$ and *fat1* + *Alox15*$^{-/-}$ mice we observed similar differences (Figure 1G,H). Thus, on *fat1* background too, inactivation of the *Alox15* gene leads to an upregulation of the Alox5 pathway. Quantification and statistical evaluation of this data are given in Table 2. Taken together, these data indicate the expected functional alterations according to the different genetic backgrounds.

3.2 | Colitis and *Alox15* deficiency in *fat1* mice

In order to investigate the effect of *Alox15* deficiency in *fat1* mice in the setting of chemically induced colitis, we first applied the TNBS model. When wild-type mice were challenged in the TNBS colitis model animals lost approximately 20% of their body weight (Figure 2A). A similar effect was observed for *Alox15*$^{-/-}$ mice, indicating that the inactivation of the *Alox15* gene did not impact colitis severity. In contrast, overexpression of the *fat1* gene (*fat1* + *Alox15*$^{+/+}$) partly protected mice from loss of body weight with only 10% weight reduction. This protective effect was completely abolished in *fat1* + *Alox15*$^{-/-}$ mice (Figure 2A). Colon length differences (Figure 2B) between *fat1* + *Alox15*$^{-/-}$ mice and *fat1* + *Alox15*$^{+/+}$ were not statistically significant ($P = .286$), but demonstrate a trend toward more severe disease activity in the *fat1* + *Alox15*$^{-/-}$ mice as compared to *fat1* + *Alox15*$^{+/+}$ mice. Furthermore, TNBS administration was associated with the presence of clinical colitis signs, such as a mushy or watery stool consistency and fecal bleeding, translating into a significantly increased disease activity index (DAI) as compared to WT mice without TNBS treatment (Figure 2C). In accordance with the aforementioned data, in *fat1* mice (*fat1* + *Alox15*$^{+/+}$) the DAI was significantly improved whereas *Alox15* deficiency on *fat1* background (*fat1* + *Alox15*$^{-/-}$) resulted in a DAI similar to wild-type animals (Figure 2C). Finally, we determined the histological inflammation score in the colons (Figure 2D,E). TNBS application strongly increased histological inflammation in wild-type mice. In contrast, in *fat1* mice (*fat1* + *Alox15*$^{+/+}$) this score was less than half as high, while *Alox15* deficiency on *fat1* background (*fat1* + *Alox15*$^{-/-}$) led to a histology score similar to the one in wild-type mice (Figure 2D,E).

Mouse Alox15 is an arachidonic acid (AA) 12-lipoxygenating ALOX15 ortholog converting AA to a 10:1 mixture of 12- and 15-HETE.[30] With the n-3 PUFA DHA the major Alox15 oxygenation product is 14-hydroxy

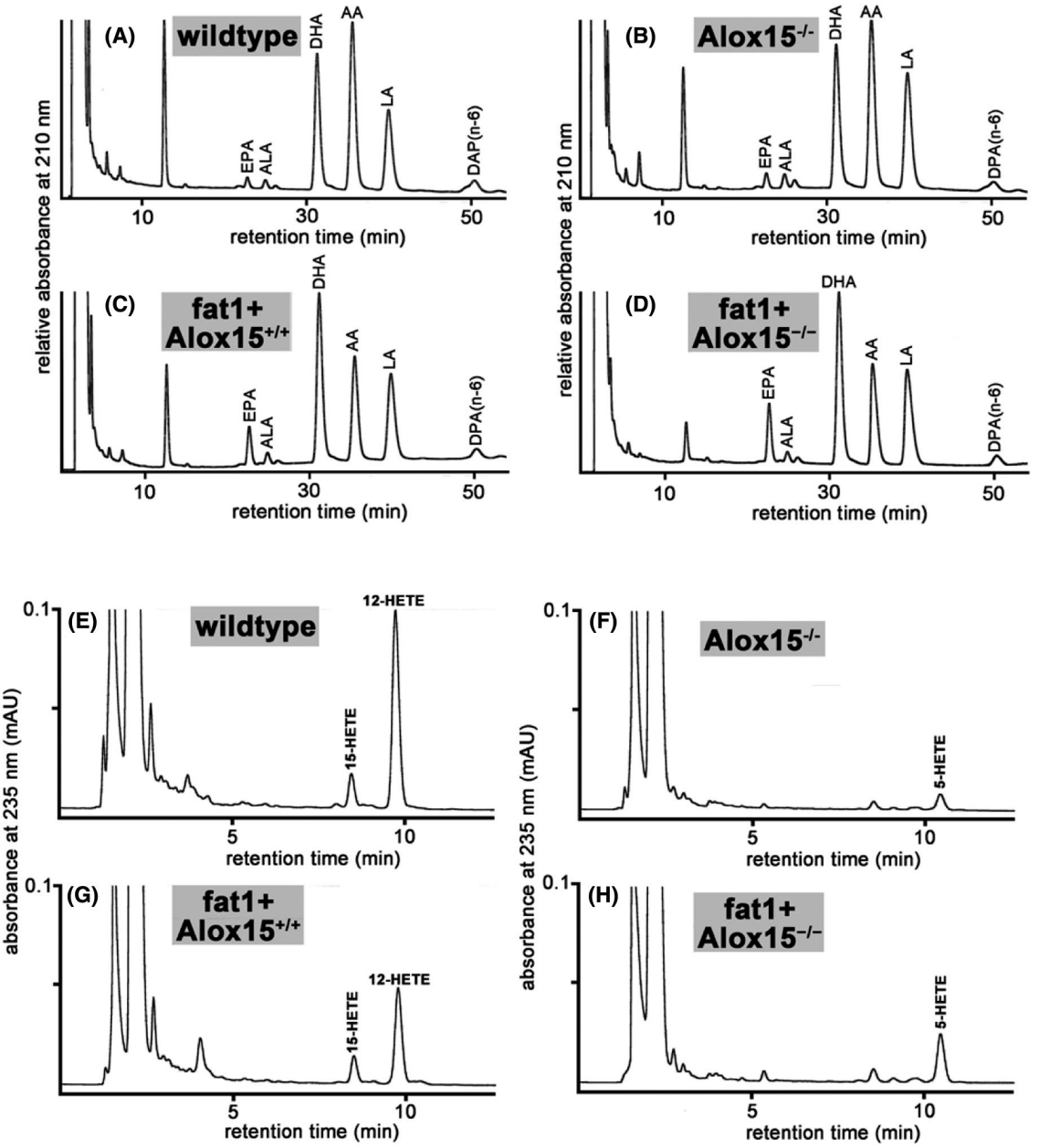

FIGURE 1 Functional characterization of genetically modified mice. A-D) PUFA composition of plasma lipids prepared from mice with different genotypes: 50 µL of blood plasma were lipid extracted[29] and the extracts were hydrolyzed under alkaline conditions. After acidification aliquots were injected into RP-HPLC. The retention times of the different PUFAs were determined by injecting known amounts of authentic standards. Analyses were carried out for 3-5 individuals of each genotype and representative chromatograms are shown. Statistic evaluation of all data is given in Table 1. E-H, Ex vivo Alox15 activity of peritoneal lavage cells prepared from mice of different genotypes: Peritoneal lavage cells were prepared and after washing, the cells were reconstituted in 0.5 ml of PBS containing 100 µmol/L AA. After a 15 min incubation period the formed hydroperoxy fatty acids were reduced (addition of 1 mg solid $NaBH_4$), protein was precipitated (addition of 0.5 ml of acetonitrile), precipitate was spun down and 0.3 ml of the protein-free supernatant were injected to RP-HPLC (see Materials and Methods). AA oxygenation products were identified by co-chromatography with authentic standards. Activity assays were carried out for three individuals of each genotype and representative chromatograms are shown. Statistic evaluation of all data is given in Table 2

docosahexaenoic acid (14-HDHA), whereas n-3 PUFA EPA is mainly oxygenated to 15-hydroxyeicosapentaenoic acid (15-HEPE).[31] As expected, 12-HETE and 15-HETE concentrations were reduced in *Alox15*$^{-/-}$ mice of either wild-type or *fat1* background (Figure 2F,G). Although the difference in 12-HETE levels between wild-type and *Alox15*$^{-/-}$ mice did not reach statistical significance these data suggest that a proportion of these oxygenated fatty acid metabolites is formed *via* the Alox15 pathway. Colonic 15-HEPE concentrations were significantly higher in *fat1* mice when compared with

TABLE 1 PUFA composition of the plasma ester lipids in mice with different genetic backgrounds

Genotype	EPA/AA		DHA/DPA		ALA/LA	
wildtype	0.014 ± 0.002	$P < .001$	4.364 ± 0.514	$P < .001$	0.036 ± 0.003	$P = .958$
fat1 + *Alox15*$^{+/+}$	0.036 ± 0.015		7.284 ± 0.254		0.038 ± 0.003	
Alox15$^{-/-}$	0.018 ± 0.004	$P < .001$	4.500 ± 0.509	$P < .001$	0.031 ± 0.006	$P = .298$
fat1 + *Alox15*$^{-/-}$	0.131 ± 0.013		8.572 ± 0.776		0.035 ± 0.007	

Note: The PUFA compositions of the plasma ester lipids were analyzed as described in Figure 1. Transgenic fat1 functions as omega-6 polyenoic fatty acid desaturase, which converts arachidonic acid (AA) to eicosapentaenoic acid (EPA), n-6-docosapentaenoic acid (DPA, n-6) to docosahexaenoic acid (DHA) and linoleic acid (LA) to alpha linolenic acid (ALA). To test the functionality of the *fat1* transgene we calculated the molar EPA/AA-ratio, the molar DHA/DPA (n-6)-ratio, and the molar ALA/LA ratios for the different genotypes, which represent the substrate/product ratios of the transgenic omega-6 fatty acid desaturase (fat1). For each genotype, the plasma fatty acid compositions of 3-5 individuals were analyzed by RP-HPLC and the experimental raw data (mean ± SEM) were statistically evaluated using the Students *t*-test.

TABLE 2 Formation of Alox-products in peritoneal lavage cells of mice with different genetic backgrounds

Genotype	12-HETE (nmol/mg protein)	15-HETE (nmol/mg protein)	5-HETE (nmol/mg protein)
wildtype	8.91 ± 1.05^{a,c}	0.65 ± 0.30	0 ± 0^{c}
Alox15$^{-/-}$	0.09 ± 0.13^{a}	0 ± 0	1.68 ± 1.25
fat1 + *Alox15*$^{+/+}$	3.67 ± 1.62^{b,c}	0.30 ± 0.36	0 ± 0
fat1 + *Alox15*$^{-/-}$	0.01 ± 0.01^{b}	0 ± 0	1.75 ± 0.44

Note: Peritoneal lavage cells were prepared from three different individuals of the four different genetic backgrounds. Product formation was normalized to the amount of cellular protein and the data (mean ± SEM) were statistically analyzed using the Students *t*-test. $^{a}P < .001$, $^{b}P = .018$, $^{c}P = .009$.

wild-type controls, probably due to the fact that *fat1* mice have significantly augmented EPA levels (Figure 2H). In contrast, in *Alox15*$^{-/-}$ mice 15-HEPE formation was strongly suppressed, suggesting that 15-HEPE is mainly formed *via* the Alox15 pathway. 14-HDHA is the major Alox15 product of DHA oxygenation and we observed lower 14-HDHA concentrations in colon tissue of *Alox15*-deficient animals of either background (Figure 2I).

Next, we tested the impact of *Alox15* deficiency on *fat1* background in the DSS-induced colitis model. Wild-type mice developed clinical colitis symptoms after 4 days of DSS administration and eight days after the onset of DSS application wild-type mice had lost about 12% of their initial body weight owing to gastrointestinal inflammation (Figure 3A) and a similar loss of body weight was observed for *Alox15*$^{-/-}$ mice. This result was somewhat surprising since previous data indicated that *Alox15* deficiency protected mice from DSS-induced colitis when the animals were kept on a regular chow diet.[21] In the experiments carried out in this study the mice were maintained on an n-6 PUFA rich diet in order to adjust the experimental protocol to that of the fat1 studies. These data suggest that the effect of *Alox15* deficiency in DSS colitis is variable and depends on the kind of diet. Transgenic *fat1* overexpression partly protected mice from DSS-induced loss of body weight[11] with loss of only 5% of their initial body weight observed here. Most interestingly, however, the protective effect of *fat1* overexpression was completely abolished (Figure 3A) when the expression of *Alox15* was systemically silenced in the *fat1* + *Alox15*$^{-/-}$ mice. Similar results were obtained when colon lengths were used as clinical readout parameter. In wild-type mice DSS treatment induced a 33% reduction of colon length (Figure 3B). In contrast, *fat1* mice showed a significantly lower colon length reduction (22%). Silencing of *Alox15* expression in *fat1* mice (*fat1* + *Alox15*$^{-/-}$) led to a more pronounced reduction of colon length comparable to the wild-type animals (Figure 3B). As shown for TNBS colitis, DSS administration led to a significantly increased disease activity index in wild-type mice (Figure 3C). In contrast, in *fat1* mice (*fat1* + *Alox15*$^{+/+}$) the DAI was less than half as high, while *Alox15* deficiency on *fat1* background (*fat1* + *Alox15*$^{-/-}$) resulted in a DAI similar to that of wild-type mice. The histological inflammation score for the different genotypes demonstrated that DSS application induced strong inflammation in wild-type mice (Figure 3D,E). In *fat1* mice (*fat*1 + *Alox15*$^{+/+}$) the score was significantly reduced. Here again, *Alox15* deficiency on *fat1* background (*fat1* + *Alox15*$^{-/-}$) led to a significant increase in inflammation severity as compared to *fat1* + *Alox15*$^{+/+}$ animals (Figure 3D,E). Taken together, these data suggest that in both types of experimental colitis (TNBS and DSS) *Alox15* deficiency on *fat1* background intensified colitis symptoms. In fact, without *Alox15* expression *fat1* mice develop a similar disease severity as compared to WT animals.

Colonic concentrations of 12-HETE and 15-HETE in DSS-treated *fat1* mice were significantly reduced when compared with samples from wild-type mice (Figure 3F,G). This

observation is consistent with the lower systemic AA concentration in these animals.[9,23] Silencing of *Alox15* expression in *fat1* background led to a further reduction of 12- and 15-HETE levels. For 15-HEPE, a principle Alox15 metabolite of EPA,[31] significantly higher levels were observed in the colon of *fat1* mice when compared with wild-type animals (Figure 3H).

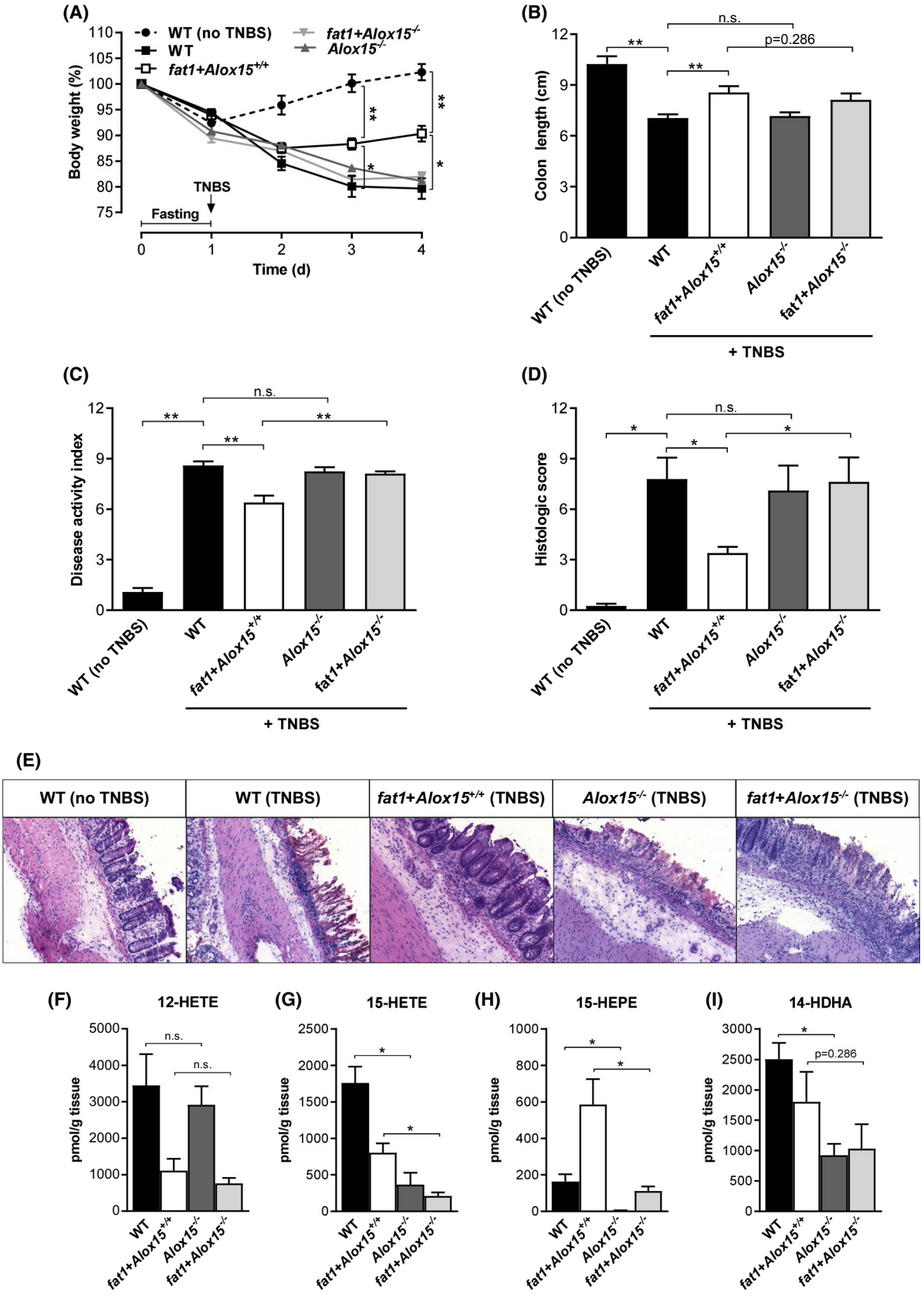

FIGURE 2 Clinical readout parameters and concentrations of free (ie, nonesterified) colonic lipid mediator in mice of different genotypes with TNBS-induced colitis. TNBS colitis was induced by the intrarectal administration of TNBS as described in Materials and Methods. Wild-type mice in the control group (WT (no TNBS)) were instilled with an equal volume of ethanol without TNBS. Female mice of different genotypes were categorized in five different groups: (a) wild-type mice (WT), no TNBS (control, n = 4); (b) wild-type mice (WT) + TNBS (n = 6); (c) *fat1* mice (*fat1* + *Alox15*$^{+/+}$) + TNBS (n = 5); (4) *Alox15*$^{-/-}$ mice + TNBS (n = 6); (5) *fat1* + *Alox15*$^{-/-}$ mice + TNBS (n = 6). Body weights were determined daily. On day 4 after initial TNBS administration, the animals were sacrificed, colons were prepared and their lengths were determined. Distal sections of the colons were used to prepare histological cross-sections for morphometric evaluations. Proximal parts were shock-frozen in liquid nitrogen and used for Alox15 metabolite quantification. A) Body weight kinetics, B) Colon length at the end of the experimental protocol, C) Disease activity index at the end of the experimental protocol, D) Histology score at the end of the experimental protocol, E) Representative images of hematoxylin and eosin-stained colon sections (magnification x100). Quantification of F) 12-HETE, G) 15-HETE, H) 15-HEPE, I) 14-HDHA levels by LC-MS/MS[26] in the colons from TNBS-treated mice at the experimental endpoint. Significant differences (*$P < .05$, **$P < .01$ by Mann-Whitney U Test) are indicated and for borderline significances, the *P* values are given

Silencing of *Alox15* expression in *fat1* mice drastically abolished 15-HEPE formation. 14-HDHA, the major Alox15 metabolite of DHA,[31] is found in large quantities in the colon tissue of DSS-treated wild-type mice and slightly lower levels were detected in *fat1* + *Alox15*$^{+/+}$ mice (Figure 3I). In contrast, we only observed low concentrations of 14-HDHA in the colon of DSS-treated *fat1* + *Alox15*$^{-/-}$ mice.

3.3 | Systemic application of 15S-HEPE protected mice from colitis

To explore the potential mechanism of the anti-inflammatory effect of Alox15 in *fat1* mice (*fat1* + *Alox15*$^{+/+}$) we systemically administered 15S-HEPE (daily intraperitoneal injection of 15S-HEPE at a dose of 50 µg/kg body weight) to wild-type mice and challenged these animals in the DSS and TNBS colitis model.

As expected, TNBS application induced a weight loss in vehicle-treated wild-type mice (10% reduction of their initial body weight (Figure 4A). Treatment with 15S-HEPE protected mice from TNBS-induced loss of body weight with only 2% weight reduction three days after TNBS instillation. These findings were confirmed by a significantly reduced colon shortening in 15S-HEPE-treated mice compared with vehicle-treated animals (Figure 4B). Colitis severity was further assessed using the disease activity index. As shown in Figure 4C, the comparison of the DAIs between vehicle- and 15S-HEPE-treated animals revealed a trend to a lower DAI due to 15S-HEPE treatment. Finally, TNBS application significantly induced histological inflammation and in accordance with the aforementioned results, the score was significantly reduced by 15S-HEPE treatment (Figure 4D,E).

Next, we explored the impact of 15S-HEPE treatment in DSS-induced colitis. Based on the body weight kinetics (Figure 5A) and on colon length comparison (Figure 5B) we also observed significant protective effects of 15S-HEPE in DSS colitis. Importantly, no alterations in clinical readout parameters were seen when 15S-HEPE was given to wild-type mice not receiving DSS as colitis inducer. Moreover, we quantified the disease activity index of 15S-HEPE- and vehicle-treated mice (Figure 5C) at the end of the experimental protocol. After DSS treatment the DAI of mice that did not receive 15S-HEPE went up to 5.5, indicating moderate colitis. In mice receiving 15S-HEPE the DAI was significantly lower, indicating a protective effect of 15S-HEPE. Finally, the histological inflammation score demonstrated inflammation induction due to DSS application and a significantly lower histological inflammation intensity due to 15S-HEPE treatment (Figure 5D,E). Taken together, these results indicate that the systemic application of 15S-HEPE protects wild-type mice from both, DSS and TNBS-induced colitis. These data are consistent with the protective effects of *fat1* overexpression and the deteriorating impact of *Alox15* deficiency in *fat1* mice.

4 | DISCUSSION

4.1 | Systemic *Alox15* knockout abolished the protective effect of *fat1* overexpression

Transgenic overexpression of *C elegans* n-6 polyenoic fatty acid desaturase *fat1* in mice protected the animals from chemically induced colitis but the molecular basis for this protective effect has not been explored in detail.[11,28] Since Alox15 products such as lipoxins and resolvins have been implicated in inflammation resolution[12,13] we reasoned that Alox15 may play a role in the protective effect of *fat1* overexpression in chemically induced colitis. To test this hypothesis, we crossed *fat1* transgenic mice with *Alox15*-deficient animals and confirmed *fat1* overexpression as well as *Alox15* deficiency by in vivo and ex vivo activity assays (Figure 1). Next, we tested the resulting *fat1* + *Alox15*$^{-/-}$ mice in two different models of chemically induced experimental colitis. Here we found that the protective effect of *fat1* overexpression was significantly reduced or even abolished when the systemic expression of *Alox15* was silenced (Figures 2,3). These data indicate that expression of *Alox15* may play a role in the protective effect of *fat1* overexpression in chemically induced colitis. To explore the underlying mechanism, we finally performed 15S-HEPE supplementation studies (Figures 4,5). 15-HEPE is the major product of mouse

Alox15 catalyzed the oxygenation of EPA.[32] EPA is present at elevated concentrations in *fat1* mice (Table 1) and we detected this metabolite at relatively high concentrations in inflamed colon specimens of TNBS-treated (Figure 2H) and DSS-treated (Figure 3H) mice. When we pre-treated wild-type mice with 15S-HEPE and subsequently challenged the

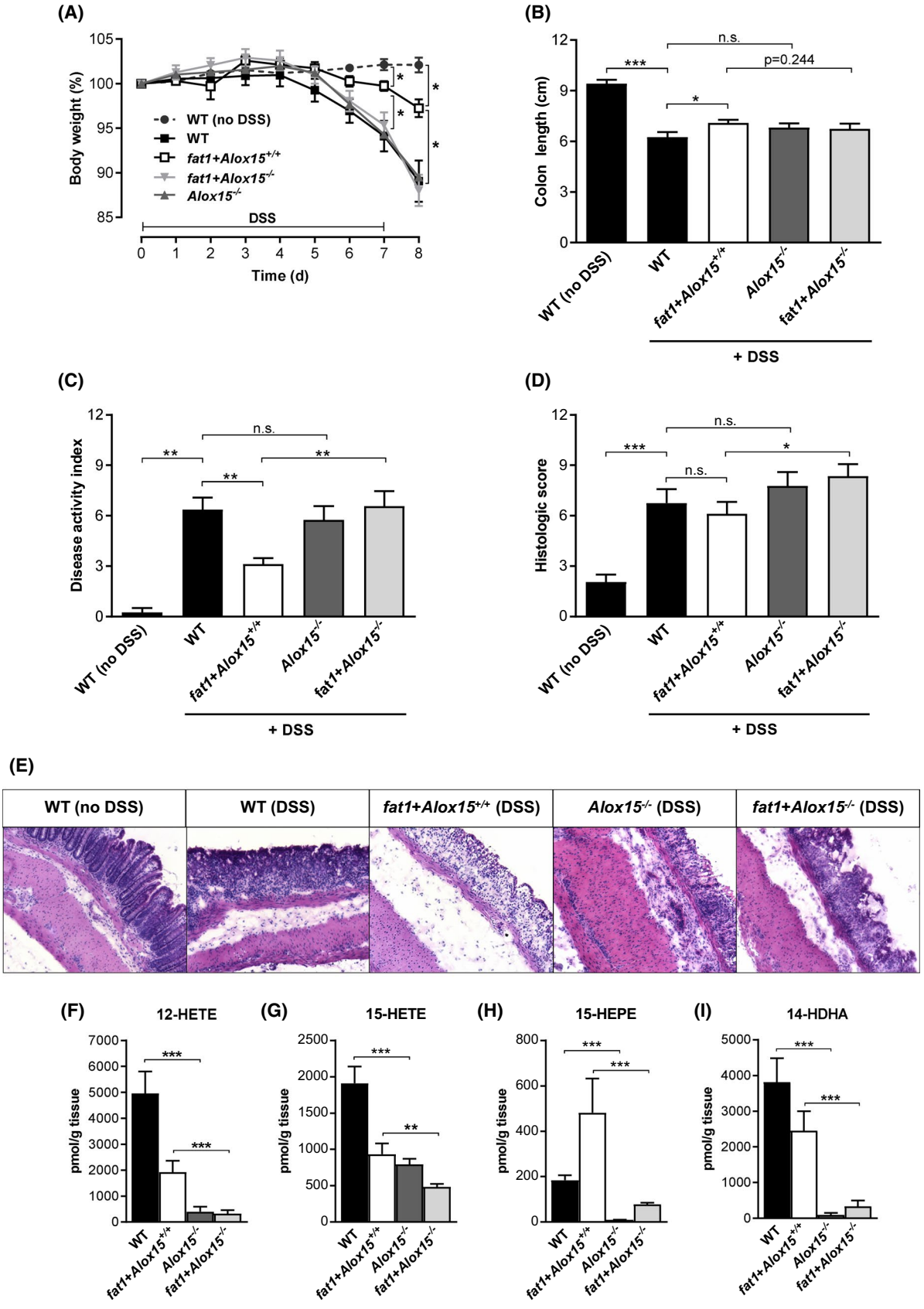

FIGURE 3 Clinical readout parameters and concentrations of free (ie, nonesterified) colonic lipid mediator in mice of different genotypes with DSS colitis. DSS colitis was induced by the administration of 2.5% DSS in the drinking water for the times indicated. Control mice received similar volumes of normal drinking water. Male mice of different genotypes were categorized in five different groups: (i) wild-type mice (WT), no DSS (control, n = 16); (ii) wild-type mice (WT) + DSS (n = 9); (iii) *fat1* mice (*fat1* + *Alox15*$^{+/+}$) + DSS (n = 9); (iv) *Alox15*$^{-/-}$ mice + DSS (n = 11); (v) *fat1* + *Alox15*$^{-/-}$ mice + DSS (n = 9). Body weights were determined daily. On day 8 after initial DSS administration, the animals were sacrificed, the colon was prepared and their lengths were determined. The colon was divided into three sections and the distal parts were taken for the evaluation of the histology scores. Proximal parts were shock-frozen in liquid nitrogen and used for Alox15 metabolite quantification. (A) Body weight kinetics, (B) Colon length at the end of the experimental protocol, (C) Disease activity index at the end of the experimental protocol, (D) Histology scores at the end of the experimental protocol, (E) Representative images of hematoxylin and eosin-stained colon sections (magnification x100). Quantification of (F) 12-HETE, (G) 15-HETE, (H) 15-HEPE, (I) 14-HDHA levels by LC-MS/MS[26] in the colons from DSS-treated mice at the experimental endpoint. Significant differences (*$P < .05$, **$P < .01$, ***$P < .001$ by Mann-Whitney U Test) are indicated

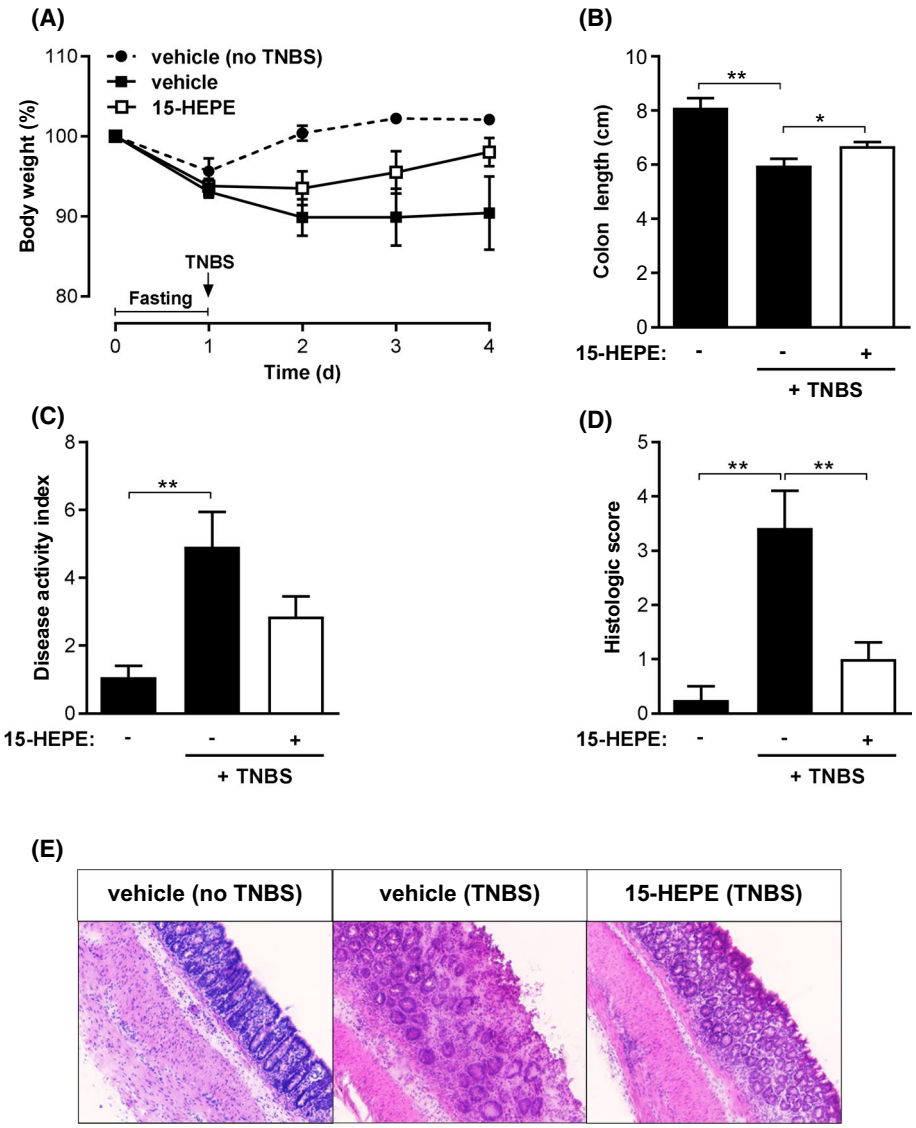

FIGURE 4 Impact of 15S-HEPE administration on TNBS colitis. Wild-type mice (n = 8 in each group) were treated daily with 15S-HEPE (intraperitoneal injection of 15S-HEPE dissolved in PBS) at a dose of 50 µg/kg body weight, starting one day before colitis induction until day 4 of the experimental protocol. Vehicle-treated animals received corresponding injections of the solvent PBS. TNBS colitis was induced by the intrarectal administration of TNBS as described in Materials and Methods. Mice in the control group (vehicle (no TNBS)) were instilled with an equal volume of ethanol without TNBS. Body weights were determined daily. On day 4 after initial TNBS administration, the animals were sacrificed, colons were prepared and their lengths were determined. A) Body weight kinetics, B) Colon length at the end of the experimental protocol, C) Disease activity index at the end of the experimental protocol, D) Histology score at the end of the experimental protocol, E) Representative images of hematoxylin and eosin-stained colon sections (magnification x100). Significant differences (*$P < .05$, **$P < .01$ by Mann-Whitney U Test) are indicated

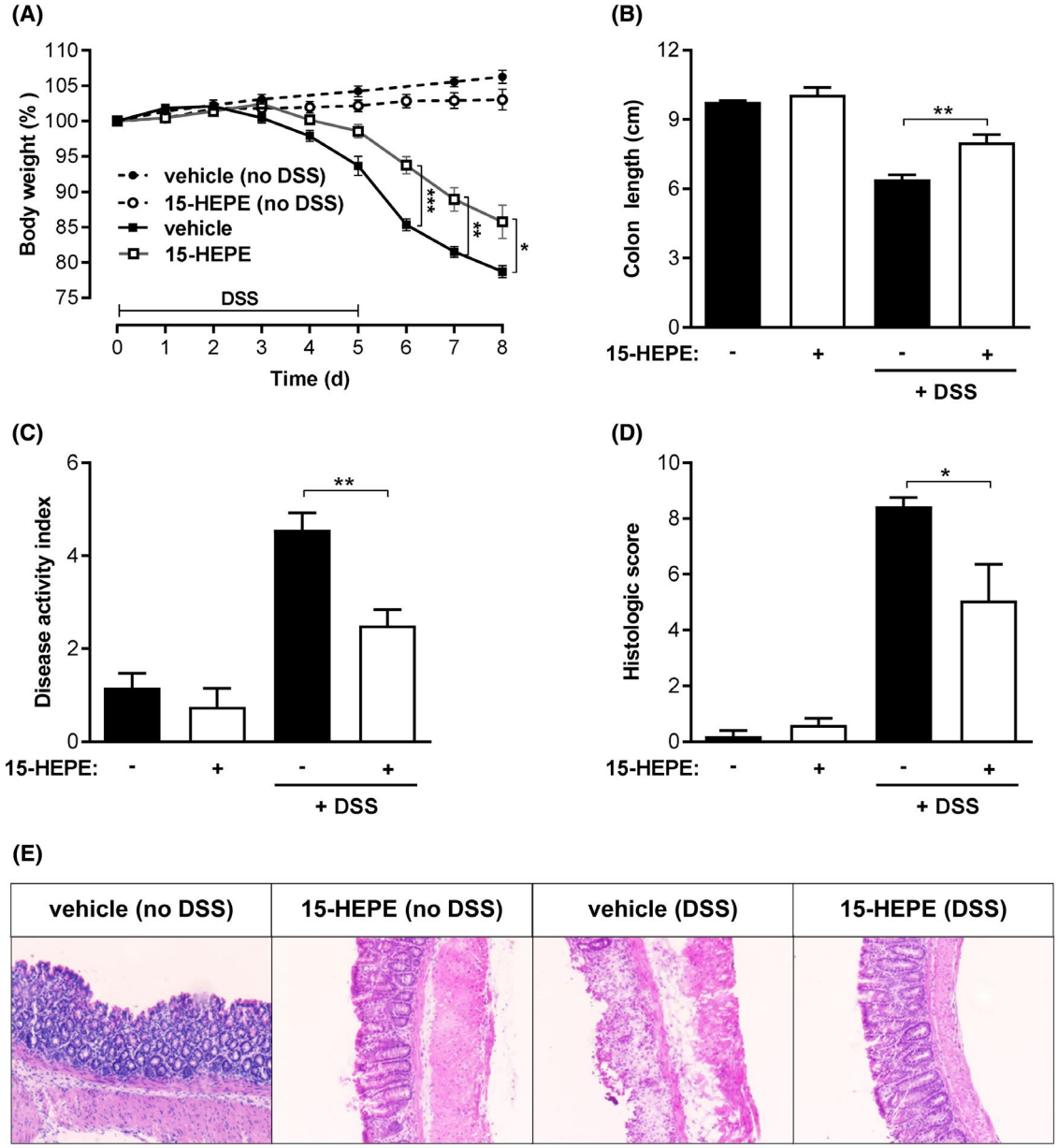

FIGURE 5 Impact of 15S-HEPE administration on DSS colitis. Wild-type mice (n = 8 in each group) were treated daily with 15S-HEPE (intraperitoneal injection of 15S-HEPE dissolved in PBS) at a dose of 50 µg/kg body weight, starting one day before colitis induction until day 8 of the experimental protocol. Vehicle-treated mice received corresponding injections of the solvent PBS. Colitis was induced by the administration of DSS (2.5% in the drinking water) and this treatment was maintained for 5 days. Then DSS-containing drinking water was removed and the animals were kept for additional 3 days with normal drinking water ad libitum. Body weights were monitored daily. On day 8 of the experimental protocol, the animals were sacrificed, the colons were prepared and the organ lengths were determined. A) Body weight kinetics, B) Colon length at the end of the experimental protocol, C) Disease activity index at the end of the experimental protocol, D) Histology score at the end of the experimental protocol, E) Representative images of hematoxylin and eosin-stained colon sections (magnification x100). Significant differences (*$P < .05$, **$P < .01$, ***$P < .001$ by Mann-Whitney U Test) are indicated

animals with TNBS (Figure 4) or DSS (Figure 5) we found that the intensity of inflammatory symptoms was strongly reduced. These data suggest that systemic 15S-HEPE application protected the mice from TNBS- and DSS-induced colitis. *Fat1* mice have increased EPA concentrations (Table 1) and this n-3 PUFA is converted by mouse Alox15 to 15-HEPE.[32] Furthermore, *fat1* overexpression protected mice from TNBS- and DSS-induced colitis and systemic expression silencing of *Alox15* abolished this protective effect (Figures 2 and 3). Taken together, these data suggest that the protective effect of *fat1* overexpression may be related to the Alox15-dependent in vivo formation of 15-HEPE. When *Alox15* expression is silenced 15-HEPE formation is strongly reduced and the protective effect is abolished.

Although 15-HEPE also exhibits anti-inflammatory activities in other inflammation models[33] it remains an open question whether the compound itself is the functionally important mediator. It might well be that 15-HEPE is further metabolized to unknown effector products. For instance, 15-HEPE can be incorporated into the cellular ester lipids yielding 15-HEPE containing phospholipids. These ester lipids may then be hydrolyzed yielding 15-HEPE containing lysophosphatids exhibiting much stronger anti-inflammatory properties than 15-HEPE itself.[34]

An alternative explanation for the anti-inflammatory role of Alox15 and its primary EPA metabolite 15-HEPE could be a role in the biosynthesis of other SPMs.[12,13] SPMs are anti-inflammatory and pro-resolving lipid mediators, which can be formed via lipoxygenases.[13] Several reports describe the principal effects of DHA- and EPA-derived SPMs in experimental models of inflammatory bowel diseases. DHA constitutes the origin of the D-series resolvins, protectins, and maresins, while EPA generates the family of E-series resolvins. In terms of DHA-derived mediators, administration of resolvin D2, aspirin-triggered resolvin D1, and maresin 1 resulted in an improvement of DSS- and TNBS-induced colitis in mice.[35,36] Similarly, EPA-derived resolvin E1 has been reported as a protective factor in both, DSS- and TNBS-induced colitis.[37-39] Furthermore, a recent study revealed that also lipid mediators from n-3 docosapentaenoic (DPA), namely protectin D1 n-3 DPA ($PD1_{n\text{-}3DPA}$) and resolvin D5 n-3 DPA, exert potent anti-inflammatory actions in intestinal inflammation in vivo.[40] Interestingly, the inhibition of 15-lipoxygenase activity by PD 146 176 not only reduced $PD1_{n\text{-}3DPA}$ concentration, but also augmented intestinal inflammation in DSS colitis[40] and thus Gobbetti et al show comparable n-3 PUFA- and Alox15-dependent effects on experimental colitis as in this study.

15-HEPE can also be further metabolized to pro-resolving SPMs and in humans ALOX12, ALOX5 and ALOX15B have been implicated in this process. The possible role of mouse Alox12 and Alox15b, the corresponding orthologs of which have been implicated in the biosynthesis of SPMs in humans, remains unclear. Mouse Alox15 converts AA mainly to 12-HETE[30] but EPA is oxygenated to 15-HEPE.[31] With DHA, 14-HDHA is the major oxygenation product.[31] Thus, it is not possible to conclude the composition of the Alox products of a given PUFA from the reaction specificity with AA. In a murine model, the interpretation of the Alox15b product pattern is even more complicated. In contrast to human ALOX15B, which oxygenates AA almost exclusively to 15-HETE,[41] the mouse ortholog converts this substrate fatty acid to 8S-HETE.[42] This metabolite cannot be converted to trihydroxy tetraenes (lipoxin isomers). Whether the reaction specificity of mouse Alox15b with EPA and DHA is similar to that of AA oxygenation (major formation of 8-HEPE from EPA and 7- or 10-HDHA from DHA) and whether this enzyme is part of the SPM biosynthesizing cascade in mice remains to be explored in the future. In this study, we specifically searched for selected SPMs (LxA_4, RvD1, RvE1, RvE2, 18S-RvE3, 18R-RvE3) using our LC-MS/MS-based method.[43] However, we were unable to detect these metabolites in colon tissue. The quantification limits of our analytical system (LLOQ) were 0.3-2.1 pg per injection (0.9-6 fmol on column), which corresponds to a tissue concentration of about 77-525 pg/g (0.2-1.5 pmol/g) wet weight. It might be that the steady-state concentrations of these compounds in the colon tissue are below the detection limits of our analytical system. Another possibility for our inability to detect significant amounts of pro-resolving mediators may be the kinetics of their biosynthesis, and their presence only during the resolution of inflammation. In our experiments we sacrificed the animals at the peak of the acute inflammatory phase (4th day of TNBS colitis, 8th day of DSS colitis). However, the inflammation dampening effect of 15-HEPE demonstrated here indicates a role of this metabolite also in early inflammation phases, and possibly even in the prevention of inflammatory processes.

4.2 | The role of Alox15 in DSS-induced colitis depends on the diet

In previous experiments[21] we have shown that Alox15 *deficiency* protected female mice from DSS-induced colitis when the mice were fed a regular chow diet. In this study, we repeated this experiment for control purposes with male mice which were kept on an n-6 PUFA enriched diet (Figure 3). Surprisingly, we observed that these mice were not protected from DSS-induced colitis. In fact, *Alox15*$^{-/-}$ mice developed similar inflammatory symptoms to wild-type controls and there was no significant difference between the two genotypes in any of the quantified readout parameters. The molecular basis for the differential effect of *Alox15* expression in different model systems has not been explored in this study but there are two methodological differences between the two model systems: (a) In the former study[21] female mice were used but males were employed in the present study. Thus, the protective effect of *Alox15* expression silencing in DSS colitis might be gender-specific for females. Similar gender-specific effects have previously been reported for *Alox5* knockout mice.[44,45] (b) During the former study the mice were kept on a regular chow diet.[21] In contrast, for the present experiments the animals were fed a chow diet that was enriched by n-6 PUFAs. Since Alox15 utilized n-6 PUFAs as oxygenation substrates it might well be that the role of this enzyme was modified by the diet. In other words, the pathophysiological role of Alox15 may depend on the n-3/n-6 PUFA ratio of the endogenous lipids, which is quite variable depending on the diet.

4.3 | Applicability for human IBD

The two animal colitis models used in our studies are frequently employed as model systems to explore pathophysiological events of human IBD. Three of the six human ALOX isoforms (ALOX15, ALOX15B, ALOX5) have been implicated in the pathogenesis of human IBD and thus, may also play a role in mouse colitis models (TNBS- and DSS-induced colitis). Human ALOX5 and its mouse ortholog exhibit similar catalytic properties and thus, the mouse enzyme may constitute a suitable model for the human ortholog. This is, however, not the case for ALOX15 and ALOX15B: Human ALOX15B converts AA predominantly to 15S-HETE[41] but 8S-HETE is the major product of mouse Alox15b.[42] Human ALOX15 is an AA 15S-lipoxygenating enzyme[46] but the mouse ortholog predominantly forms 12-HETE.[30] This difference also impacts the lipoxin/SPM synthesizing capacity of the two enzyme orthologs. Although the two human enzymes exhibit a lipoxin synthase activity human ALOX15 is more efficient under strictly comparable in vitro conditions.[47] Important in the context of the murine data presented here, and despite differences in the reaction specificity of AA oxygenation, mouse and human ALOX15 orthologs catalyze almost exclusive n-6 oxygenation of EPA to 15-HEPE. This data points toward a possible role of 15-HEPE in humans to alleviate intestinal inflammation.

ACKNOWLEDGMENTS

We thank Ines Eichhorn and Birgit Bogdanoff for expert technical assistance and Fabian Nolte and Janina Waritschlager for their help with the sample preparation. We thank Jing X Kang from Massachusetts General Hospital and Harvard Medical School for providing the *fat1* mouse strain used in this study. This work was supported by research grants of the German Research Council (DFG) to HK (KU961/11-1, KU961/13-1) and KHW (WE2908/11-1).

CONFLICT OF INTEREST

All authors declare that they do not have any competing financial interests according to the policy of the journal. There are no other conflicts of interest to be disclosed.

AUTHOR CONTRIBUTIONS

N. Rohwer, H. Kühn, and K.-H. Weylandt: conception and design of the study, N. Rohwer, C.-Y. Chiu, D. Huang, C. Smyl, M. Rothe, KM Rund, and N. H. Schebb: experimental setup, lipidomics analyses data collection, generation of tables and figures; N. Rohwer, H. Kühn, and K.-H. Weylandt: drafting of the manuscript. All co-authors contributed to the final preparation of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Rohwer N, Chiu C-Y, Huang D, et al. Omega-3 fatty acids protect from colitis via an Alox15-derived eicosanoid. *The FASEB Journal*. 2021;35:e21491. https://doi.org/10.1096/fj.202002340RR