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Short-term high fat feeding induces inflammatory responses of tuft cells and mucosal barrier cells in the murine stomach

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Summary. Feeding mice with a high fat diet (HFD) induces inflammation and results in changes of gene expression and cellular composition in various tissues throughout the body, including the gastrointestinal tract. In the stomach, tuft cells expressing the receptor GPR120 are capable of sensing saturated long chain fatty acids (LCFAs) and thus may be involved in initiating mechanisms of mucosal inflammation. In this study, we assessed which cell types may additionally be affected by high fat feeding and which candidate molecular mediators might contribute to mucosa-protective immune responses. A high fat dietary intervention for 3 weeks caused an expansion of tuft cells that was accompanied by a higher frequency of mucosal mast cells and surface mucous cells which are a known source of the insult-associated cytokine interleukin 33 (IL-33). Our data demonstrate that both brush and mucosal mast cells comprise the enzyme ALOX5 and its activating protein FLAP and thus have the capacity for synthesizing leukotriene (LT). In HFD mice, several tuft cells showed a perinuclear colocalization of ALOX5 with FLAP which is indicative of an active LT synthesis. Monitoring changes in the expression of genes encoding elements of LT synthesis and signaling revealed that transcript levels of the leukotriene C4 synthase, LTC4S, catalyzing the first step in the biosynthesis of cysteinyl (cys) LTs, and the cysLT receptors, cysLTR2 and cysLTR3, were upregulated in mice on HFD. These mice also showed an increased expression level of IL-33 receptors, the membrane-bound ST2L and soluble isoform sST2, as well as the mast cell-specific protease MCPT1. Based on these findings it is conceivable that upon sensing saturated LCFAs tuft cells may elicit inflammatory responses which result in the production of cysLTs and activation of surface mucous cells as well as mucosal mast cells regulating gastric mucosal function and integrity.

Key words: Tuft cell, Surface mucous cell, Mucosal mast cell, High fat diet, GPR120, Cysteinyl leukotriene, IL-33

Introduction

Consumption of HFDs is considered as a risk factor for various diseases, however, the mechanisms for the detrimental effects of HFDs seem to be more complicated than the simple concept of energy imbalance and remain poorly understood (Duan et al., 2018). Following the ingestion of HFDs, inflammation can develop in various organs throughout the body, including the gastrointestinal tract (Ma et al., 2019). Typically, inflammation is considered as a normal bioprotective response to pathogen exposure (Murakoshi et al., 2021). This process involves the release of inflammatory and protective molecules and is part of the immune response against infection. The rapid production of eicosanoids and cytokines, especially cysteinyl leukotrienes (cysLTs) and interleukins, such as interleukin 33 (IL-33), following an infection by immune and epithelial cells indicates their important role

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for the initiation of inflammatory reactions (von Moltke et al., 2012; Buzzelli et al., 2015; Seo et al., 2017). The notion that apparently saturated LCFAs can act as inflammatory stimuli (Ma et al., 2019), causing effects that typically occur during infections, raises the question about the mechanisms underlying this reaction.

Since the alimentary tract is first exposed to high fat compounds in food, it seems plausible that the processes may primarily occur at sites within the gastrointestinal system where dietary lipids accumulate. Thus, it seems conceivable that cells of the surface epithelial layer in the stomach may represent appropriate contributors to mucosal defensive functions (Werther, 2000). This applies particularly to cells capable of sensing food constituents and cells releasing relevant signal molecules, such as tuft cells and mucosal mast cells as well as surface mucous cells (Phillipson et al., 2008; De Winter et al., 2012; Kurashima et al., 2013; Ali et al., 2020). Tuft cells, also named brush, caveolated, multivesicular, or fibrillovesicular cells (Luciano and Reale, 1992), are ideally positioned to act as chemosensory sentinels which can respond to diverse luminal compounds and can relay information via a spectrum of intercellular signaling molecules, including prostaglandins and acetylcholine (Schneider, 2021).

For the small intestine it was recently found that tuft cells in the intestinal epithelium play an important role in initiating inflammatory reactions in response to infections by parasites and bacteria. Tuft cells contribute to these processes by releasing interleukin 25 (IL-25) and cysLTs (Gerbe et al., 2016, Howitt et al., 2016, von Moltke et al., 2016; Nadjsombati et al., 2018; McGinty et al. 2020). CysLTs are synthesized from arachidonic acid via the arachidonate 5-lipoxygenase (ALOX5) pathway which requires activation through the 5-lipoxygenase activating protein (FLAP) (Gonzalez-Periz and Claria, 2007). It was recently demonstrated that upon a helminth infection the defensive responses in the intestine require tuft cell-derived cysLTs and IL-25, which induce an activation of group 2 innate lymphoid cells (ILC2s) (von Moltke et al., 2016; McGinty et al. 2020). Activation of ILC2s in turn further mediates a systemic innate protection e.g. by secreting IL-13 and priming mucus production, thus promoting tissue remodeling and self-renewal of intestinal stem cells (Campbell et al., 2019; Zhu et al., 2019).

However, an effector function of gastric tuft cells and the underlying circuit in the context of HFD feeding is still uncertain. In view of the fact that tuft cells in the stomach express GPR120 (FFAR4), a G protein coupled receptor (GPCR) for medium and long chain fatty acids (MCFAs, LCFAs) (Hirasawa et al., 2005; Tanaka et al., 2008; Cartoni et al., 2010; Matsumura et al., 2009), it is conceivable that these cells are responsive to saturated LCFAs (Widmayer et al., 2015). The data indicate that tuft cells are affected by a HFD; a significant increase in the cell number was observed which occurred already within few weeks and persists for months (Widmayer et al., 2015). Moreover, exposure to LCFAs for a few

minutes induced an increased phosphorylation of the extracellular signal-regulated kinases 1/2 (ERK1/2) in tuft cells which was accompanied by upregulated mRNA and protein levels of cyclooxygenase 2 (COX-2), the inducible key enzyme for prostaglandin biosynthesis (Widmayer et al., 2019).

In view of this information, the question arises whether tuft cells may have the molecular capacity for contributing to inflammatory reactions in the stomach induced after feeding of a HFD and to coopt with associated cells. Therefore, we fed mice a HFD for 3 weeks and studied the effects on tuft, mucosal mast, and surface mucous cells for a putative role in local defense responses against the fat dietary insult.

Materials and methods

Mice and nutritional experiments

To study early HFD-induced effects on the gastric corpus epithelium, a short-term high fat feeding protocol has been applied as described previously (Widmayer et al., 2015). A feeding period for 3 weeks was chosen because after this relatively short period of time already substantial alterations in gene expression, inflammatory features and cellular composition of the stomach epithelium, including expansion of GPR120-expressing tuft cells capable of sensing saturated LCFAs in the HFD, have been reported (Widmayer et al., 2015; Inagaki-Ohara et al., 2016). In brief, 4-week old weaned male C57BL/6 mice were housed in groups of 5-6 littermates and received diets with differing fat calorie contents for a feeding period of 3 weeks in a facility of the Central Unit for Animal Research at the University of Hohenheim. Two control groups were provided a standard laboratory chow containing 9% calories from fat (CF) (3.06 kcal/g, 58% from carbohydrates and 33% from protein; V1534-300 R/M-H, ssniff Spezialitäten GmbH) and two HF groups were fed a HFD containing 60% calories from fat (5.24 kcal/g, 20% from carbohydrates and 20% from protein, D12492 Research diets). Of each CF and HF cohort, one group was used for immunohistochemical examinations and the other for quantitative RT-PCR analyses.

Mice had access to chow and water *ad libitum*. Body weights were determined weekly. Under these HFD conditions no significant change in body weight of chow fed (CF) and high fat fed (HF) mice developed as reported previously (Widmayer et al., 2015). For tissue preparation, mice were killed via inhalation of lethal doses of carbon dioxide. Experiments were carried out in accordance with the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. The work was approved by the Committee on the Ethics of Animal Experiments at the Regierungspräsidium Stuttgart (V318/14 PHY) and the University of Hohenheim Animal Welfare Officer (T125/14 PHY, T126/14 PHY).

Tissue preparation

For immunohistochemistry and qPCR analyses stomachs were removed and washed in ice-cold phosphate-buffered saline (PBS) (0.85% NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4). For cryosectioning, the fundus was removed and the whole stomach fixed in 4% buffered paraformaldehyde (in 150 mM phosphate buffer, pH 7.4) for 15 min or 2h at 4°C followed by cryoprotection in 25% sucrose at 4°C overnight. Then, stomachs were embedded in Tissue Freezing Medium (Leica Microsystems) and quickly frozen using liquid nitrogen. Sections (8 µm) were cut on a CM3000 cryostat (Leica Microsystems) and attached to Superfrost Plus microslides (Menzel Gläser). For RNA isolation, corpus regions were carefully excised and immediately transferred into a collection tube, frozen in liquid nitrogen and stored at -70°C until use.

Immunohistochemistry

Cryosections were air-dried, rinsed in PBS for 10 min and incubated with blocking solution (PBS with 10% normal donkey serum (NDS), with or without (ST2 staining) 0.3% Triton X-100) for 60 min at room temperature. After washing in PBS three times, sections were treated with primary antisera prepared in the blocking solution at 4°C overnight.

For immunoreactivity to ST2, a citrate-antigen-retrieval was performed. Therefore, frozen sections were incubated in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 45 min at 4°C. Then, sections were immersed in the same sodium citrate buffer for 2 min at 100°C.

Primary antibodies were used in the following dilutions: rabbit anti-TRPM5 (1:600, courtesy of T. Gudermann and V. Chubanov), goat anti-5-HT (serotonin) (1:1000, ab66047, Abcam), rabbit anti-TFF1 (1:200, GTX-333, GeneTex), sheep anti-MCPT1 (1:200, AF5146, R&D systems), goat anti-FLAP (ALOX5AP) (1:300, NB300-891, Novus Biologicals), rabbit anti-ALOX5 (1:200, #MA5-38050, Thermo Fisher Scientific), goat anti-IL-33 (1:200; AF3626, R&D systems), and rabbit anti-ST2 (1:50, BSS-BS-2382R, Biozol). Then, slides were washed three times in PBS. Primary antibodies were visualized using appropriate secondary antibodies either conjugated to Alexa 488 (Dianova) or to Alexa 568 (Thermo Fisher Scientific), diluted 1:500 in blocking solution for 2h at room temperature. After three rinses in PBS, sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI)-containing solution (1 µg/ml in PBS, Sigma Aldrich) for 3 min at room temperature to visualize nuclei, then rinsed in water and finally mounted in Mowiol (10% polyvinylalcohol 4-88 (Sigma), 20% glycerol in PBS). Specificity of all antibodies was proven by stainings on consecutive sections in which the respective primary antibody was omitted. In control experiments no immunoreactivity was observed.

Microscopy and imaging

Immunofluorescence was examined and documented with a Zeiss Axiophot microscope (Carl Zeiss MicroImaging). Images were captured using a SensiCam CCD camera (PCO Computer Optics), adjusted for contrast in AxioVision LE Rel. 4.3 (Carl Zeiss MicroImaging) and arranged in PowerPoint (Microsoft) or Adobe Photoshop (Adobe Systems).

RNA isolation, cDNA synthesis, and quantitative RT-PCR

Total RNA was harvested using the NucleoSpin RNA kit (Macherey-Nagel). To ensure complete DNA removal, DNase digestion (DNase, Macherey-Nagel) was conducted. RNA (1.5 µg) was reverse transcribed into complementary DNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) primed with oligo(dT). RNA integrity of samples was confirmed by the amplification of the housekeeping gene encoding the ribosomal protein L8 with intron spanning primers to verify successful DNA removal. Quantitative PCR experiments were performed as previously described (Widmayer et al., 2015). In brief, mRNA levels were assessed using the Light Cycler (Roche Diagnostics). The qPCR reaction mixture (10 µl) consisted of GoTaq qPCR Master Mix (Promega) and primer sets. The oligonucleotide primer sequences (5'−3') were as follows: LTC4S sense CGT CTT CCG AGC CCA GGT AAA C, LTC4S as CGA ACA GGT AGA ACA GTC CGC ACA (nt 241−362 from GenBank accession number NM_008521, the expected size of PCR products, 122 bp); cysLTR1 sense CAT TGC CTC TCC GTG TGG TCT ATT, cysLTR1 as GAG GCG GCA CAA AAA GTC ACC (NM_021476, nt 878-948, 71 bp); cysLTR2 sense CTT CTT GCT CCC ATT TCT CAC GC T, cysLTR2 as ATC CCC ACA TGA ATC TTT ATC CCA (NM_133720, nt 1240-1456, 217 bp); cysLTR3 sense GCC TTG ACG GAC TTG CTG TAT CTG, cysLTR3 as GAG GTT GAA GTG GAA GCC GAA GC (NM_001001490, nt 749-874, 126 bp); IL-33 sense GGA AAA GAC CAA GAG CAA GAC CA, IL-33 as GCT TCT TCC CAT CCA CAC CG (NM_133775, nt 522-615, 94 bp); ST2L sense GGA GAG ACC TGT TAC CTG GGC AAG, ST2L as GTC CCC AAC CTG TAG TCG GCT T (NM_001025602, nt 1627-1850, 224 bp); sST2 sense ATG CCC CCA AAG AGG ACG CT, sST2 as GCT TGG CGG CTT TTT ATG TAT GG (NM_001025602, nt 472-698, 226 bp); MCPT1 sense ATT CCC TTG CCT GGT CCC TCT G, MCPT1 as TTT CTC TCC AGT TTT CCC CCA GC (NM_008570, nt 417-497, 81 bp); and L8 sense GTG CCT ACC ACA AGT ACA AGG C, L8 as CAG TTT TGG TTC CAC GCA GCC G (BC043017, nt 548−771, primers intron spanning, 224 bp, 375 bp genomic contamination). Each assay included (in duplicate) 90 ng of cDNA and a non-template control reaction. Quantitative PCR conditions were: 95°C for 2 min, 95°C for 15 s, 60°C for 15 s, 72°C

for 15 s with 45 cycles. A melting curve analysis was included to ensure that only a single, specific amplicon had been produced. Relative amounts of transcripts were normalized to L8 which remained constant in all samples. Amplification of only one product was additionally confirmed by agarose electrophoresis. LightCycler Software 3.5 (Roche Diagnostics) results were exported as tab-delimited text files and imported into Microsoft Excel. Data were calculated using the $2^{-\Delta Ct}$ method and expressed as mean ± S.D.

Cell quantification

Digital microscopic images of sections through the corpus mucosa and cardia were acquired at 20x or 40x magnification. Counts of TRPM5 positive tuft cells and 5-HT labeled mucosal mast cells were expressed as cells per 0.5 mm^2 (n=5 mice per group). To determine surface mucous cell frequency, the number of IL-33 immunoreactive cells in 5 foveolae of 200 μm length was counted (n=5 mice per group). For quantification of ALOX5, nuclei of FLAP positive tuft cells were assessed by scoring signal intensities of 30 cells (n=4 mice per group). Nuclei were considered weak when the fluorescent signal was barely above background, those with a clear labeling were rated moderate to strong. The number of positive cells were expressed as a mean ± SD.

Statistical analysis

Significant differences were analyzed by the unpaired t-test with GraphPad Prism using the GraphPad web site https://www.graphpad.com. Statistical significance was set at *P*<0.05.

Results

Expansion of gastric tuft cells and mucosal mast cells in HFD mice

In the stomach GPR120-expressing tuft cells are candidate fat responsive cells and are associated with mucosal mast cells (Hass et al., 2007; Widmayer et al., 2015). To approach whether feeding mice with a HFD for 3 weeks may affect both cell populations, we first analyzed the localization and frequency of tuft and mucosal mast cells in chow fed (CF) and high fat fed (HF) mice. Immunohistochemical visualization utilizing the marker TRPM5 for tuft cells (Kaske et al., 2007) and 5-HT for mucosal mast cells (Li et al., 2014) revealed that under control conditions both cell populations were rare in the gastric mucosa and resided in the upper glandular region constituting the gastric mucosal barrier (Fig. 1A). Tuft cells were located primarily in the gland isthmus and mucosal mast cells in the gastric pit region (Fig. 1A). After feeding mice for 3 weeks with a HFD significant changes were observed. Both cell types were intermingled and distributed throughout the entire top part of gastric glands and the number of cells

significantly increased (Fig. 1B). A quantification of the cell number revealed a 1.5 fold (P=0.0010) increase of tuft cells and a 1.8 fold (P=0.0026) increase of mucosal mast cells (Fig. 1C, D). Of note, in HFD mice TRPM5 and 5-HT labeling was also visible in cells lining the luminal surface of gastric pits. To additionally investigate influences on the organization of the uppermost glandular region, immunofluorescence analyses for the trefoil factor 1 (TFF1) were performed, which is a secreted peptide necessary for gastric homeostasis. In our early setting of HFD feeding no apparent difference in surface mucus staining was observed. In CF and HF mice, TFF1 labeling was found to be equally distributed among a subset of mucous cells on the surface and along gastric pits (Fig. 1E, F).

Effect of high fat feeding on specific components of the eicosanoid biosynthetic and signaling pathway

To approach the question whether tuft cells in the stomach might have the capacity to initiate or contribute to inflammatory reactions, we analyzed their potential to produce cysLTs. Therefore, tuft cells were assessed whether they comprise proteins of the ALOX5 pathway including the ALOX5 activating protein FLAP. As a first step, immunohistochemical analyses were performed using tissue sections from HFD mice. The results indicate that numerous elongated and rounded cells with FLAP immunoreactivity were located in the upper gland region of the corpus (Fig. 2A-F). All tuft cells labeled by their marker TRPM5 were found to be FLAP immunoreactive (Fig. 2A-C). In close proximity to tuft cells, additional mostly smaller cells were FLAP positive (albeit mostly weaker). Because the morphology and labeling pattern of these cells mimicked that of mucosal mast cells, we further assessed tissue sections for the expression of 5-HT with FLAP and 5-HT with the mucosal mast cell-associated protease MCPT1 (Li et al., 2014), respectively. The results demonstrate that a subpopulation of FLAP immunoreactive cells was positive for 5-HT (Fig. 2D-F) and that all 5-HT positive cells showed strong positive labeling for MCPT1 indicating that they in fact represent mucosal mast cells (Fig. 2G-I). Further assessments of stomach sections of HFD mice revealed numerous FLAP immunoreactive cells infiltrating the submucosal thickenings and interglandular spaces compared to CF mice (Fig. 3A,B). These cells are likely of myeloid origin and may promote tissue inflammation.

An activation of the ALOX5 pathway is caused by binding of ALOX5 to the nuclear membrane and its interaction with FLAP, accordingly, changes in protein expression and localization are considered to be indicative for an activation of the signaling pathway and a functional interaction of ALOX5 with FLAP (Dixon et al., 1990; Ring et al., 1997). To explore these aspects, we next compared the distribution and staining patterns for ALOX5 and FLAP in CF and HF mice. In mice fed a HFD, most tuft cells displayed a clear ALOX5 labeling
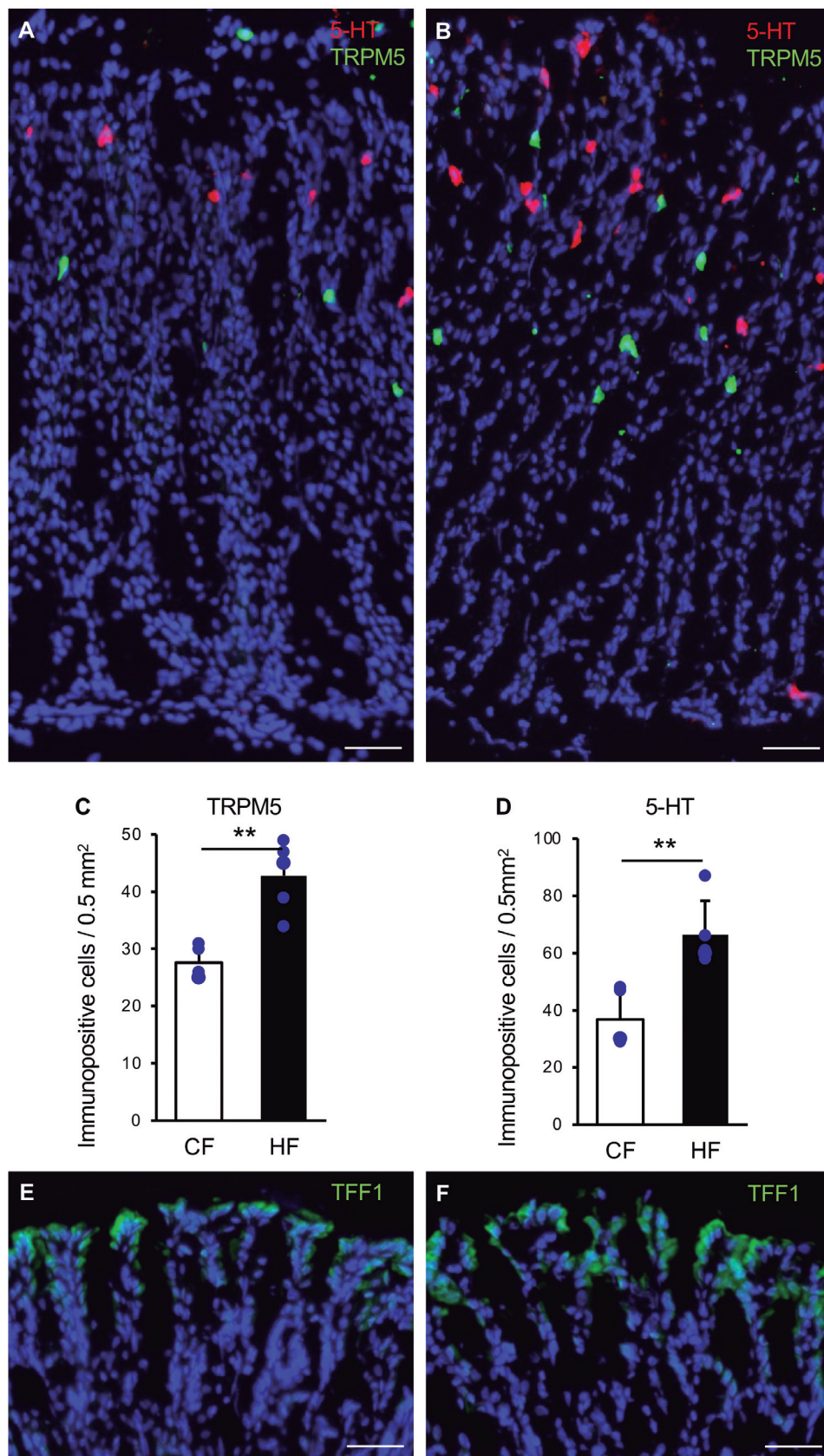
Fig. 1. Effects of feeding mice with a HFD for 3 weeks on tuft cells, mucosal mast cells and surface mucous cells. **A.** Distribution of tuft cells (TRPM5, green) and mucosal mast cells (5-HT, red) in the normal stomach. **B.** High fat feeding induced expansion of tuft cells and mucosal mast cells in the upper third of gastric glands. Quantification of TRPM5-positive tuft cells **(C)** and 5-HT-immunoreactive mucosal mast cells **(D)** in chow fed (CF) and high fat fed (HF) mice per 0.5 mm^2. Data in **(C)** to **(D)** are means ± S.D. from n=5 mice per group with each circle representing a separate mouse. Statistically significant results determined by the unpaired t-test are indicated by *$P<0.05$, **$P<0.005$, ***$P<0.0001$. Localization of TFF1 in gastric pit mucous cells in CF mice **(E)** closely resembles that of HF mice **(F)**. Scale bars: A, B, E, F, 50 µm.

compared to tuft cells of control mice (Fig. 4A,B). While in HFD mice ALOX5 immunostaining was intense and expanded throughout the entire nucleus, in CF mice the ALOX5 labeling was generally weaker and diffusely distributed among nuclei (Fig. 4C,D) or produced a punctate nuclear fluorescence in tuft cells (Fig. 4F-H). Differences in ALOX5 immunoreactivity were further assessed by categorizing staining intensity of 30 tuft cell nuclei in "none to weak "versus "moderate to strong". The results indicate that the number of moderately to
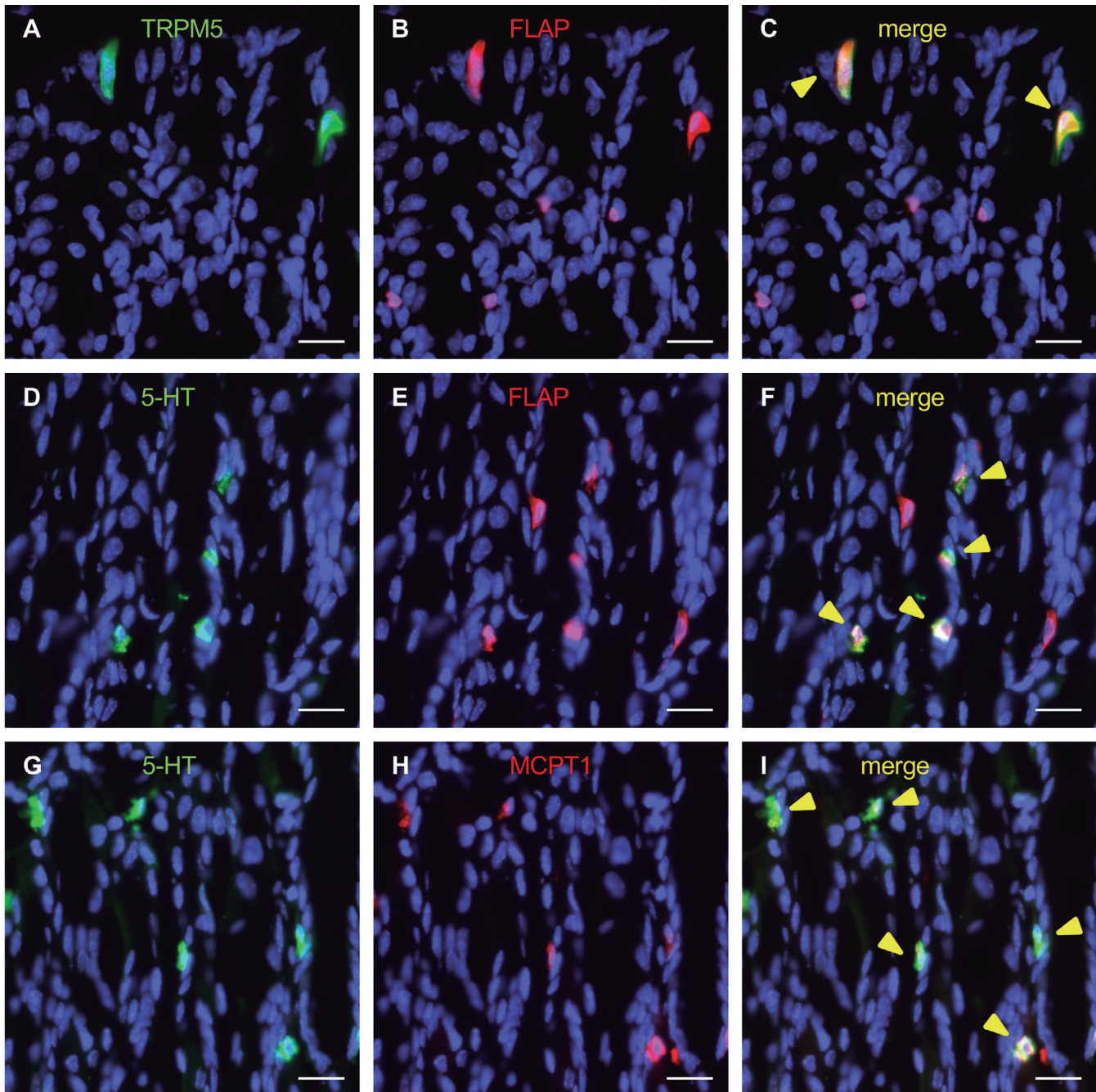

Fig. 2. Localization and characterization of FLAP-expressing cells in the stomach corpus mucosa of a HFD mouse. **A-C.** FLAP immunoreactivity **(B)** was present in tuft cells identified by TRPM5 **(A)**. **D-F.** In this upper glandular region also mucosal mast cells, labeled by 5-HT **(D)**, were FLAP-positive **(E)**. Both cell populations were often arranged in clusters, but also scattered singly throughout the epithelium. **G-I.** Mucosal mast cells were defined by immunofluorescence staining for mucosal mast cell protease MCPT1. Arrowheads point to colabeled cells (merge). Scale bars: 20 μm.

strongly labeled tuft cells was doubled in HFD mice compared to control mice (CF: 10.8±2.6 versus HF: 22.3±1.7), whereas in CF mice the proportion of none to weakly stained tuft cells dominated (CF: 19.3±2.6 versus HF: 7.8±1.7) (P=0.0003) (Fig. 4E). Moreover, in some tuft cells of mice fed with a HFD ALOX5 immunolabeling was visible at the nuclear membrane and a colocalization with FLAP was evident (Fig. 4I-K). The perinuclear colocalization of ALOX5 and FLAP and the pronounced nuclear ALOX5 staining intensity in HFD mice may suggest an interaction of ALOX5 with its cofactor FLAP possibly causing ALOX5 pathway activation in turn promoting leukotriene formation. Similar to these oxyntic mucosal tuft cells, also tuft cells on the columnar epithelial side at the gastric groove showed this distinctive pattern of ALOX5 labeling. Whereas in CF mice clusters of tuft cells beneath the limiting ridge exhibited a faint immunoreactivity for ALOX5, in HFD mice the signal intensity for ALOX5 in tuft cells was more pronounced (Fig. 4L,M).

Next, we determined if high fat feeding may affect expression levels of the rate limiting enzyme for the production of cysLTs, the leukotriene C4 synthase (LTC4S) which converts LTA4 to LTC4. Determining the level of mRNA by qPCR analyses showed significantly higher transcript levels of LTC4S (P=0.0234) in HFD mice compared to mice fed the control diet (Fig. 5A). Subsequently, the expression levels of leukotriene receptors, as a surrogate for functional actions of cysLT, were investigated. The results of the qPCR analyses, depicted in Fig. 5C,D, show significantly higher transcript levels for the cysLT receptor types cysLTR2 (P=0.0077) and cysLTR3 (also named OXGR1 or GPR99) (P=0.0321) in HFD mice compared to mice fed the control diet. The expression level of the receptor type cysLTR1 (P=0.3742) was apparently not affected (Fig. 5B). Taken together these results indicate that high fat feeding leads to higher numbers of tuft cells and mucosal mast cells and to upregulated expression levels of leukotriene C4 synthase and specific receptor types for cysLTs. These changes may have implications for the promotion of stomach inflammation.

Effect of high fat feeding on the expression of IL-33 and IL-33 producing cells

Besides the cysLTs, IL-33 is considered as a key element for the initiation and maintenance of an inflammatory environment (Boudaud et al., 2018). IL-33 is a member of the IL-1 family of cytokines and represents a crucial stomach mediator released from a subset of mucus producing cells (surface mucous cells) upon an external insult (Schmitz et al., 2005; Pichery et
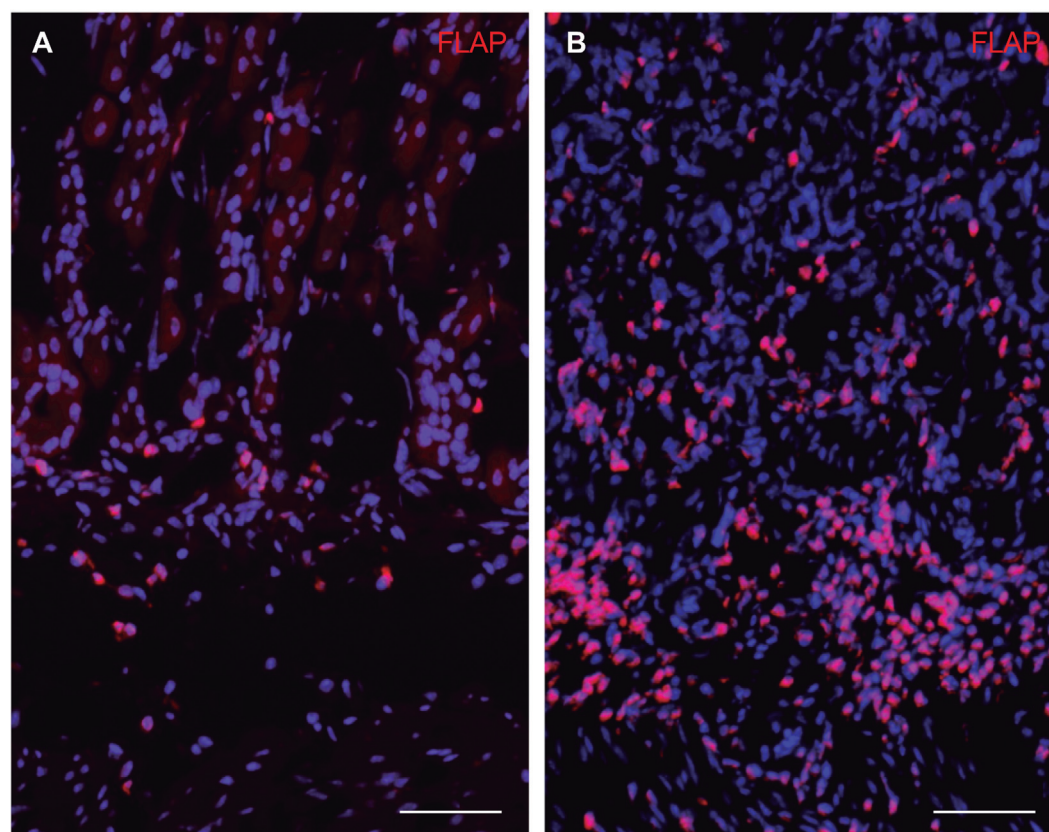

Fig. 3. High fat feeding causes infiltration of immune/inflammatory cells. **A.** In the submucosal layer of CF mice scattered FLAP positive cells were found which expanded in density in thickened submucosal and interglandular spaces of HFD mice **(B)**. Scale bars: 50 μm.
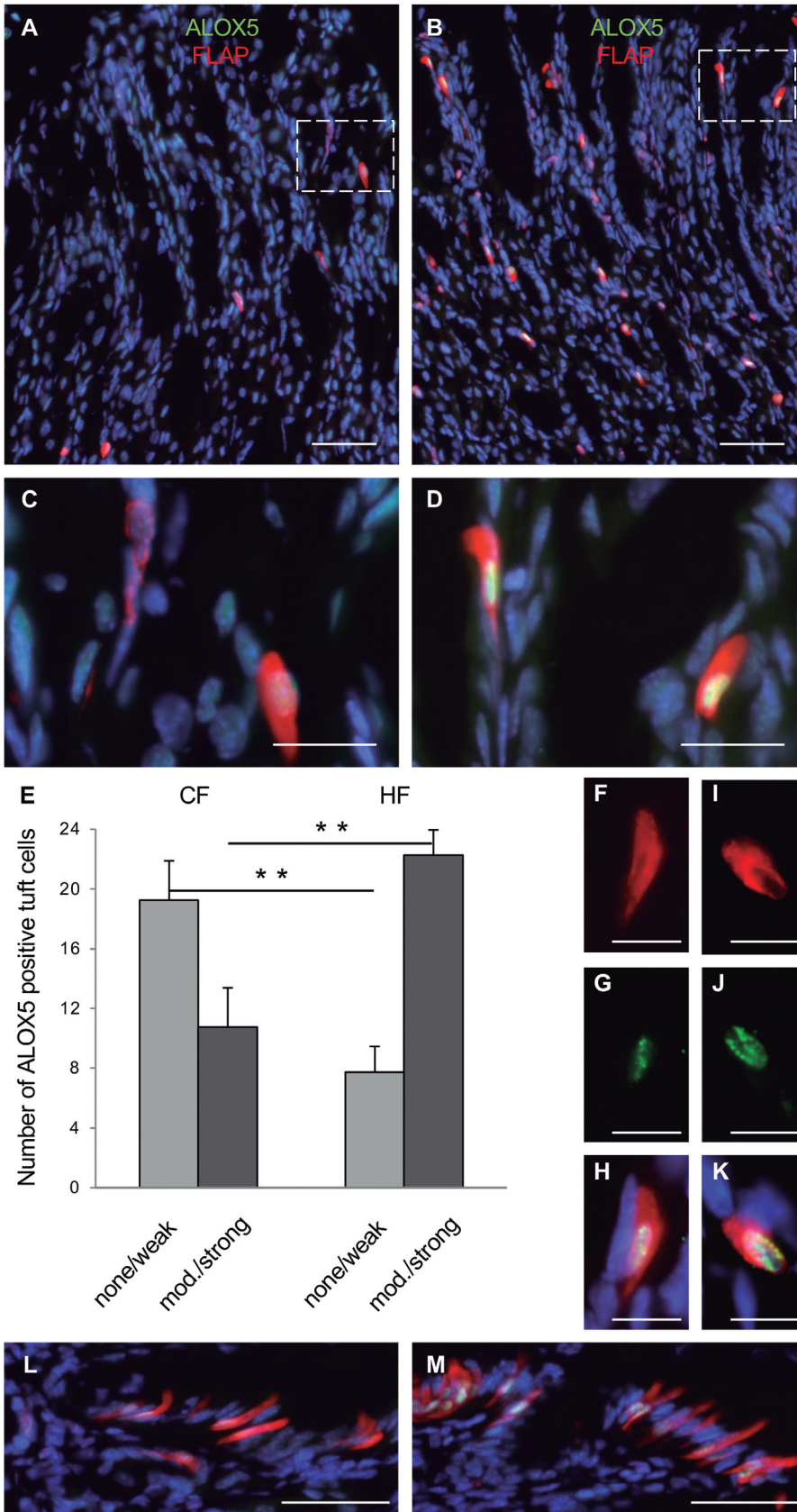
Fig. 4. Impact of HFD feeding on the distribution and labeling pattern for ALOX5 and FLAP in tuft cells. **A.** Staining for ALOX5 in CF mice mostly led to weakly labeled nuclei of tuft cells. **C.** Inset shows enlarged view of an ALOX5 negative tuft cell and a tuft cell with a diffuse nuclear ALOX5 fluorescence. **B.** In the oxyntic mucosa HFD feeding caused an intense ALOX5 staining of tuft cells. Square indicates enlarged area shown in **(D)** to emphasize the expanded ALOX5 immunolabeling of tuft cells' nuclei. **E.** Classification of ALOX5 positive nuclei into "none to weak" (light grey bars) versus "moderate to strong" (dark grey bars) resulted in a shift in the ratio of more moderate to strong signals in HFD mice. Data are represented as mean ± S.D. *$P<0.05$, **$P<0.005$, ***$P<0.0001$. **F-H.** Chow feeding produced a punctate nuclear ALOX5 fluorescence in tuft cells. **I-K.** Change in subcellular distribution of ALOX5 in some tuft cells of HFD mice: ALOX5 shifted from inner nuclear locales to the nuclear membrane where ALOX5 colocailized with FLAP. **L.** Immunohistochemistry for ALOX5 of tuft cells at the gastric groove shows clustered tuft cells with rather weakly labeled nuclei in CF mice which are intensely stained in HFD mice **(M)**. Scale bars: A, B, L, M, 50 μm; C, D, 20 μm; F-K, 10 μm.

al., 2012, Buzzelli et al., 2015). To this end, we next approached the question whether expression of IL-33 might change upon high fat feeding by determining the relative levels of IL-33 mRNA by qPCR. The results indicate that after high fat feeding for 3 weeks the level of IL-33 transcripts was significantly reduced in comparison to mice fed a standard chow (P=0.0047) (Fig. 6A).

To determine whether reduced IL-33 expression levels correspond to changes in the frequency of surface mucous cells, histological sections of the gastric mucosa were assessed. As documented in Fig. 6B and D, in the normal oxyntic mucosa of chow fed mice IL-33 fluorescence labeling was restricted to surface mucous cells of gastric pits and displayed a nuclear localization. In mice fed with a HFD the intensity and distribution of IL-33 immunostaining was significantly changed (Fig. 6C). At higher magnification strong IL-33 immuno-labeling was visible within the cytoplasm of surface mucous cells (Fig. 6E) suggesting that the protein is released from the nucleus of damaged or activated epithelial cells. In addition to foveolar cells, IL-33 immunoreactivity was also observed in some cells located at and/or near the base of gastric glands (Fig. 6C). A quantification of the IL-33 positive cells at gastric pits revealed a marked increase for the number of IL-33 positive cells in foveolae of HFD mice (P=0.0063) (Fig. 6F).

High fat feeding affects expression of IL-33 receptors and mucosal mast cell-specific protease

The biological actions of IL-33 on target cells are mediated via its specific receptor complex. This is composed of the membrane-bound receptor ST2L and the IL-1R accessory protein (IL-1RAcP) (Chackerian et al., 2007). IL-33 can be neutralized by binding to the soluble antagonistic decoy receptor sST2. In order to approach the question whether the responsiveness of target cells may change as a result of high fat feeding we investigated the mRNA levels of ST2L and sST2 by qPCR. The results, documented in Fig. 7A and B, demonstrate that in HFD mice the concentration of mRNA for the receptor ST2L (P=0.0059) and the soluble binding protein sST2 (P=0.0005) is increased. Since ST2L is constitutively expressed by mast cells (Miller, 2011), we next analyzed whether mucosal mast cells may express the receptor for IL-33. Therefore, tissue sections were assessed for ST2 and the mucosal mast cell-associated protease MCPT1 performing double-labeling experiments. The result, depicted in Fig. 7C, indicates that gastric mucosal mast cells are ST2-positive cells. Since activation of mast cell-specific genes is considered as a measure of mucosal mast cell function (Liu et al., 2015), we next determined the expression level of the mucosal mast cell-associated protease MCPT1 by qPCR. This analysis revealed that the expression of the inflammatory protease MCPT1 was substantially increased in stomach samples from HFD mice compared with CF mice (P=0.0199) (Fig. 7D). In conclusion, these data show that high fat feeding induces alterations in IL-33 receptor expression and identify mucosal mast cells as putative target cells for IL-33.

Discussion

Saturated LCFAs have proinflammatory effects and extensive feeding of HFDs is consequently associated
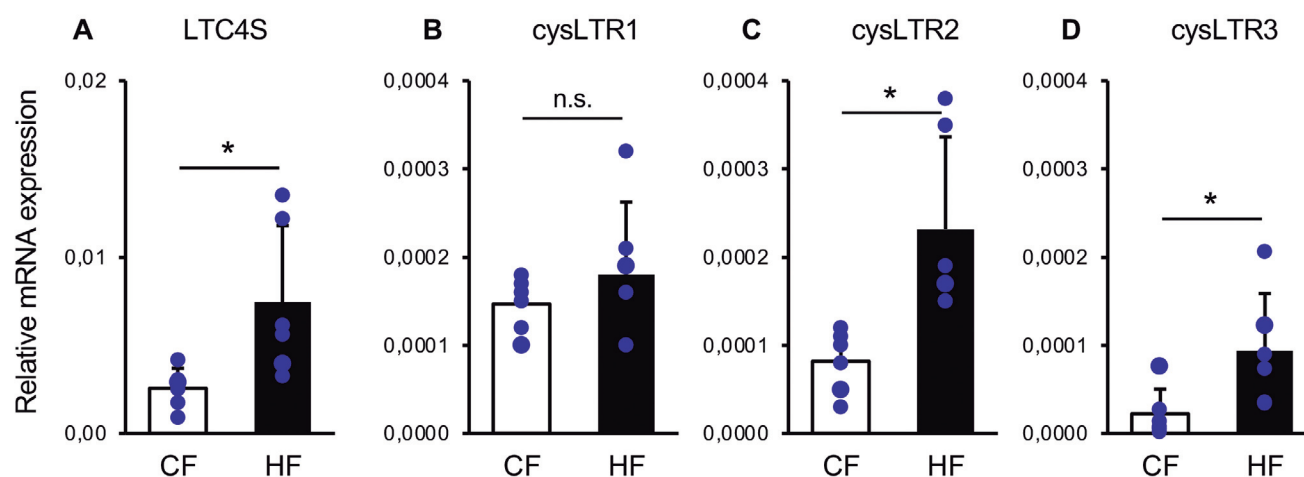

Fig. 5. Relative mRNA expression for leukotriene synthesis and receptor genes in the corpus of mice fed a standard chow diet (CF) or high fat diet (HF) for 3 weeks as measured by qPCR. High fat feeding elicited an induction of mRNA expression of the cysLTs rate-limiting enzyme LTC4S **(A)** and cysLT receptors cysLTR2 **(C)** and cysLTR3 **(D)**. Levels of cysLTR1 remained unchanged **(B)**. Data were calculated using the $2^{-\Delta Ct}$ method and expressed as mean ± S.D. For normalization L8 was used showing no significant change. Each circle represents a separate mouse with n=6 mice per group. Statistically significant results are indicated by *P<0.05, **P<0.005, ***P<0.0001, and n.s., not significant.

with inflammatory reactions throughout the body including the gastrointestinal tract (Ma et al., 2019). Exactly how these reactions are elicited by LCFAs is a matter of debate (Hidalgo et al., 2021), however, LCFAs exert their actions in part through membrane receptors which were first described in intestinal cells (Hirasawa et al., 2005). In the stomach, representing the first site of the alimentary tract exposed to dietary lipids, tuft cells express GPR120 (Janssen et al., 2012; Widmayer et al., 2015), the receptor for MCFAs and LCFAs (Hirasawa et al., 2005; Tanaka et al., 2008; Matsumura et al., 2009; Cartoni et al., 2010). In the course of high fat feeding an early upregulation of GPR120 expression was observed as well as a significant increase in the number of GPR120 expressing tuft cells in the upper glandular epithelium (Widmayer et al., 2015). These findings indicate that a feeding period of 3 weeks substantially alters gene expression and cellular composition of the stomach epithelium. Moreover, the present results indicate that high fat feeding for this relatively short time period can cause inflammatory features characterized by an infiltration of immune cells in interglandular and basal spaces of the gastric mucosa, thus confirming earlier studies (Inagaki-Ohara et al., 2016). Based on the consideration that tuft cells in the gastric epithelium are responsive to LCFAs it seemed conceivable that tuft cell responses may be involved in mediating the early phase of a reaction cascade leading to inflammatory responses. This notion is supported by the results of this study demonstrating that all tuft cells of HFD fed mice exhibited strong positive staining for FLAP, the accessory protein necessary for the activation
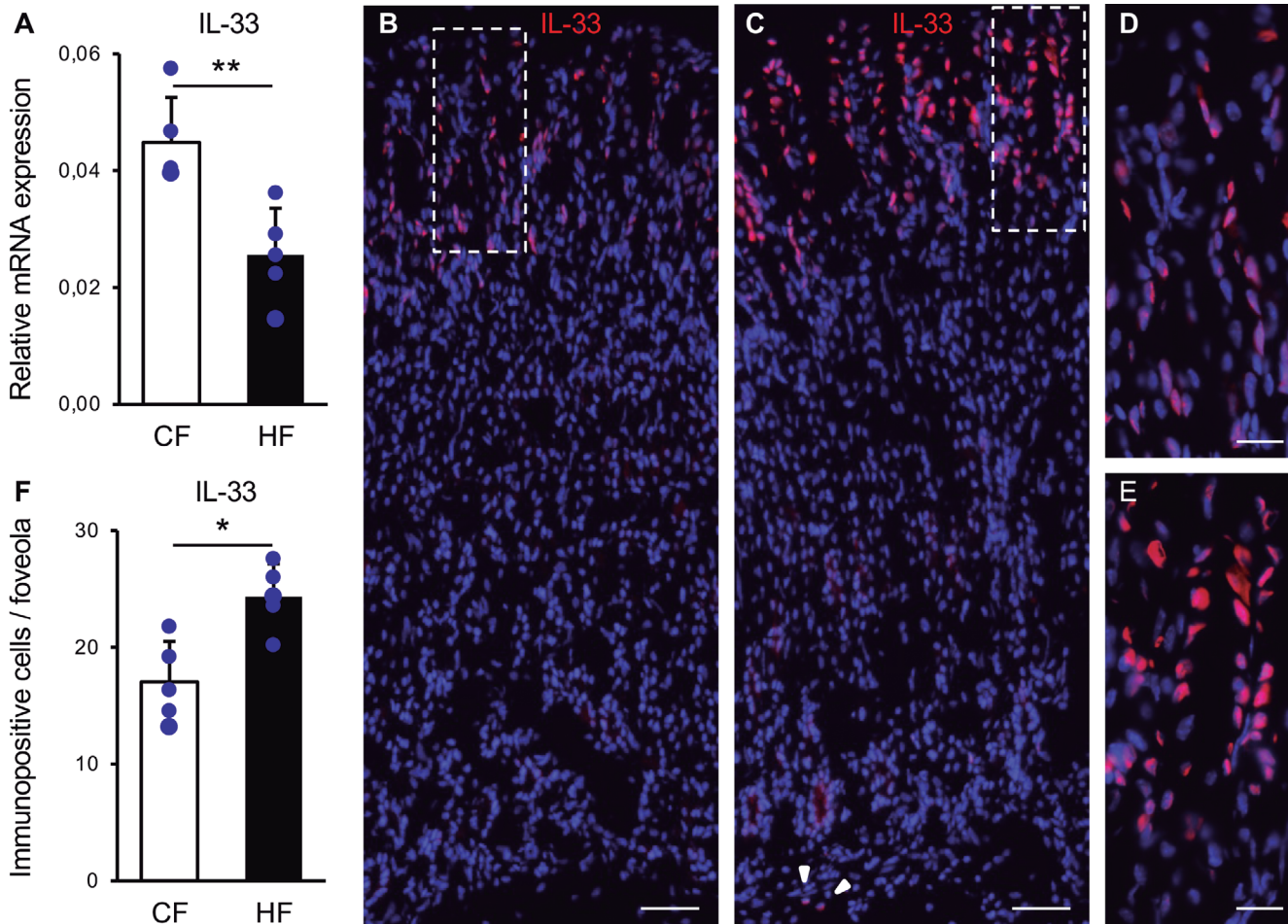

Fig. 6. Expansion of IL-33-expressing surface mucous cell density and reduction of IL-33 mRNA expression owing to high fat feeding. **A.** Relative mRNA expression levels of IL-33 in chow fed (CF, n=5) and high fat (HF, n=5) fed mice assessed by qPCR. **B.** Distribution of IL-33 immunoreactive surface mucous cells in the stomach of CF mice. **D.** IL-33 is predominantly localized to cell nuclei. **C.** In HFD mice, gastric pits harbored numerous intensely stained IL-33 surface mucous cells. **E.** IL-33 staining often appeared cytoplasmic. **F.** Cell counts of IL-33 positive surface mucous cells in CF and HF mice per 200 μm-long apical invaginations. Data in **(A)** were standardized to the housekeeper L8, calculated using the $2^{-\Delta Ct}$ method and expressed as mean ± S.D. Data in **(F)** are means ± S.D. from 5 foveolae of n=5 mice per group. Each circle represents a separate mouse. Statistically significant results are indicated by *P<0.05, **P<0.005, ***P<0.0001. Scale bars: B, C, 50 μm; D, E, 20 μm.

of ALOX5, the key enzyme for the synthesis of arachidonic acid-derived LTs (Dixon et al., 1990). LTs are an important group of proinflammatory factors (Salmon and Higgs, 1987) capable of initiating and further promoting inflammation (Evans et al., 2008; Haeggström and Funk, 2011). Indeed, after high fat feeding for 3 weeks an overall pronounced nuclear staining of ALOX5 in tuft cells was observed and, in some cells, a localization ALOX5 at the outer nuclear membrane was apparent, which is important for an interaction with FLAP to induce LT synthesis (Dixon et al., 1990). Our results further provide first evidence that tuft cells may be capable of generating cysLTs. The contribution of tuft cells to the early HFD-induced

Fig. 7. Changes of membrane-bound and soluble ST2 expression following high fat feeding, ST2 protein localization to mucosal mast cells and upregulation of mast cell-specific MCPT1. Relative expression of mRNA levels of STL2 **(A)**, sST2 **(B)**, and MCPT1 **(D)** in chow fed (CF) and high fat fed (HF) mice was determined by qPCR. **C.** Immunofluorescence analysis showing the presence of ST2 immunoreactivity in mucosal mast cells, detected by MCPT1. Data in **(A, B, D)** were normalized to L8, calculated using the $2^{-\Delta Ct}$ method and expressed as mean ± S.D. Each circle represents a separate mouse with n=5-6 mice per group. Statistically significant results determined by the unpaired t-test are indicated by *$P<0.05$, **$P<0.005$, ***$P<0.0001$. Scale bars: 10 μm.

mucosal inflammatory response is supported by the finding that in high fat fed mice genes encoding important elements of the cysLT signaling, including LTC4S, cysLTR2 and cysLTR3, were upregulated. These genes encode the rate limiting enzyme for the production of cysLTs (Lam et al., 1994) and the receptors for cysLTC4, cysLTD4 and cysLTE4 (Heise et al., 2000; Kanaoka et al., 2013). Thus, the colocalization of FLAP and ALOX5 in the outer nuclear membrane of tuft cells and the increased expression of LTC4S, cysLTR2 and cysLTR3 are indicative for an active synthesis machinery for cysLTs in tuft cells and cysLTs-driven actions.

The notion that tuft cells have an important function as local sentinels in various epithelia (Tizzano et al., 2010; Krasteva et al., 2011; Deckmann et al., 2014) was strongly supported by recent reports indicating an exclusive role for tuft cells in the local defense strategy for worm clearance (Gerbe et al., 2016; Howitt et al., 2016; von Moltke et al., 2016; Nadjsombati et al., 2018). Subsequentially, it emerged that tuft cells orchestrate mucosal inflammation of hollow organs, even via different signaling pathways. For example, in the intestine and airways responses to helminths and allergens require tuft cell-derived cysLTs for regulatory responses (Bankova et al., 2018; McGinty et al., 2020), whereas tuft cell-mediated responses to protists apparently do not include cysLT-driven actions (McGinty et al., 2020). Based on our findings, we suggest an important role for cysLTs in response to the fat dietary insult and propose that saturated LCFAs activate gastric tuft cells via GPR120 to initiate inflammatory processes by inducing the synthesis of cysLTs which in turn exert their biologic effects on cells with receptors for cysLTs, i.e. the receptor types cysLTR2 or cysLTR3. While both cysLT receptors have been found in various cell types, in the context of the present study it is interesting to note that airway tuft cells themselves express the cysLT receptor cysLTR3 which was found to be involved in the regulation of allergen-induced tuft cell expansion and function (Bankova et al., 2018). Whether the receptor cysLTR3 has a similar role in tuft cells of the stomach is of great interest, since its presence on gastric tuft cells could allow feedback reactions and for example could elicit amplified cysLT-driven tuft cell responses.

Although it is currently unclear which cells types may be responsive to tuft cell-derived cysLTs, it is conceivable that cysLTs may activate surface mucous cells to induce the release of the cytokine IL-33 which represents a crucial alarm molecule within the stomach (Schmitz et al., 2005; Pichery et al., 2012, Buzzelli et al., 2015). Surface mucous cells are epithelial barrier cells which constitutively contain IL-33 that is typically localized to the nucleus and rapidly released during necrosis (Lefrançais et al., 2012). After acute epithelial damage or pathogenic infection, surface mucous cells can rapidly upregulate IL-33 expression and actively secrete IL-33 (Buzzelli et al., 2015). In an ongoing
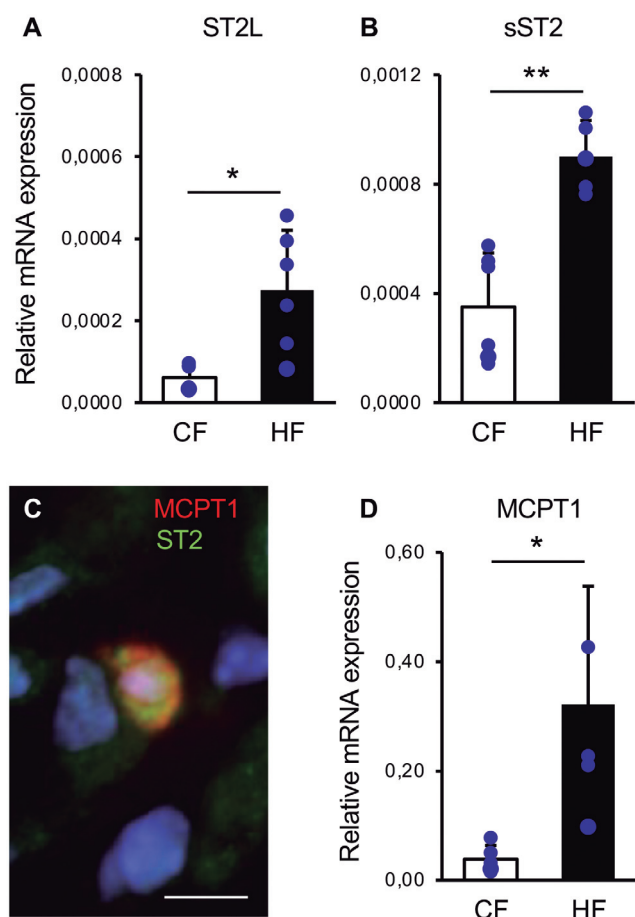
inflammatory process IL-33 protein is released from the nucleus and then localized in the cytoplasm (Buzzelli et al., 2015). After 3 weeks on the HFD, we observed that IL-33 was in fact present in the cytoplasm of surface mucous cells. Hence, at this stage of inflammation, already a chronic state seems to be reached with reduced IL-33 mRNA levels, but higher numbers of surface mucous cells. Such changes are characteristic for a chronic injury e.g. induced by Helicobacter pylori infection (Buzzelli et al., 2015). Thus, it seems conceivable that responses to gastric insults require rapid transcriptional changes in IL-33 expression resulting in IL-33 mRNA and protein expression not being congruent.

High fat-driven relocalization of IL-33 in surface mucous cells and the transcriptional alterations of IL-33 and its receptors, ST2L and sST2, emphasizes an active role for IL-33 during a fat dietary insult and suggests that IL-33 might be necessary for the maintenance of mucosal homeostasis, restitution and repair (Buzzelli et al., 2015). In the later course of high fat feeding, hyperplasia of surface mucous cells might develop allowing an enhanced production of the protective mucin Muc5ac co-secreted with the trefoil factor family TFF1 peptide from surface mucous cells supporting an appropriate gastric mucus barrier.

Considering a role for IL-33 in high fat feeding it was important to identify IL-33 responsive cells expressing its receptor ST2 (Schmitz et al., 2005). The present findings that ST2 was expressed in mucosal mast cells and that the mast cell activation marker MCPT1 was upregulated in high fat feeding experiments implicates that mucosal mast cells are candidate target cells for IL-33 and that IL-33 presumably activates mast cells as was previously reported for mast cells in the airways (Liu et al. 2015). Under normal conditions the bone marrow-derived mucosal mast cells constitutively express the mast cell specific protease MCPT1, however, within the intestine of parasitized mice the MCPT1 levels were found to be significantly upregulated (Huntley et al., 1990). Since an increased level of MCPT1 is prevented upon blockade of either IL-33 or ST2 (Liu et al. 2015), the upregulated MCPT1 (β-chymase) activity in HFD mice may point to an IL-33-induced defensive function of mucosal mast cells following the fat dietary insult. As a consequence, mucosal mast cells may release various kinds of other biologically active products (Albert-Bayo et al., 2019), thus further promoting gastric inflammatory/immune reactions.

In conclusion, the results of this study show that a high fat dietary intervention of only 3 weeks already leads to changes in the cellular composition of the gastric mucosal barrier layer affecting tuft cells, surface mucous cells, and mucosal mast cells. The pronounced hyperplasia of these cell types implicates their participation in regulatory responses to maintain mucosal barrier integrity and to limit the insult evoked by the excess of dietary fat. In this proposed reaction circuit, tuft cells activated by LCFAs release cysLTs which may act on surface mucous cells to elicit the secretion of IL-33 which in turn activates mucosal mast cells to presumably produce potent chemotactic factors and enzymes, thereby preventing further damage of the epithelium.

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