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Original Article

ORIGINAL ARTICLE

Anti-inflammatory chemoprevention attenuates the phenotype in a mouse model of esophageal adenocarcinoma

Theresa Baumeister[1,†], Jonas Ingermann[1,†], Sabrina Marcazzan[1,2,3], Hsin-Yu Fang[1], Rupert Oellinger[4], Roland Rad[1,4], Thomas Engleitner[4], Karin Kleigrewe[5], Akanksha Anand[1], Julia Strangmann[1], Roland M.Schmid[1], Timothy C.Wang[6] and Michael Quante[1,7,*]

[1]Department of Medicine II, Klinikum rechts der Isar, Technical University Munich (TUM), Ismaninger Str. 22, München, Germany, [2]Chair of Biological Imaging, School of Medicine, Technische Universität München, Munich, Germany, [3]Helmholtz Zentrum München, Institute of Biological and Medical Imaging, Neuherberg, Germany, [4]Institute of Molecular Oncology and Functional Genomics, Klinikum rechts der Isar, Technical University Munich (TUM), Ismaninger Str. 22, München, Germany, [5]Bavarian Center for Biomolecular Mass Spectrometry (BayBioMS), Technical University of Munich, Freising 85354, Germany, [6]Department of Medicine, Columbia University Irving Medical Center, New York, NY, USA and [7]Innere Medizin II, Universitätskliniken Freiburg, Universität Freiburg, Freiburg, Germany

*To whom correspondence should be addressed. Tel: +49 761 270-32766; Fax +49 761 270-18200; Email: michael.quante@uniklinik-freiburg.de
†These authors contributed equally to this work.

Abstract

Barrett's esophagus (BE) is the main known precursor condition of esophageal adenocarcinoma (EAC). BE is defined by the presence of metaplasia above the normal squamous columnar junction and has mainly been attributed to gastroesophageal reflux disease and chronic reflux esophagitis. Thus, the rising incidence of EAC in the Western world is probably mediated by chronic esophageal inflammation, secondary to gastroesophageal reflux disease in combination with environmental risk factors such as a Western diet and obesity. However, (at present) risk prediction tools and endoscopic surveillance have shown limited effectiveness. Chemoprevention as an adjunctive approach remains an attractive option to reduce the incidence of neoplastic disease. Here, we investigate the feasibility of chemopreventive approaches in BE and EAC via inhibition of inflammatory signaling in a transgenic mouse model of BE and EAC (L2-IL1B mice), with accelerated tumor formation on a high-fat diet (HFD). L2-IL1B mice were treated with the IL-1 receptor antagonist Anakinra and the nonsteroidal anti-inflammatory drugs (NSAIDs) aspirin or Sulindac. Interleukin-1b antagonism reduced tumor progression in L2-IL1B mice with or without a HFD, whereas both NSAIDs were effective chemoprevention agents in the accelerated HFD-fed L2-IL1B mouse model. Sulindac treatment also resulted in a marked change in the immune profile of L2-IL1B mice. In summary, anti-inflammatory treatment of HFD-treated L2-IL1B mice acted protectively on disease progression. These results from a mouse model of BE support results from clinical trials that suggest that anti-inflammatory medication may be effective in the chemoprevention of EAC.

Introduction

In recent decades, the incidence of esophageal adenocarcinoma (EAC) increased at a rate of 4–10% annually in regions of the Western world (1), although this increase has leveled off somewhat in recent years (2). Nevertheless, EAC remains with a very poor prognosis and a median survival of less than 1 year. The main known precursor for EAC is Barrett's esophagus (BE), a

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Abbreviations

ASA	acetylsalicylic acid
BE	Barrett's esophagus
COX	cyclooxygenase
EAC	esophageal adenocarcinoma
HED	human equivalent dose
HFD	high-fat diet
IL-1RA	IL-1 receptor antagonist
IMC	immature myeloid cell
NSAIDs	nonsteroidal anti-inflammatory drugs

premalignant, inflammation-dependent metaplastic transformation of esophageal tissue above the squamous columnar junction (3). The principal risk factor for BE is chronic reflux of both gastric and duodenal contents, known as gastroesophageal reflux (4). Despite the increasing use in recent decades of powerful acid suppressant medications (proton-pump inhibitors) to treat gastroesophageal reflux disease, the prevalence of EAC has not decreased, indicating the need for newer approaches for the prevention and treatment of BE and EAC.

Thus, although much of the focus in the past has been on suppressing acid secretion and reflux, it is now appreciated that the development of BE is largely driven by chronic esophageal inflammation. The development of chronic esophagitis is the result not only of acid injury but also of exposure to bile acids in the reflux that support local inflammation by the activation of cyclooxygenase (COX) (5,6). COX signaling also plays a role in the reaction to oxidative stress. Thus, treatment with antioxidants could in theory provide protection against BE and EAC, possibly reducing DNA damage and oxidative processes induced by inflammation (7,8).

Indeed, it is now recognized that the development of chronic inflammation may be exacerbated by obesity and the Western diet. In addition to male gender, age and tobacco use (9), obesity may cause gastroesophageal reflux disease, and subsequently BE and EAC. Obesity is now endemic in the Western world and increasing rapidly (10), and a Western diet can also provoke chronic inflammation in the GI tract (11). Moreover, in a transgenic mouse model of BE (L2-IL1B), a high-fat diet (HFD) led to an accelerated tumor phenotype via worsening of chronic inflammation (12). Increased serum cytokine (IL-1b and IL-8) and adipokine levels are associated with an increased risk of developing BE and EAC, supporting the notion of an impact of systemic inflammation on disease onset and progression (13,14). Altogether, the data strongly suggest that BE and EAC formation seems to depend not only on reflux-induced tissue damage but also on inflammatory processes (5,15).

The current approach of endoscopic surveillance appears to be inadequate as a strategy to prevent cancer, in that endoscopic screening fails to identify most patients that will develop EAC. Chemoprevention as a primary or adjunctive approach to endoscopic therapy remains an attractive option to reduce neoplastic progression. However, progress in the development of chemoprevention therapy for BE has been severely limited in the past by the absence of a tractable preclinical model of BE and EAC. Recently, this gap has been addressed by the development of the L2-IL1B transgenic mouse model of BE that recapitulates the human histopathologic progression to EAC (3,5). Considering the strong association of gastrointestinal cancer with inflammatory conditions, and the remarkable influence of inflammation on tumor development, we tested anti-inflammatory treatments on disease progression in L2-IL1B mice. To also account for the role of diet induced inflammatory conditions, we treated L2-IL1B mice with either regular chow or a HFD. The treatment comprised either the IL-1b receptor antagonist (IL-1RA or Anakinra) or a nonsteroidal anti-inflammatory drug (NSAID)—either acetylsalicylic acid (ASA; Aspirin) or Sulindac, one of the most effective NSAIDs for cancer chemoprevention.

Materials and methods

Mouse model

All animal experimental work performed in Germany was carried out with the approval of the Regierung Oberbayern according to the animal experimental permits (Tierversuchsanträge) 55.2.1.54-2532-125-12 and 55.2-1-54-2532-24-2016. L2-IL1B mice express human IL-1B under control of the EBV-L2 promoter, leading to continuous inflammation in the esophagus BE and EAC (5). L2-IL1B mice were backcrossed with C57BL/6J mice (wild type = wt). After weaning and routinely performed genotyping, the mice were randomly assigned to experimental cohorts.

Anakinra treatment

L2-IL1B mice were put on either HFD or regular lab chow at the age of 6 weeks. Mice were fed lab chow or HFD only (chow $n = 11$; HFD $n = 11$) or treated with Anakinra (chow $n = 5$; HFD $n = 5$) from 6 weeks until euthanasia with 39 weeks of age. Male and female mice were equally distributed in the groups if possible. At the age of 18 and 24 weeks, an osmotic pump (Minipump 2006, Alzet) was implanted and filled with 150 µg/µl Anakinra (Kineret, Swedish Orphan Biovitrum AB). The pump, which is operated via an osmotic gradient, produces the desired filling quantity via the infusion cannula into the tissue at a flow rate of 0.15 µl/h after an incubation and refilling time of at least 6 h. The 2006 model guarantees a continuous flow over a period of 6 weeks at a fill level of 200 µl. The Anakinra concentration in the pump is 150 µg/µl, so that 22 µg cytokines/h are delivered. The infusion took place over 12 weeks, so that the pump had to be replaced once after 6 weeks. To ensure an immediate start of infusion after implantation, the pumps were incubated for 12 h at 37°C after replenishment in NaCl 0.9%. The pumps dispensed 30 mg Anakinra (Kineret, Swedish Orphan Biovitrum AB) per kg bodyweight per day to treat the mice for a total of 12 weeks. The respective human equivalent dose (HED) for a dose of 30 mg/kg/day in mice is calculated as 2.43 mg/kg based on the formula by Neir and Jacob (16) For implantation, mice were narcotized with 0.5 mg/kg medetomidin, 5 mg/kg midazolam and 0.05 mg/kg fentanyl injected intraperitoneally. Shortly before the cut, 10 mg/kg rimadyl was injected subcutaneously. After surgery, the mice were awakened using 2.5 mg/kg atipamezol, 0.5 mg/kg flumazenil and 1.2 mg/kg naloxon and allowed to recover on a heating plate (experimental setup; Figure 1A and B).

Nonsteroidal anti-inflammatory drug treatment

For the treatment with NSAIDs, male and female mice were fed HFD after weaning and genotyping from 6 weeks of age. Mice were fed HFD (HFD $n = 5$) or treated with ASA (HFD $n = 3$) or Sulindac (HFD $n = 14$) from 6 weeks until euthanasia with 26 weeks of age. Drug doses were determined by literature research and were defined as 9 mg/kg for ASA (17) and 64 mg/kg Sulindac (18) with respective HED of 0.729 mg/kg for ASA and 5.184 mg/kg for Sulindac based on the formula by Neir and Jacob (16). Drug concentration calculations were based on a median intake of 6-ml water and 4-g food per mouse per day as described by Bachmanov *et al.* (19). ASA was purchased from the veterinary pharmacy at the Wissenschaftszentrum Weihenstephan (Suispirin, Animedica). Aspirin was prepared freshly three times a week, sterile-filtered and given to the mice starting at the age of 6 weeks *ad libitum* in the drinking water (17). For Sulindac treatment, L2-IL1B mice were treated with HFD enriched with 0.032% Sulindac (S8139-5G, Sigma) by the manufacturer Ssniff from 6 weeks of age until euthanasia (17) (Experimental setup, Figure 2A). Additional Material and Methods are described in Supplementary Material, available at *Carcinogenesis* Online.

Results

Anakinra attenuates the HFD-induced dysplastic phenotype

L2-IL1B mice were put on either HFD or regular laboratory chow at the age of 6 weeks. At the age of 18 and 24 weeks, the

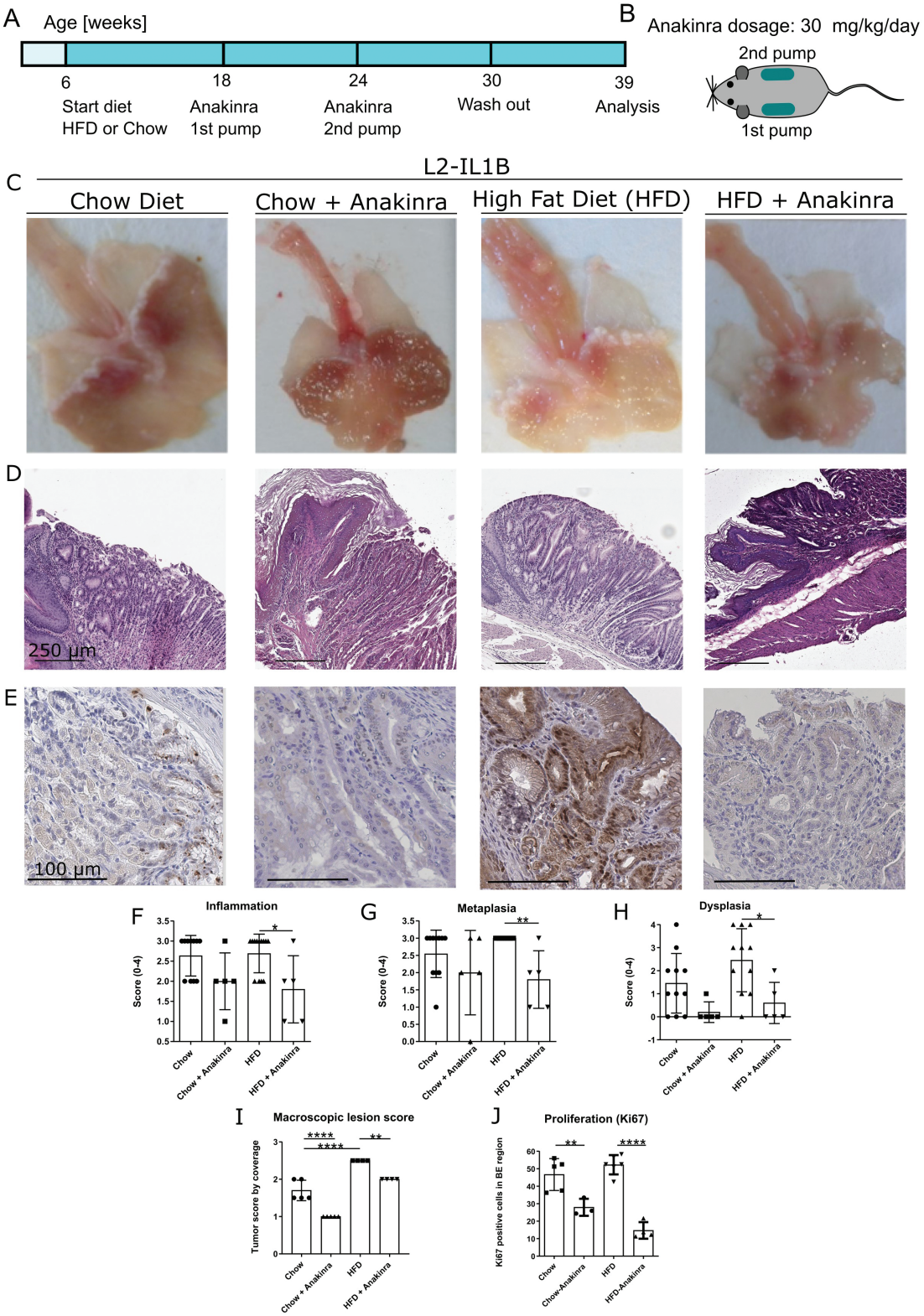

Figure 1. Macroscopic and histologic analysis of Anakinra-treated L2-IL1B mice samples using HE and Ki67 staining. **(A)** Representative timeline of Anakinra treatment in L2-IL1B mice with starting experimental diets after weaning and genotyping at 6 weeks of age, insertion of first and second pumps at 18 and 24 weeks, washout from week 30 and euthanasia at week 39. **(B)** Scheme of insertion of osmotic pumps. **(C)** Representative macroscopic pictures of chow- and HFD-treated mice with or without Anakinra treatment. **(D)** Representative histologic pictures of chow- and HFD-treated mice with or without Anakinra treatment. Bars 250 µm. **(E)** Representative pictures of Ki67 IHC of all mouse cohorts. Bars 100 µm. **(F)** Inflammation scores. **(G)** Metaplasia scores. **(H)** Dysplasia scores of chow and HFD mice with and without Anakinra treatment (inflammation HFD–HFD Anakinra $P = 0.0316$; metaplasia HFD–HFD Anakinra $P = 0.0099$; dysplasia HFD–HFD Anakinra $P = 0.0344$) (chow $n = 5$; HFD $n = 5$; chow + Anakinra $n = 11$; HFD + Anakinra $n = 11$). All graphs show significantly decreasing scores when comparing HFD to HFD + Anakinra treatment. **(I)** The Macroscopic tumor lesion score in the BE (esophagus and cardia) region decreases in both chow and HFD mouse cohorts when treated with Anakinra, while scores are

mice were implanted with an osmotic pump (Minipump 2006, Alzet). The pumps dispensed 30 mg/kg/day of Anakinra (Kineret, Swedish Orphan Biovitrum AB) for a total of 12 weeks. The respective HED for a dose of 30 mg/kg/day in mice is calculated as 2.43 mg/kg based on the formula of Neir and Jacob (16). At 39 weeks of age, the mice were sacrificed for experimental analysis (experimental setup, Figure 1A and B). Macroscopically, the tumor lesion score increased in mice treated with HFD compared with chow diet as described previously (Figure 1C and I) (12). Histopathology of all mice cohorts is showed in Figure 1D. In the L2-IL1B mice fed HFD, inflammatory scores were significantly reduced by Anakinra (Figure 1D and F). Importantly, metaplasia and dysplasia scores were also significantly reduced by Anakinra treatment in L2-IL1B mice fed HFD. Within the chow-fed cohort, the effect of Anakinra treatment was not significant (Figure 1D and F–H). Anakinra treatment reduced the macroscopic tumor score in chow- and HFD-treated mice (Figure 1C and I). In addition, a highly significant effect of Anakinra treatment on cell proliferation was observed in both diets by immunohistochemistry for Ki67 (Figure 1E and J), or by Cyclin D1, specific for G1 phase of the cell cycle (Supplementary Figure 1A and B, available at *Carcinogenesis* Online).

Sulindac but not ASA prevents dysplastic development of metaplasia

Given our findings (12) that HFD increased the inflammatory phenotype in L2-IL1B mice, and that the most pronounced anti-inflammatory effect of Anakinra could be detected in L2-IL1B mice treated with HFD, we next tested treatment with NSAIDs in cohorts of L2-IL1B mice fed HFD only. Mice were treated with Sulindac by providing HFD enriched with 0.032% Sulindac (S8139-5G, Sigma) from 6 weeks of age on until the time of sacrifice at 26 weeks of age. Treatment with ASA was performed by providing ASA *ad libitum* in the water bottles to L2-IL1B mice fed HFD starting at 6 weeks of age until 26 weeks of age (experimental setup, Figure 2A). Drug doses per mouse were based on the literature and were set at 9 mg/kg for ASA (17) and 64 mg/kg Sulindac (18), with respective HED of 0.729 mg/kg for ASA and 5.184 mg/kg for Sulindac based on the formula of Neir and Jacob (16). Drug concentration calculations were based on a median intake of 6-ml water and 4-g food per mouse per day as described by Bachmanov *et al*. (19). Uptake of Sulindac and ASA and conversion to their active substances, Sulindac sulfide and salicylic acid, were confirmed in the treated mice by mass spectrometry (Figure 2B). Macroscopically, we observed a significant influence of the Sulindac treatment on the tumor lesion score combining all affected regions (Figure 2C and D). Although the effect of Sulindac on histologic end points was significant for both inflammation and dysplasia, the effect of ASA on dysplasia did not reach significance (Figure 2E and F–H). While showing similar results as the Sulindac treatment, the lack of statistical power in the ASA treatment could be justified by the low sample size in this group. No significant effect of the treatments could be detected in metaplasia scores. Both treatments significantly increased the number of mucus producing cells in the BE region at the squamous columnar junction (Figure 2I and J). Only Sulindac had a significant effect on proliferation analyzed by immunohistochemistry for Ki67 (Figure 2K and L), whereas both treatments had a significant effect on the expression of Cyclin D1, a G1 phase–specific proliferation marker (Supplementary Figure 1C and D, available at *Carcinogenesis* Online). It is probable that significance for ASA treatment in Ki67 staining was not reached due to the low sample size compared with HFD and Sulindac treatment.

NSAIDs change the immune phenotype

HFD was previously shown to accelerate the BE phenotype in L2-IL1B mice through alterations of the immune microenvironment (12). We analyzed immune cell populations in BE tissue and blood via flow cytometry as previously described (flow trees: Supplementary Figures 2 and 3, available at *Carcinogenesis* Online) (20). Anti-inflammatory treatment with ASA or Sulindac changed the immune cell profile as predicted. Interestingly, only Sulindac triggered a HFD-induced systemic immune effect leading to increased γδT and NKT cells in the blood, whereas ASA did not appear to have such an influence at a systemic level (Figure 3A, B, D and E). We also detected an increased recruitment of likely protective γδT and NKT cells into the BE tissue in HFD-fed mice treated with Sulindac compared with control mice fed HFD (Figure 3C and F). Similar to our previous observations (12), there was a significant decrease of tumor-promoting neutrophils in the BE region of Sulindac-treated mice compared with untreated L2-IL-1B HFD mice (Figure 3G and H). Interestingly, this effect was not reflected in the blood of the mice, where neutrophilic granulocytes were significantly more abundant in L2-IL1B HFD mice treated with ASA compared with both HFD controls and Sulindac-treated mice (Supplementary Figure 4A, available at *Carcinogenesis* Online).

Importantly, immature myeloid cells (IMCs) were significantly decreased in the BE tissue of L2-IL1B HFD mice treated with Sulindac compared with control L2-IL1B mice on HFD (Figure 3I and J). Furthermore, T helper cells were increased significantly in the BE region of ASA-treated mice compared with L2-IL1B HFD controls, whereas Sulindac only showed a trend toward increased T helper cells (Figure 3K and L). Levels of macrophages and cytotoxic T cells were not altered significantly in either BE tissue or blood, and IMC and T helper cell levels did not change across groups in blood (Supplementary Figure 4A and B, available at *Carcinogenesis* Online). Consistent with the effect of Sulindac on the immune cell profile of the mice, Sulindac treatment resulted in significant decreases in caspase-1 activity, suggesting a reduction in inflammasome activity (Figure 3M and N). Similarly, ASA treatment showed an effect on neutrophils, IMCs in BE and a reduced caspase-1 expression. However, the smaller sample size in the ASA treatment cohort impeded valid comparison of treatment groups.

significantly increasing comparing HFD to chow (chow–HFD $P \leq 0.0001$; chow–chow Anakinra $P \leq 0.0001$; HFD–HFD Anakinra $P = 0.0013$). **(J)** Analysis of Ki67 IHC shows a decrease of proliferating Ki67-positive cells with Anakinra treatment in both diets. Data presented as percentage of positive cells in BE region (chow–chow Anakinra $P = 0.0075$; HFD–HFD Anakinra $P \leq 0.0001$) (chow $n = 3$; HFD $n = 4$; chow + Anakinra $n = 5$; HFD + Anakinra $n = 6$). Ki67 expression decreases in both chow and HFD mouse cohorts when treated with Anakinra. (F–J) For statistical analysis, ordinary one-way ANOVA with Tukey's test to correct for multiple analyses was performed to test for significant differences. Plotted is mean with SD *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

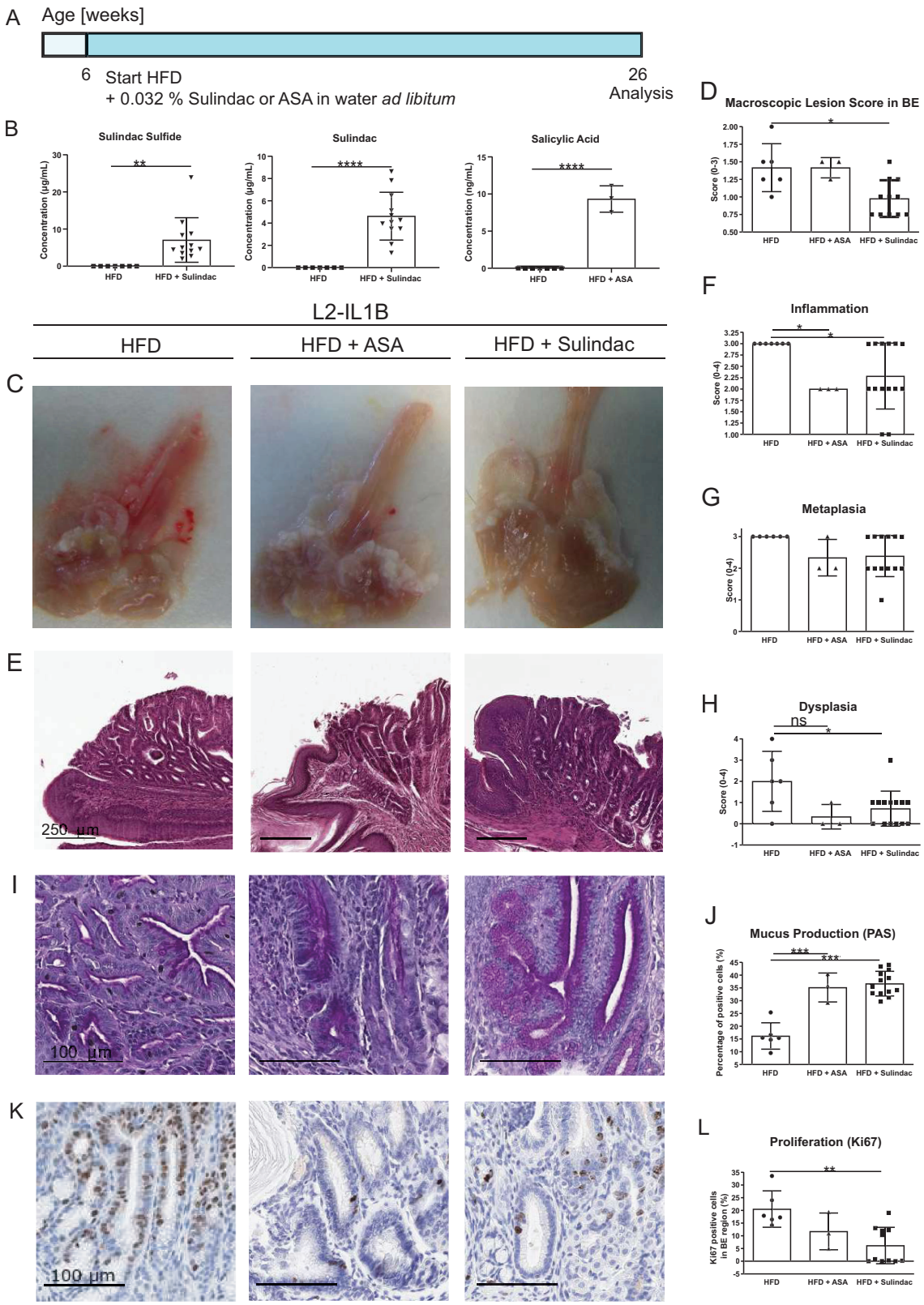

Figure 2. Macroscopic and histologic analysis of ASA- and Sulindac-treated L2-IL1B HFD mice samples. **(A)** Representative timeline of Sulindac and ASA treatment in L2-IL1B HFD mice, with treatment and experimental diet starting after weaning and genotyping at 6 weeks of age until euthanasia at 26 weeks of age. **(B)** Mass spectrometry analysis of serum levels of Sulindac and its active compound Sulindac sulfide as well as salicylic acid, the active compound of ASA in HFD-fed mice with or without ASA or Sulindac treatment. The graphs show that Sulindac sulfide, Sulindac and salicylic acid levels are significantly increased in the respective treated HFD mice compared with HFD controls (Sulindac sulfide $P = 0.0068$; Sulindac $P < 0.0001$, salicylic acid $P < 0.0001$). ASA levels could not be detected due to its short half-life and fast conversion to salicylic acid. **(C)** Representative macroscopic pictures of HFD-treated mice with or without ASA or Sulindac treatment. **(D)** The macroscopic lesion

Sulindac but not ASA treatment induces downregulation of inflammatory and oncogenic pathways in HFD L2-IL1B mice

RNA-sequencing of the BE tissue region and subsequent pathway enrichment analyses was performed in HFD mice without treatment and mice treated either with Sulindac or ASA. ASA treatment did not have a strong impact on mice fed HFD, whereas mice treated with Sulindac clearly cluster away as shown by principal component analysis (Figure 4A). Treatment with Sulindac induces changes in gene expression for pathways relevant for RNA and peptide processing. Manual curation of genes in significantly enriched pathways shows three superordinate disease relevant signaling mechanisms driving differences between HFD mice and HFD mice treated with Sulindac: inflammatory, oncogenic and disease-associated and antimicrobial response signaling (Figure 4B–D). The antimicrobial response group included genes involved in antimicrobial defense, LPS sensing and TLR4 signaling (Figure 4B). The inflammatory signaling group could be characterized by a Sulindac-driven downregulation of the inflammatory response, cytokine and neutrophil signaling and of genes involved in IL-17, EGFR and Erbb, PI3K-Akt and TGFβ signaling (Figure 4C). Interestingly, Col1a3, Ubb, Gdf9, Fermt2 and Furin, which are specifically involved in the response to TGFβ stimulus, were enriched in Sulindac-treated mice, whereas TGFβ expression itself was downregulated. Within the oncogenic and disease-associated signaling group, we observed influence of Sulindac on cell adhesion, proliferation and differentiation pathways and MAPK signaling besides regulation of genes characteristically involved in carcinogenesis (Figure 4D).

Discussion

Considering the strong association of gastrointestinal cancer and especially EAC with chronic inflammation, and the known influence of inflammation on tumor development, it is plausible that anti-inflammatory agents might be beneficial in both prevention and treatment of EAC (21,22). The increase in BE and EAC in developed countries over the last 50 years points to an important role for environmental influences. We have recently demonstrated that progression of BE to EAC might be secondary to the increased inflammation related to a consumption of a Western-style or HFD (23). HFD accelerated BE progression in the L2-IL1B mouse model through an altered inflammatory microenvironment. In our initial report on the L2-ILB mice, we noted marked upregulation of IL-6, a downstream target of IL-1β and an attenuation of the phenotype by genetic ablation of IL-6 (5).

Indeed, we here utilized the IL-1 receptor antagonist (IL-1RA) as a proof of principle to interfere with IL-1 receptor activation (24). Recombinant IL-1RA (Anakinra) is accepted as a treatment for patients with other inflammatory conditions such as rheumatoid arthritis (25). Additionally, a phase II trial has shown that patients with smoldering or indolent multiple myeloma might benefit from the treatment with Anakinra (26). Not surprisingly, IL-1RA treatment in our mouse model of IL-1B overexpression showed a reduction of dysplasia, serving as a proof of concept that the secondary inhibition of inflammation can reduce tumor formation. Nevertheless, IL-1RA treatment might not be feasible for chronic administration and prevention in BE patients, due to issues of cost and off-target effects, so other anti-inflammatory drugs should be considered for chemoprevention.

Therefore, anti-inflammatory treatment was explored with two effective NSAIDs, ASA and Sulindac, which have been used for COX inhibition in several previous trials for other conditions. ASA is an inhibitor of COX-1 and COX-2, but most probably acts additionally via different unknown pathways. It has been shown that ASA has a delaying effect on the development of neuroblastoma in a mouse model (27). In human patients, a protective effect of ASA has been described for colorectal carcinoma (28). Sulindac is a COX-inhibitor with a stronger preference for COX-2 and fewer side effects, and also has an effect on additional signaling pathways (29). Results from mouse studies suggest a protective effect of Sulindac on colon cancer (18). We observed a protective effect of both NSAIDs on the development of dysplasia in our BE mouse model, with a stronger effect in Sulindac-treated animals. Both ASA and Sulindac reduced the number of proliferating cells assessed by Ki67 and Cyclin D1 IHC. Importantly, Cyclin D1 was found to be highly expressed in this mouse model and is associated to high oncogenic potential (30). Of note, this effect was observed in L2-IL1B HFD mice, which were proven to have accelerated disease development due to alterations of the immune microenvironment (12), suggesting that such exogenous inflammatory conditions could be eliminated by NSAID treatment, whereas the intrinsic inflammation due to the perpetual IL-1B expression was not changed.

In Sulindac-treated mice, we observed a local and systemic increase in γδT cells, which have been reported to exert beneficial antitumor effects in other cancers (31–33). Moreover, there was a significant increase in NKT cells in the blood in Sulindac-treated mice. NKT cells have been reported to have cytotoxic and antitumorigenic properties mediated by IFN-γ and IL-2 signaling (34,35) and are associated with prolonged survival in adenocarcinoma (36). It seems plausible that NKT cells were activated in response to Sulindac treatment and may have contributed to the suppression of tumor growth in L2-IL1B mice. NK cells have been proposed as a favorable prognostic marker in Stage II-III esophageal cancer (37). We observed in our L2-IL1B mouse model that increases in neutrophils are generally accompanied by reduced NK/NKT cells. In contrast to NK/NKT cells, neutrophils have been shown to prevent NK/NKT cell-mediated clearance of tumor cells and are unfavorable in tumor prognosis (38).

Although myeloid cells can carry out both tumor-promoting and immunosuppressive functions (39), a subtype comprising CD11b+Ly6G^{high}Ly6C^{low} neutrophils and CD11b+Ly6G^{low}Ly6C^{high}

score of the BE region of L2-IL1B mice significantly decreases in Sulindac-treated HFD mice compared with untreated mice (HFD–HFD Sulindac $P = 0.0162$; HFD–HFD ASA $P = 0.0646$). **(E)** Representative histologic pictures of HFD-fed mice with or without ASA or Sulindac treatment. Bars 250 μm. **(F)** Inflammation score. **(G)** Metaplasia score. **(H)** Dysplasia score of HFD mice with and without ASA and Sulindac treatment (inflammation HFD–HFD ASA $P = 0.0344$; HFD–HFD Sulindac $P = 0.0484$; metaplasia HFD–HFD Sulindac $P = 0.0852$; dysplasia HFD-HFD Sulindac $P = 0.0377$; HFD–HFD ASA $P = 0.0668$). Inflammation significantly decreases in both treatment cohorts compared with controls. Also, dysplasia scores decrease in both treatment cohorts compared with controls, but the decrease fails to reach significance in HFD mice treated with ASA, due to the low sample size. Representative pictures of **(I)** periodic-acid–Schiff staining and **(K)** Ki67 IHC in HFD mice with and without ASA and Sulindac treatment. **(J)** Mucus production assessed by PAS staining significantly increases in both ASA and Sulindac-treated HFD mice compared with HFD-only mice (HFD–HFD ASA $P = 0.0001$; HFD–HFD Sulindac $P \leq 0.0001$). **(L)** Proliferation represented by Ki67 IHC analysis significantly decreases in Sulindac-treated HFD mice compared with HFD-only mice ($P = 0.0029$). IHC expression is represented by the percentage of positive cells in BE region. (B, D, F–H, J, L) For statistical analysis, one-way ANOVA with Tukey's test to correct for multiple comparisons was performed. Plotted are individual values and mean with SD $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

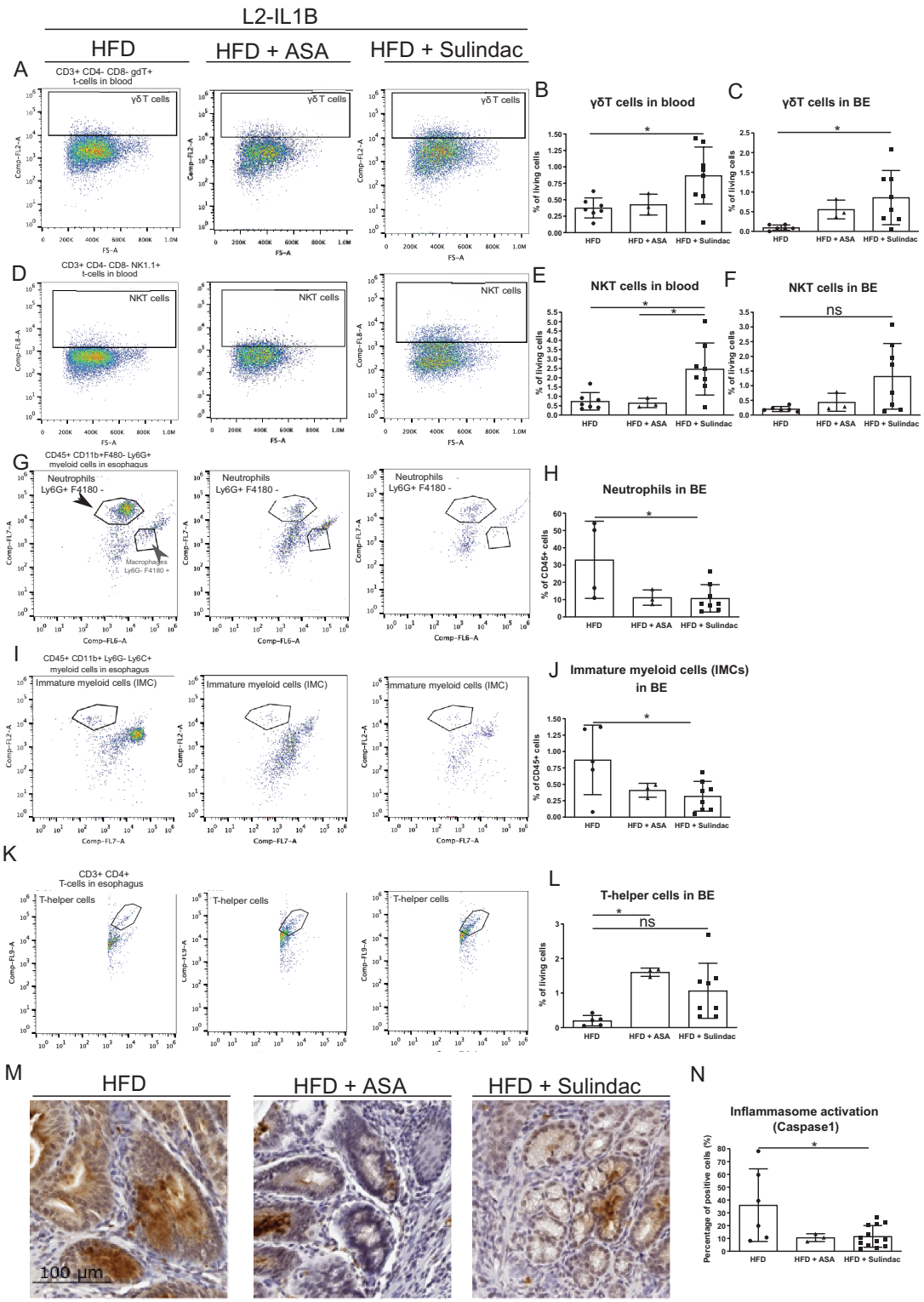

Figure 3. ASA and Sulindac treatments change the inflammatory microenvironment in L2-IL1B HFD mice. Representative gating of **(A)** γδT cells, **(D)** NKT cells, **(G)** neutrophilic granulocytes (neutrophils), **(I)** immature myeloid cells (IMCs) and **(K)** T helper cells in the blood of L2-IL1B mice fed HFD. The percentage of living γδT cells in **(B)** blood (HFD–HFD Sulindac $P = 0.0225$) and the **(C)** BE region (HFD–HFD Sulindac $P = 0.0323$) is significantly increasing in HFD Sulindac compared with HFD-treated mice. NKT cells in **(E)** blood (HFD–HFD Sulindac $P = 0.0119$; HFD Sulindac–HFD ASA $P = 0.0429$) **(F)** and BE region (HFD–HFD Sulindac $P = 0.0538$) are increasing in HFD Sulindac compared with HFD-treated mice, while the increase does not reach significance in BE. The percentage of living CD45-positive neutrophilic granulocytes/neutrophils **(H)** in the BE region (HFD–HFD Sulindac $P = 0.0369$) and **(J)** immature myeloid cells (IMCs) in the BE region (HFD–HFD Sulindac $P = 0.0340$) is significantly decreasing in HFD Sulindac compared with HFD-treated mice. Living T helper cells **(L)** in the BE region (HFD–HFD Sulindac $P = 0.0164$; HFD–HFD ASA $P = 0.0571$) of L2-IL1B HFD mice with or without ASA or Sulindac treatment are increasing in both ASA and Sulindac treatment compared with HFD, but the increase does not reach significance in Sulindac. Representative pictures of **(M)** Caspase1 IHC in HFD mice with and without Sulindac and ASA treatment. **(N)** Caspase 1 activation is significantly reduced in HFD Sulindac compared with HFD mice ($P = 0.0199$). IHC expression is represented by the percentage of positive cells in BE region. ANOVA test with Tukey's *post hoc* testing was performed to test for significant differences. Plotted are individual values and mean with SD $^*P < 0.05$.

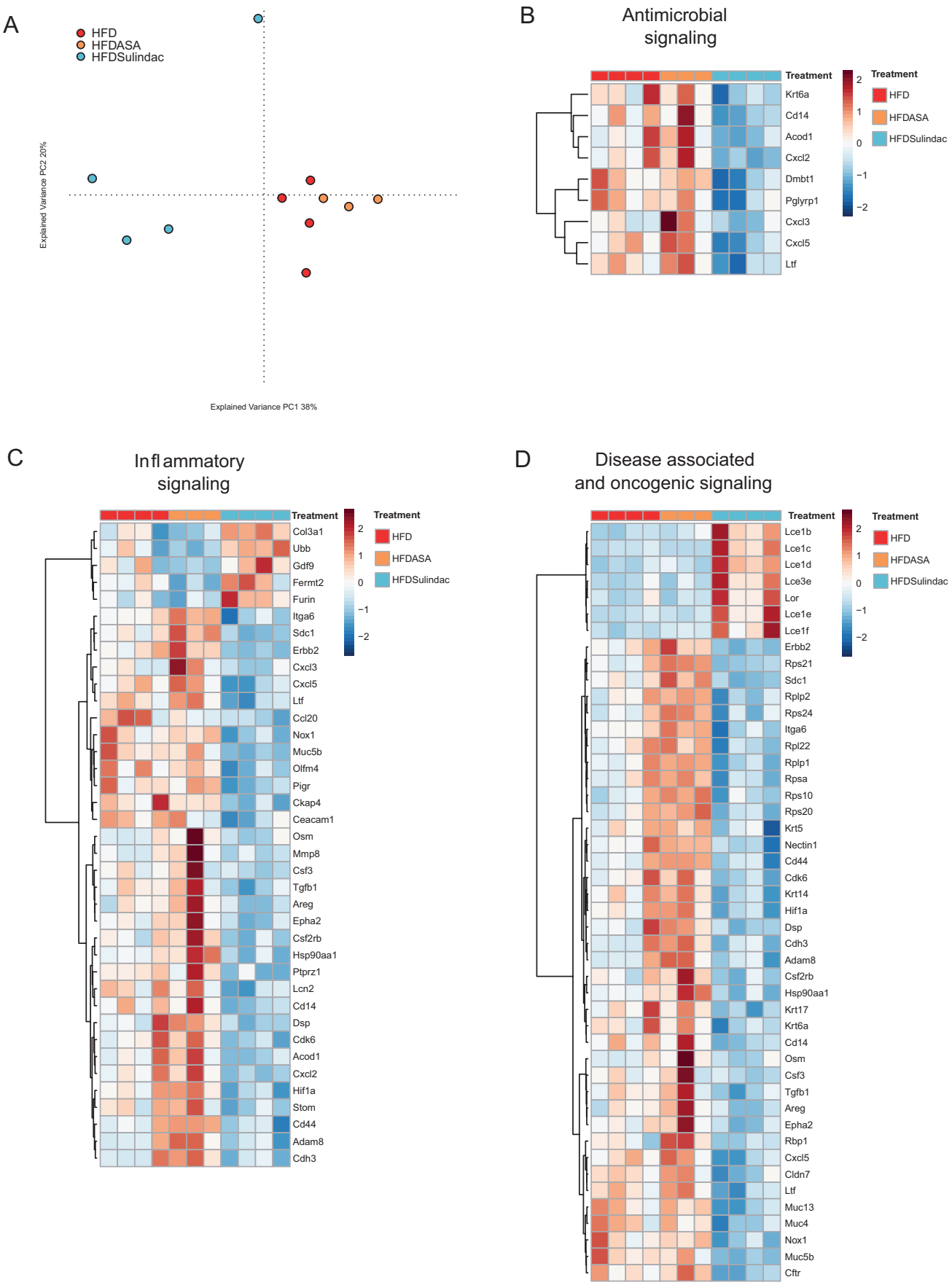

Figure 4. Sulindac treatment had a suppressive effect on inflammatory and oncogenic signaling pathways compared with ASA treatment and untreated L2-IL1B HFD mice. RNA-sequencing was performed for L2-IL1B HFD mice without treatment and mice treated either with Sulindac or with ASA (L2-IL1B HFD $n = 4$; L2-IL1B HFD

IMCs probably contributes to epithelial growth and progression. Both ASA and Sulindac reduce IMC and neutrophil influx in the BE region of L2-IL1B mice on HFD, thereby potentially suppressing tumor progression. However, the effect of ASA treatment in this study did not reach significance most probably due to low sample size. Previously, we observed during disease progression an accumulation of IMCs and neutrophils through HFD (12) and bile acid (DCA) treatment in L2-IL1B mice (5), pointing to the importance of neutrophils in disease progression. Although neutrophils can occasionally be tumor antagonizing, increased levels of neutrophils have been reported frequently in association with a higher tumor grade and poor prognosis (40–42). Interestingly, we could detect significant increases of neutrophils in the blood of L2-IL1B HFD mice treated with ASA compared with both HFD controls and Sulindac-treated mice. This observation provides a possible explanation for the reduced effect of ASA on disease progression in L2-IL1B HFD mice compared with Sulindac.

We could also detect an increase in T helper cells in the BE tissue of both L2-IL1B HFD NSAID treatment cohorts, which reached significance in ASA-treated mice, whereas a trend toward an increase was observed in Sulindac-treated mice. T helper cells play an essential role in anti-tumor immunosurveillance and appeared to be the most important T cell population in healthy esophagus and BE tissue in humans, whereas their frequency diminishes in EAC tissue (43,44). The increase of T helper cell levels in ASA and Sulindac-treated mice suggests a possible role in enhanced tumor-immunosurveillance. In conclusion, we suspect that the altered immune cell profiles in NSAID-treated mice could plausibly explain mechanistically how ASA and even more Sulindac treatment attenuated the phenotype of our L2-IL1B model. This interpretation would be consistent with the significant decrease in caspase-1 expression in Sulindac-treated mice. Caspase-1 cleaves and activates interleukin-1 and is part of the inflammasome complex, which is responsible for intracellular inflammatory responses. Indeed, RNAseq data showed that Sulindac treatment significantly suppressed inflammatory, oncogenic and interestingly also antimicrobial signaling. Interestingly, Sulindac treatment suppressed acute inflammatory responses including cytokine signaling and neutrophil activation, which we previously showed to be important in acceleration of disease progression in L2-IL1B mice fed HFD compared with a control diet (12).

The data indicate differential effects of both treatments on the immune cell phenotype of the mice despite their exploratory nature in the ASA treatment group. Those results vindicate further mechanistic investigation of the effects of specific NSAIDs on the pathogenicity of BE and EAC. The L2-IL1B mouse model resembles human pathogenesis of BE and EAC (3) and seems a good tool to analyze such treatment effects. Nevertheless, it is limited by the fact that mice do not have gastroesophageal reflux, and therefore, the inflammation-based aspects of disease development have to be evaluated with the acknowledgment of an IL-1b-driven inflammatory process. Surgical canine and rat models could also be used to examine pharmacological tumor prevention (45–48) but may harbor other difficulties.

ASA has been reported to act protectively in different cancer types. The AspECT trial investigated the influence of ASA on malignant disease progression of BE in patients and showed that ASA improved overall outcomes in patients with BE, but not disease-specific outcome, especially when combined with esomeprazole (49). Further clinical studies will be needed to validate the effects that we observed in our mouse model, especially since we employed low dose aspirin, which did not result in a significant reduction of the dysplastic phenotype. Of note, the AspECT trial used 300 or 325 mg of aspirin (ASA) per day, which result in a significantly improved outcome in overall survival only in combination with proton-pump inhibitors. Nevertheless, treatment with aspirin alone in BE patients did not have a significant better outcome compared with placebo treatment, similar to our results from the mouse model, despite the higher dose of aspirin used. Thus, further studies are needed to determine if aspirin should be utilized for EAC prevention in humans.

In contrast, Sulindac suppressed pro-inflammatory IL-17 signaling, probably via downregulation of TGFβ signaling, in addition to its known inhibition of COX-2 and NF-κB signaling (50). IL-17 mediates antimicrobial defense mechanisms, inflammatory pathways and attraction of myeloid cells, such as neutrophils to inflammatory sites, via production of Cxcl-2 and Cxcl-5 (51), crucial mechanism in the L2-IL1B mouse model to promote esophageal carcinogenesis (12). Moreover, PI3K-Akt and MAPK signaling pathways involved in proliferation differentiation, inflammatory signaling and oncogenic signaling were downregulated in Sulindac-treated mice (52–54), suggesting a profound suppressive effect of Sulindac on pro-inflammatory and pro-oncogenic signaling. Studies have shown that Sulindac supports tumor regression by reducing the influx of macrophages in a mouse model of breast cancer, supporting the notion of Sulindac as a modulator of the tumor microenvironment (55). Considering the results from the AspECT trial (49) and our results here with ASA and Sulindac, we postulate that NSAIDs in general are a promising approach for chemoprevention in BE patients. Further investigations needed to determine whether in fact Sulindac is the more promising chemopreventive agent, and the optimal design and end points for future prevention studies in BE patients.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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Data availability

Data available on request. Detailed tables showing RNA-sequencing results (differential expression analysis, gene enrichment analysis via EnrichR, pathway annotation of the genes presented in Figure 4) are available on request. Raw sequencing data are available under the accession number PRJEB42619.

Sulindac $n = 4$; L2-IL1B HFD ASA $n = 3$). Pairwise differential expression analysis was performed followed gene set enrichment analysis for significantly regulated genes using the web-based gene enrichment analysis tool EnrichR. **(A)** Principal component analysis shows that HFD Sulindac mice cluster separately from both other groups, indicating a different effect of Sulindac treatment compared with ASA treatment on gene expression in mice on HFD. In contrast, HFD and HFD ASA mice cluster in close proximity. Significantly regulated genes can be further grouped into three disease relevant classes, which are depicted as heatmaps. **(B)** Antimicrobial defense mechanisms, **(C)** inflammatory pathways and **(D)** disease-associated and oncogenic pathways. All three groupings show a Sulindac-driven downregulation of gene expression and thus pathway activity.

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