Inflammasome adaptor ASC suppresses apoptosis of gastric cancer cells by an IL-18 mediated inflammation-independent mechanism

Virginie Deswaerte¹,²,* Paul Nguyen³,⁴,* Alison West¹,² Alison F. Browning¹,² Liang Yu¹,², Saleela M. Ruwanpura¹,² Jesse Balic¹,² Thaleia Livis¹,² Charlotte Girard⁵, Adele Preaudet³,⁴, Hiroko Oshima⁶, Ka Yee Fung³,⁴, Hazel Tye¹,² Meri Najdovska¹,² Matthias Ernst⁷, Masanobu Oshima⁶, Cem Gabay⁵, Tracy Putoczki³,⁴, and Brendan J. Jenkins¹,²

¹Centre for Innate Immunity and Infectious Diseases, Hudson Institute of Medical Research, Clayton, Victoria, Australia; ²Department of Molecular Translational Science, School of Clinical Sciences, Monash University, Clayton, Victoria, Australia; ³Inflammation Division, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia; ⁴Department of Medical Biology, University of Melbourne, Parkville, Victoria, Australia; ⁵Division of Rheumatology, University Hospital of Geneva, and Department of Pathology and Immunology, University of Geneva School of Medicine, Geneva, Switzerland; ⁶Division of Genetics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan; ⁷Olivia Newton-John Cancer Research Institute, La Trobe University School of Cancer Medicine, Heidelberg, Victoria, Australia.

*These authors contributed equally.

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Corresponding author: Brendan J. Jenkins, Centre for Innate Immunity and Infectious Diseases, Hudson Institute of Medical Research, 27-31 Wright Street, Clayton, Victoria 3168, Australia. Tel: +61 3 85722740; E-mail: Brendan.Jenkins@hudson.org.au

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Abstract

Inflammasomes are key regulators of innate immunity in chronic inflammatory disorders and autoimmune diseases, but their role in inflammation-associated tumorigenesis remains ill-defined. Here we reveal a pro-tumorigenic role in gastric cancer (GC) for the key inflammasome adaptor ASC and its effector cytokine IL-18. Genetic ablation of ASC in the $gp130^{F/F}$ spontaneous mouse model of intestinal-type GC suppressed tumorigenesis by augmenting caspase-8-like apoptosis in the gastric epithelium, independently from effects on myeloid cells and mucosal inflammation. This phenotype was characterized by reduced activation of caspase-1 and NF-κB activation and reduced expression of mature IL-18, but not IL-1β, in gastric tumors. Genetic ablation of IL-18 in the same model also suppressed gastric tumorigenesis, whereas blockade of IL-1β and IL-1α activity upon genetic ablation of the IL-1 receptor had no effect. The specific pro-tumorigenic role for IL-18 was associated with high IL-18 gene expression in the gastric tumor epithelium compared to IL-1β, which was preferentially expressed in immune cells. Supporting an epithelial-specific role for IL-18, we found it to be highly secreted from human GC cell lines. Moreover, IL-18 blockade either by a neutralizing anti-IL-18 antibody or by CRISPR/Cas9-driven deletion of ASC augmented apoptosis in human GC cells. In clinical specimens of human GC tumors, we observed a significant positive correlation between elevated mature IL-18 protein and ASC mRNA levels. Collectively, our findings reveal the ASC/IL-18 signaling axis as a candidate therapeutic target in GC.
Introduction

Gastric cancer (GC) is the third most lethal cancer worldwide, and is among a growing number of cancers associated with precursory chronic inflammatory responses (1-3). The predominant histologic subtype of GC is intestinal-type, and despite the causal correlation between chronic gastric inflammation triggered by pathogenic microbes (i.e. Helicobacter pylori) and intestinal-type GC (2,4), the identity of innate immune regulators within the host gastric mucosa that promote GC remains unclear. Consistent with an altered host immune response predisposing to GC, gene polymorphisms for the pro-inflammatory cytokine interleukin (IL)-1β, which are associated with augmented gene expression, increase the risk of human GC (5,6), and transgenic overexpression of IL-1β in mice triggers gastric inflammation and tumors (7). In addition, clinical studies on the related IL-1 cytokine family member IL-18, which can display opposing anti- or pro-tumorigenic effects dependent upon the tissue and cellular context in various cancers (8), demonstrate that IL-18 levels are increased in GC patients and serve as a poor prognostic marker (9-11). While experimental data from human GC cell lines also suggest that IL-18 may contribute to the malignant progression of tumors (9,12,13), a definitive role for IL-18 in GC remains unproven.

Inflammasomes have recently been identified as multiprotein complexes that are essential for the production of mature and bioactive IL-1β and IL-18 proteins. Accordingly, they have attracted much attention as key factors of the immune system with the potential to influence susceptibility to many autoimmune and inflammatory diseases, as well as cancers, where IL-1β and IL-18 are implicated (14-17). These cytokines are initially produced as inactive proIL-1β and proIL-18 forms, with proIL-1β expression upregulated following ligand-mediated activation of pattern recognition receptors (PRRs) such as Toll-like receptor (TLR) or nucleotide-binding oligomerisation domain-containing (NOD) family members, whereas proIL-18 is constitutively expressed. Subsequently, their inflammasome-mediated secretion as
bioactive cytokines is controlled by members of the nucleotide-binding domain and leucine-rich repeat containing protein (NLR) family, namely NLR pyrin domain containing 1 (NLRP1), NLRP3, NLRP6, NLRP12, NLR CARD domain containing 4 (NLRC4) and NLR apoptosis inhibitory protein (NAIP), as well as the cytosolic DNA sensor Absent in melanoma 2 (AIM2) (15,18). Specifically, each of these NLRs and AIM2 form the core of distinct inflammasome complexes, whereby upon sensing host- and/or microbial-derived ligands, they associate with the key adaptor protein Apoptosis-related speck-like protein containing a CARD (ASC) to facilitate activation of caspase-1, which catalyses the maturation of proIL-1β and proIL-18 precursors into bioactive secreted cytokines (15).

Although ASC is critical for inflammasome-mediated pathologies involving IL-1β and/or IL-18, investigations into the definitive role of ASC in tumorigenesis are still in their infancy and have been largely restricted to intestinal and skin carcinogenesis, with contrasting findings (19). Here, we reveal that elevated PYCARD (hereafter referred to as ASC) mRNA and mature IL-18, but not IL-1β, protein levels are a coincident feature of gastric tumors from both GC patients and the gp130F/F intestinal-type GC mouse model (20). Furthermore, genetic ablation of either Asc or Il18 in gp130F/F mice suppressed gastric tumor growth, independent of inflammation, which was associated with augmented neoplastic epithelial cell death. Interestingly, at the molecular level suppressed gastric tumorigenesis in both gp130F/F:Asc−/− and gp130F/F:Il18−/− mice was characterized by reduced activation levels of NF-κB. In further support of these findings, we also demonstrate in vitro that CRISPR/Cas9-mediated genetic ablation of ASC in human GC cells suppressed their colony-forming potential (i.e. growth) which was associated with reduced secretion of mature IL-18, increased cellular apoptosis, and reduced activation levels of NF-κB. Collectively, these findings support the existence of a novel pro-tumorigenic ASC inflammasome/IL-18 axis in GC.
Materials and Methods

Mice

The \textit{gp130}^{F/F} mice (20), along with mice homozygous null for \textit{Asc} (\textit{Pycard}) (21), \textit{Il1r} (22) or \textit{Il18} (23) were used to generate \textit{gp130}^{F/F}:\textit{Asc}^{-/-}, \textit{gp130}^{F/F}:\textit{Il1r}^{-/-} and \textit{gp130}^{F/F}:\textit{Il18}^{-/-} mice, respectively, on a mixed 129Sv x C57BL/6 background. Experiments comparing different mouse strains included genetically- and age-matched littermates, including where appropriate, wild-type (\textit{gp130}^{+/+}) control mice. Mice were housed under specific pathogen-free conditions on a 12-hour light/dark cycle, and all animal studies were approved by the Monash University Monash Medical Centre “A” Animal Ethics Committee and the Walter and Eliza Hall Institute Animal Ethics Committee.

Human Biopsies

Gastric biopsies were collected at the Xin Hua Hospital (Shanghai, China) from patients, upon formal written informed consent, undergoing upper gastrointestinal endoscopy or surgical resection. Clinicopathological features and demographics of GC patient cohorts are described in Supplementary Table S1. Biopsies were snap-frozen in liquid nitrogen. Studies were approved by the Xin Hua Hospital Human Research Ethics Committee and undertaken in accordance with the appropriate ethics guidelines. Patient studies were conducted in accordance with the World Medical Association Declaration of Helsinki statement on the ethical principles for medical research involving human subjects.

Laser Microdissection

Tumor epithelial and stroma samples from \textit{gp130}^{F/F} mice were collected from OCT-embedded frozen sections stained with toluidine blue using laser microdissection (Leica). Total RNA was extracted from microdissected samples using the miRNeasy microkit (Qiagen), and then reverse
transcribed with the PrimeScript RT Reagent Kit (Takara). Quantitative RT-PCR (qPCR) was performed as described below.

**RNA Isolation and Gene Expression Analyses**

Total RNA was isolated from snap-frozen mouse and human stomach tissues using TRI Reagent® Solution (Sigma) followed by on-column RNeasy® Mini Kit RNA clean-up and DNase treatment (Qiagen). qPCR was performed on cDNA with Taqman® Gene Expression Assays (mouse *Il1b*, *Il1r*, *Il18*, *Il18r1*, *Pycard*; ThermoFisher Scientific) or SYBR Green chemistry (Life Technologies) using the Applied Biosystems 7300, 7900HT Fast, and Viia7 Real-Time PCR Systems (ThermoFisher Scientific). Data acquisition and analyses were undertaken using the Sequence Detection System Version 2.4 software (Applied Biosystems). Forward and reverse primer sequences for mouse *18S rRNA*, *Il1b*, *Tnfa*, *Cxcl1*, *Cxcl2*, *Ccnd1*, *Ccnd2* and *c-myc* have been previously published (24). Sequences for other mouse and human primers will be given upon request.

**The Cancer Genome Atlas (TCGA)**

Gene expression data and clinical information from TCGA GC patients were obtained from the open access TCGA data portal (https://portal.gdc.cancer.gov/projects/TCGA-STAD). The alignment of sample identifiers yielded 18 primary GC cases for which there was available tumor and matched non-tumor data. We used reads per kilobase of exon model per million mapped reads (RPKM) to quantify *ASC* expression levels from RNA sequencing (RNA-Seq) data generated from each GC patient within TCGA.

**ELISA and Immunoblotting**

Total protein lysates from snap-frozen tissues were prepared at room temperature with two
incubations of 20 minutes at 37°C before and after homogenization (25). ELISA for human total IL-1β (R&D Systems), and mouse and human total IL-18 (MBL International Corporation) were performed according to the manufacturer’s instructions. Mature and free human IL-18 was detected with a recently-developed sandwich ELISA (26), and mature mouse IL-18 was detected by ELISA using equivalent methodology for the mature human IL-18 ELISA. All ELISAs were performed using 50µg of protein lysate per well of 96-well plates. Immunoblotting was performed with antibodies against mouse (AdipoGen) and human (Cell Signaling Technologies) caspase-1, IL-18 (BioVision), phospho(p)NF-κB p65 (Ser536) (Cell Signaling Technologies) and α-Tubulin (Abcam), and protein bands were visualized using either the Odyssey Infrared Imaging System (LI-COR) for IL-18 (p24/p18) and α-Tubulin, or enhanced chemiluminescence for caspase-1 (p45/p20). The bands were quantified using ImageJ software (National Institutes of Health, USA).

**Histology, Immunohistochemistry and Immunofluorescence**

Formalin-fixed and paraffin-embedded tissue sections were stained with hematoxylin and eosin (H&E) for histological evaluation. The terminal deoxynucleotidyl transferase (tdT)–mediated dUDP nick-end labeling (TUNEL) assay (Millipore), as well as immunohistochemistry to detect proliferating cell nuclear antigen (PCNA) and cleaved caspase-8 (Cell Signaling Technologies), pNF-κB p65 (Ser536) (Santa Cruz Biotechnology), and CD45, B220, CD3 and CD68 (BD BioSciences), were performed as before (24,27). Positive-stained cells were counted manually (n = 20 high-power (x40) fields) with a random offset, or percentage area of positive staining was acquired using ImageJ software. Dual immunofluorescence was performed on paraffin-embedded stomach tissues using fluorescent-conjugated primary antibodies, and Alexa Fluor secondary antibody. Antigens were detected with antibodies against E-cadherin and active caspase-3 (Cell Signaling Technologies), pNF-κB p65 (Ser536) (Santa Cruz
Biotechnology) and CD45 (BD BioSciences), as previously described (27). Slides were examined by confocal microscopy (Nikon) and analyzed for red and green fluorescence. Where appropriate, stereological techniques were applied to enumerate dual-stained cells (27).

**Flow Cytometric Sorting and Analyses of Gastric Single Cell Suspensions**

Single cell suspensions of dissected mouse stomach tumor-bearing tissue were prepared by collagenase digestion as described previously (24). For cell sorting, single cells were collected and stained with fluorescent-conjugated antibodies against CD45 (Biolegend) and EpCAM (eBioscience), as well as propidium iodide, and then sorted using a FACS Aria instrument (BD Biosciences). For analyses, single cell populations were stained with fluorescent-conjugated antibodies against CD3, CD69, CD19, CD4, CD11c, B220, CD86, CD45 CD11b, CD8, CD11b (eBioscience), as well as Gr-1 and F4/80 (BD BioSciences). Apoptosis of human AGS cells was assessed with an Annexin V:FITC Apoptosis Detection Kit (BD Pharmingen). Stained cells were acquired on a FACSCanto instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star) as described previously (24).

**Human Cell Lines and Colony-forming Assays**

Human GC cell lines AGS (American Type Culture Collection) and MKN1 (Japanese Collection of Research Bioresources Cell Bank) were maintained in RPMI supplemented with 10% heat-inactivated FCS, 1% penicillin-streptomycin and 1% L-glutamine (GIBCO). Cell lines were authenticated via short tandem repeat profiling (PowerPlex HS16 System kit, Promega) in our laboratory after receipt in 2013. For experiments, cell lines were passaged for under 3 months at a time between freeze/thaw cycles. Cell lines were routinely tested throughout the time of experiments for mycoplasma contamination (MycAlert™ PLUS Mycoplasma Detection Kit, Lonza). For clonogenic assays, AGS and MKN1 cells were seeded
in 6-well plates (1×10⁴ cells/well) with RPMI/10% FCS media with or without either anti-hIL-18-IgA antibody (1µg/ml; InvivoGen) or anakinra (1µg/ml; Swedish Orphan Biovitrum AB), the latter a specific IL-1 receptor (IL-1R) antagonist that blocks the activity of IL-1β and IL-1α. After 10 days of culture, colonies (containing ≥ 50 cells) were stained and fixed with a solution of 0.005% crystal violet (Sigma-Aldrich) in 10% methanol, and colonies counted.

**CRISPR-driven ASC and Caspase-1 Gene Knockout**

Using published protocols (28), self-complementary oligonucleotides (Sigma-Aldrich) for human ASC (hASC-sgRNA1-O1-caccgCGAGGGTCACAAACGTTGAG; hASC-sgRNA1-O2-aaacCTCAACGTTTTGACCCCTCGc; hASC-sgRNA2-O1-caccgCATGTCGCAGCAGCAGTGGAG; hASC-sgRNA2-O2-aaacCTAACGTGCTGCAGCATGc) and human caspase-1 (hCASP1-sgRNA1-O1-caccgAAAGCTGTTTATCCGTTCCA; hCASP1-sgRNA1-O2-aaacTGGAACGGATAAACAGCTTTc; hCASP1-sgRNA2-O1-caccgGCTCCCTAGAAAGAAGCTCAA; hCASP1-sgRNA2-O2-aaacTTGAGCTTCTTCTAGGGAGCc) were ligated into the LentiCRISPRv2 construct (Addgene). The single-guided (sg) RNA sequences are designed and constructed for human ASC to allow for sgRNA targeting of constitutive exonic coding regions (Exon 1 and Exon 3). Lentivirus was produced by transfecting vectors into Lenti-X/H293T cells with LentiCRISPR:psPAX2:pmD2.G at a ratio of 4:3:1. Virus was harvested 48 hours after transfection, filtered and used to infect AGS cell cultures containing 5µg/ml polybrene. Infected cells were selected with puromycin, and cells infected with non-target control sgRNA vector were used as negative controls.

**Bone-marrow Chimeric Mice**
The $gp130^{FF}$ and $gp130^{FF}:Asc^{+/-}$ mice were lethally-irradiated (single 9.5Gy dose) and reconstituted with $5 \times 10^6$ unfractionated donor bone marrow cells from the indicated genotypes as described previously (24).

**Statistical Analyses**

Statistical analyses were performed using GraphPad PrismV6.0 software. Data normality was assessed using the D’Agostino and Pearson omnibus K2 normality test, and the appropriate tests to identify statistical significance ($P < 0.05$) between the means of two or multiple groups are presented in the relevant figure legends. Data are expressed as the mean ± standard error of the mean (SEM).
Results

ASC expression is elevated in human GC, and genetic ablation of ASC in $gp130^{F/F}$ mice suppresses gastric tumor growth

In human GC, gene expression for the key inflammasome adaptor, ASC, was significantly elevated in tumors from 2 independent GC patient cohorts (Fig. 1A and 1B, Supplementary Table S1), thus implicating ASC in disease pathogenesis. To interrogate the role of ASC in GC, we generated $gp130^{F/F}$ mice deficient for ASC ($gp130^{F/F}:Asc^{-/-}$). At 10-12 weeks of age, which is 4-6 weeks after the onset of antral gastric hyperplasia and tumor formation in $gp130^{F/F}$ mice, the stomach size, tumor mass and incidence were comparable between $gp130^{F/F}$ and $gp130^{F/F}:Asc^{-/-}$ mice (Supplementary Fig. S1A-E). However, compared to age- and sex-matched $gp130^{F/F}$ littermates, 20-24 week old (wo) and 30-34wo $gp130^{F/F}:Asc^{-/-}$ mouse stomachs displayed a markedly reduced hyperplastic response and were visibly smaller and significantly reduced in mass by ~30% and ~40%, respectively (Fig. 1C and 1D, Supplementary Fig. S1F and S1G), with no observable gender bias. Furthermore, although histological assessment of $gp130^{F/F}$ and $gp130^{F/F}:Asc^{-/-}$ mouse stomachs revealed similar gastric adenomatous hyperplastic lesions with no evidence of low grade dysplasia nor carcinoma *in situ*, the gastric antral tumor mass in $gp130^{F/F}:Asc^{-/-}$ mice was significantly reduced by ~40% at 20-24 weeks and ~60% at 30-34 weeks compared to age-matched $gp130^{F/F}$ mice (Fig. 1D, Supplementary Fig. S1H). In addition, the total incidence of gastric lesions in 20-24wo and 30-34wo $gp130^{F/F}:Asc^{-/-}$ mice was also significantly reduced compared to $gp130^{F/F}$ mice at the corresponding ages, which corresponded with smaller hyperplastic lesions in $gp130^{F/F}:Asc^{-/-}$ mice (Fig. 1D and 1E, Supplementary Fig. S1I and S1J).

Protein levels of activated caspase-1 p20 subunit, the downstream effector of ASC inflammasomes, were significantly increased 1.7- to 2-fold in stomachs of 20-24wo $gp130^{F/F}$ compared to $gp130^{+/+}$ wild-type mice (Supplementary Fig. S2A), and were significantly
reduced by 7.1-fold (compared to 2.6-fold for procaspase-1) in \(gp130^{FF}:Asc^{-/-}\) versus \(gp130^{FF}\) tumors (Supplementary Fig. S2B). These data therefore confirm that the elevated inflammasome activity during gastric tumorigenesis in \(gp130^{FF}\) mice is reduced in the absence of ASC.

**ASC-driven gastric tumorigenesis in \(gp130^{FF}\) mice is independent of inflammation**

Since ASC inflammasome activation can instigate potent inflammatory responses, we investigated whether suppressed tumorigenesis in \(gp130^{FF}:Asc^{-/-}\) mice was associated with reduced gastric inflammation. Histological assessment of H&E-stained gastric tissue sections from 20-24wo mice revealed that the presence of chronic inflammatory infiltrates comprising primarily plasma and lymphoid cells was comparable in the submucosa and mucosa regions of tumors from both genotypes (Fig. 1F). Immunohistochemical analyses also indicated comparable numbers of CD45+ leukocyte infiltrates, and CD68+ macrophage, B220+ B cell and CD3+ T cell immune subsets, in the gastric mucosa of \(gp130^{FF}\) and \(gp130^{FF}:Asc^{-/-}\) tumors (Fig. 2A and 2B, Supplementary Fig. S3A-D). Consistent with these observations, flow cytometry on tumor-bearing stomachs of 20-24wo \(gp130^{FF}:Asc^{-/-}\) and \(gp130^{FF}\) mice confirmed similar frequencies of gastric immune cell subsets, namely CD11b+Gr-1+ myeloid-derived suppressor cells (MDSC), CD11c+CD11b+ dendritic cells, CD11b+F4/80+ monocytes/macrophages, and B and T cell populations (Fig. 2C). Also, the activation status of B (B220+CD86+) and T (CD4+CD69+ and CD8+CD69+) cells, which are the predominant infiltrating immune cells in \(gp130^{FF}\) and \(gp130^{FF}:Asc^{-/-}\) gastric tumors, was comparable (Fig. 2C). The similar frequency and activation status of distinct immune cell subsets was also confirmed in perigastric lymph nodes of \(gp130^{FF}\) and \(gp130^{FF}:Asc^{-/-}\) mice (Supplementary Fig. S3E).

The unaltered inflammation was also coincident with similar mRNA levels of numerous inflammatory genes in gastric tumors from 20-24wo \(gp130^{FF}\) and \(gp130^{FF}:Asc^{-/-}\) mice (Fig.
3A). We also assessed whether the suppressed gastric tumorigenesis was associated with reduced expression of various genes encoding angiogenic factors implicated in GC, namely Cxcl1, Cxcl2, Vegf, Mmp2 and Mmp9. However, their mRNA levels were not significantly reduced in gp130<sup>F/F</sup>:Asc<sup>−/−</sup> tumors (Supplementary Fig. S3F), suggesting that ASC does not promote tumor angiogenesis in gp130<sup>F/F</sup> mice.

Since ASC is expressed in both the gp130<sup>F/F</sup> gastric tumor epithelium and immune cell-containing stroma, albeit significantly higher in the tumor epithelium (Fig. 3B), we assessed whether ASC-expressing myeloid cells contributed to gastric tumorigenesis by generating reciprocal bone marrow (BM) chimeras between irradiated 8wo gp130<sup>F/F</sup> and gp130<sup>F/F</sup>:Asc<sup>−/−</sup> mice that were subsequently aged. The size of hyperplastic stomachs and tumor mass from 20-24wo gp130<sup>F/F</sup> recipients reconstituted with gp130<sup>F/F</sup>:Asc<sup>−/−</sup> donor BM was comparable to control gp130<sup>F/F</sup> mice reconstituted with autologous gp130<sup>F/F</sup> BM (Fig. 3C and 3D). Similarly, the reciprocal reconstitution of gp130<sup>F/F</sup>:Asc<sup>−/−</sup> recipients with gp130<sup>F/F</sup> BM had no effect on gastric tumor growth (Fig. 3C and 3D). Collectively, these data indicate that ASC-expressing myeloid cells do not promote gastric tumorigenesis.

**Suppressed gastric tumorigenesis in gp130<sup>F/F</sup>:Asc<sup>−/−</sup> mice is characterized by augmented tumor cell apoptosis**

We have previously demonstrated that gastric tumorigenesis in gp130<sup>F/F</sup> mice is associated with a high PCNA proliferative index compared to wild-type mice (24). However, immunostaining of gastric tumor sections from 20-24wo gp130<sup>F/F</sup> and gp130<sup>F/F</sup>:Asc<sup>−/−</sup> mice revealed comparable PCNA<sup>+</sup> cell numbers, indicating that suppressed tumorigenesis in gp130<sup>F/F</sup>:Asc<sup>−/−</sup> mice was not characterized by reduced tumor cell proliferation (Fig. 4A). Similarly, the expression of cell cycle regulatory genes Ccnb1, Ccnd1, Ccnd2, c-myc and Cdc42 was comparable in gp130<sup>F/F</sup>:Asc<sup>−/−</sup> and gp130<sup>F/F</sup> tumors (Supplementary Fig. S3G).
We next investigated whether ASC contributed to increased survival of neoplastic gastric epithelial cells, a cellular process which is associated with gastric tumorigenesis in gp130F/F mice (20). Indeed, TUNEL+ and cleaved caspase-8+ apoptotic cell numbers were significantly increased (~3-fold) in gastric tumors of 20-24wo gp130F/F:Asc−/− compared to gp130F/F mice (Fig. 4B and 4C). Dual immunofluorescence staining for cleaved caspase-3 and the epithelial marker E-cadherin further confirmed a significant increase in the number of apoptotic epithelial cells in gastric tumors of gp130F/F:Asc−/− versus gp130F/F mice (Fig. 4D, Supplementary Fig. S4A).

Since activation of the pro-tumorigenic transcription factor NF-κB within the intestinal epithelium has been linked with suppressing epithelial cell apoptosis in colitis-associated cancer (29), we investigated whether a similar function for NF-κB could be assigned to the gastric epithelium during tumorigenesis. Indeed, in gastric tumors of 20-24wo gp130F/F mice, immunofluorescence indicated both nuclear and cytoplasmic staining for the phosphorylated (Ser536) p65 subunit of NF-κB primarily in the mucosal glandular epithelium, with little to no pNF-κB p65 staining in the immune/inflammatory cell-rich submucosal or lamina propria regions (Supplementary Fig. S4B). Furthermore, in the smaller gp130F/F:Asc−/− tumors displaying increased apoptosis, immunoblotting revealed significantly reduced pNF-κB p65 levels (Supplementary Fig. S4C). In addition, immunohistochemistry confirmed that the reduced phosphorylation (i.e. activation) of NF-κB associated with a significantly lower number of pNF-κB p65-positive cells in the mucosal epithelium, compared to gp130F/F tumors (Fig. 4E). By contrast, immune/inflammatory cell aggregates found within the matching submucosal areas of gp130F/F and gp130F/F:Asc−/− gastric tumors contained comparable low numbers of pNF-κB p65-positive cells (Supplementary Fig. S4D). Collectively, these data suggest that ASC deficiency in the gp130F/F gastric tumor epithelium augments caspase-8-mediated cell death, which correlates with reduced NF-κB activation.
Increased production of IL-18, but not IL-1β is associated with ASC-mediated gastric tumorigenesis in gp130F/F mice

To elucidate a role for the ASC inflammasome effector cytokines IL-1β and/or IL-18 in gastric tumorigenesis, we initially measured IL-1β and IL-18 expression levels in gastric tumors of 10-12wo and 20-24wo gp130F/F mice. Although Il1b mRNA levels were significantly increased by up to ~50-fold in tumor and ~6-fold in adjacent tumor-free gastric antrum tissues of gp130F/F mice compared to normal gastric antrum tissue of gp130+/+ mice (Fig. 5A), immunoblotting indicated that protein levels of pro (31kD) and mature (17kD) forms of IL-1β were only increased (albeit significantly) by up to ~3-fold in gp130F/F tumor and/or tumor-free lysates compared to gp130+/+ gastric antrum lysates, with the highest increase in 20-24wo gp130F/F tumors (Fig. 5B and 5C). By contrast, despite a <3-fold increase in Il18 mRNA levels in gp130F/F gastric tumor and/or non-tumor tissues compared to gp130+/+ antrum tissue (Fig. 5A), immunoblots revealed that the mature 18kD form of IL-18, but not the immature 24kD proIL-18, was specifically upregulated by up to 27-fold in both 10-12wo and 20-24wo gp130F/F tumor and tumor-free tissues (Fig. 5B and 5C).

In light of these findings suggesting increased processing of mature IL-18 protein, we next assessed whether IL-18 specifically acted downstream of ASC to promote gastric tumorigenesis. Indeed, immunoblotting revealed that mature IL-18 protein levels were significantly reduced in tumor lysates from 20-24wo gp130F/F:Asc−/− versus gp130F/F mice, which was not observed for mature IL-1β (Fig. 5D). The reduced processing of IL-18 in gp130F/F:Asc−/− gastric tumor lysates was also confirmed by an ELISA specific for mature IL-18 (Supplementary Fig. S5A). Moreover, gastric hyperplasia and tumor burden were significantly reduced in 20-24wo, but not 10-12wo, gp130F/F:Il18−/− mice lacking IL-18 compared to gp130F/F mice (Fig. 6A-D, Supplementary Fig. S5B-E), which mimicked the
suppressed gastric tumor phenotype of \( gp130^{FF:Asc^{-/-}} \) mice. By contrast, in \( gp130^{FF} \) mice the genetic ablation of IL-1R, leading to blockade of IL-1β (and IL-1α) activity, had no significant impact on tumor burden, as evidenced by comparable tumor mass \((gp130^{FF}, 0.130 \pm 0.017g \text{ versus } gp130^{FF:Il1r^{-/-}}, 0.121 \pm 0.064g)\), tumor incidence and overall stomach size in 20-24wo \( gp130^{FF} \) and \( gp130^{FF:Il1r^{-/-}} \) mice (Supplementary Fig. S5F-K). Since the long-term, daily administration of tumor-bearing \( gp130^{FF} \) mice with the IL-1R antagonist anakinra (high dose, 100mg/kg) also had no effect on tumorigenesis, these observations suggest that IL-1β does not play a major role contributing to tumorigenesis in this model. In addition, treatment of human AGS and MKN1 GC cells with anakinra had no effect on anchorage-dependent and -independent colony formation (Supplementary Fig. S5L).

Similar to \( gp130^{FF:Asc^{-/-}} \) mice, ameliorated gastric tumorigenesis in \( gp130^{FF:Il18^{-/-}} \) mice was associated with significantly elevated numbers of apoptotic TUNEL⁺ (Fig. 6E) and caspase-8⁺ (Fig. 6F) cells, and reduced numbers of cells expressing activated NF-κB (Supplementary Fig. S6A), within the mucosal epithelium. By contrast, we observed no changes in the level of proliferation, infiltration of inflammatory cells, or expression of cell cycle, angiogenic and inflammatory genes (Supplementary Fig. S6B-D and S7A-C), in the tumor epithelium. Collectively, these findings support a pro-tumorigenic role for ASC in GC that is mediated largely by IL-18.

**Elevated gastric epithelial IL-18 expression augments GC cell growth**

Consistent with elevated \( Asc \) gene expression in the gastric epithelium of \( gp130^{FF} \) mice (Fig. 3B), mRNA levels for \( Il18 \) or its \( Il18r1 \) receptor gene were significantly increased in EpCam⁺ epithelial compared to CD45⁺ immune (or stroma) cells in \( gp130^{FF} \) gastric non-tumor or tumor tissues (Fig. 7A, Supplementary Fig. S7D). By contrast, while \( Il1r \) expression levels were generally unchanged in the gastric epithelial and immune compartments, \( Il1b \) was
lowly-expressed in epithelial versus immune cells or stroma in \(gp130^{F/F}\) gastric tumors (Fig. 7A, Supplementary Fig. S7D). In support of these findings, ELISA confirmed that total and mature IL-18 protein levels were significantly increased in tumor compared to matched non-tumor tissue lysates from GC patients, with total IL-18 levels higher than those for total IL-1\(\beta\) (Fig. 7B). Importantly, the elevated levels of IL-18, but not IL-1\(\beta\), positively correlated with increased ASC mRNA levels in GC patient tumors (Fig. 7C). Furthermore, secreted (i.e. mature) IL-18 protein was detected by ELISA in human GC cell line supernatants, whereas as expected, secreted IL-1\(\beta\) protein was undetectable due to the absence of steady-state cellular proIL-1\(\beta\) levels (Fig. 7D).

The therapeutic utility of targeting IL-18 in human GC was supported by the observation that treatment of AGS and MKN1 human GC cells with an IL-18 neutralizing antibody suppressed anchorage-dependent and -independent colony formation, and augmented apoptosis (Fig. 7E-G). Furthermore, CRISPR/Cas9-mediated ASC ablation in AGS cells suppressed colony formation which coincided with reduced secretion of mature IL-18 (but not IL-1\(\beta\)) and activation of caspase-1 and NF-\(\kappa\)B, and conversely elevated apoptosis (Fig. 7H-K). Notably, these findings mimic those observed upon CRISPR/Cas9-mediated knock-out of caspase-1 in AGS cells (Supplementary Fig. S8A-C), and therefore support the notion that elevated IL-18 production (downstream of ASC and caspase-1) in human GC cells promotes cell-autonomous growth, which can be readily targeted therapeutically.
Discussion

Over recent years, studies largely restricted to melanoma, skin and colon carcinogenesis have identified complex and contrasting tumor-promoter and tumor-suppressor roles for the inflammasome adaptor ASC, which reveal a functional dependency by ASC-associated inflammasomes on cell type (i.e. myeloid versus epithelial), tissue specificity and disease stage (19). For instance, in experimentally-induced skin carcinogenesis, myeloid-derived ASC expression favours tumorigenesis, whereas ASC expression in keratinocytes negates tumor formation (30,31). In addition, modulating ASC expression in primary and metastatic human melanoma demonstrated opposing anti- and pro-tumorigenic functions, respectively, for ASC, indicating that the role of ASC in cancer can depend on disease stage (32). With respect to the gut, ASC-deficient mice are more susceptible to azoxymethane (AOM)/dextran sodium sulfate (DSS)-induced colitis-associated carcinogenesis (CAC), thus supporting an anti-tumorigenic role for ASC (33). By contrast, a pro-tumorigenic role for ASC in colorectal cancer (CRC) is suggested in AhR-/ and ApcMin/+ spontaneous CRC models, whereby ASC deficiency ameliorates tumor formation (34).

Here, in GC we reveal a pro-tumorigenic role for ASC, whose elevated expression was observed in tumors of ~75% of intestinal-type GC patients, that is independent of gastric inflammation. Specifically, we demonstrate that suppressed gastric tumorigenesis in gp130F/F:Asc-/- mice is associated with reduced caspase-1 activation and elevated numbers of caspase-3- and caspase-8-expressing cells within the tumor epithelium, and thus uncover a hitherto unknown function for ASC inflammasomes in eliciting an anti-apoptotic response in neoplastic gastric epithelial cells involving caspases-3 and -8. Furthermore, our discovery that ASC ablation in gastric epithelial (cancer) cells suppresses their growth potential which correlates with reduced NF-κB activation (and IL-18 processing/production), along with elevated apoptosis, suggests a role for NF-κB as a signaling facilitator of the anti-apoptotic/pro-
survival function for ASC in the gastric (tumor) epithelium. Indeed, this notion is consistent with the emergence that NF-κB promotes the initiation and/or progression of numerous epithelial cancers, as evidenced by the observation that during CRC, in which ASC has been assigned a pro-tumorigenic role (34), NF-κB activation in the intestinal epithelium promotes tumorigenesis by suppressing apoptosis (29). Therefore, in the context of GC, our findings invoke the existence of potential signaling cross-talk between activation of the canonical ASC inflammasome (via NF-κB) and caspase-8 apoptotic machinery to dampen the latter, thus augmenting tumor growth. Furthermore, our observations revealing that the predominate NF-κB signal is localised to epithelial and not immune cells within gastric tumors is consistent with our proposed tumor (epithelial) cell-autonomous anti-apoptotic role for the ASC inflammasome/IL-18 axis, and thus provide an explanation (at least in part) for why inflammation is not affected in the \(gp130^{F/F}::Asc^{-/-}\) tumors.

Our current findings also expand upon the traditional role of ASC that has been linked to cell death facilitated by canonical inflammasome-mediated pyroptosis via caspase-1, or more recently the formation of a non-canonical, apoptosis-inducing ASC apoptosome (with AIM2 and NLRP3) via interaction with caspase-8, independent of inflammatory-related caspase-1 activation (35-37). Regarding the former, it has recently emerged that ASC inflammasome/caspase-1-dependent cleavage and activation of the pore-forming effector protein, gasdermin D, is a critical event in pyroptosis (38). Although the pathophysiological role of gasdermin D-mediated pyroptosis, including in the context of cancer, is ill-defined, our data presented here suggest that the novel anti-apoptotic arm of the ASC inflammasome/caspase-1 axis in gastric epithelial cells which protects against caspase-8-mediated cell death occurs independent of gasdermin D processing by the ASC inflammasome. Considering that the mechanistic basis governing this dual mode of ASC-mediated cell death (i.e. apoptosis and pyroptosis) remains to be fully elucidated, our notion regarding the
independent role of gasdermin D in GC (and potentially other cancers) warrants further investigation.

We also note that the above-mentioned pro-apoptotic and pro-pyroptotic functions previously assigned to ASC most likely explain its tumor suppressor actions in certain cancers, which have also been linked to methylation-induced silencing of ASC (39). In this respect, a recent study indicated that ASC is methylated in approximately one-third of GC cases, although the histologic subtype (i.e. intestinal or diffuse) involved and the effect on ASC expression in the tumors analyzed were not reported (40).

Another key finding of our study was the identification of IL-18, but not IL-1β, as a major downstream inflammasome effector cytokine which propagates the growth potential of GC cells. Previous studies have shown that stomach-specific over-expression of human IL-1β in transgenic mice induces gastric inflammation-associated carcinogenesis via the recruitment of MDSCs (7), and in human GC elevated gastric IL-1β production is associated with inflammation (41). Notably, our demonstration that IL-18, like ASC, promotes tumor growth independent of inflammation in \(gp130^{FF}\) mice contrasts these findings for IL-1β. Here, in \(gp130^{FF}\) gastric tumors we show that gene expression for IL-18 and its IL-18R1 receptor, as well as for ASC, is elevated in epithelial compared to inflammatory/immune cells, which contrasts the low and high gene expression levels for IL-1β in epithelial and inflammatory cells, respectively. Together with the high basal production of IL-18 in supernatants of human GC cell lines, along with the growth inhibitory effect of a neutralizing IL-18 monoclonal antibody on human GC cells, our findings support a direct, cell-autonomous effect of IL-18 (downstream of ASC) on GC cells. We therefore propose that the functional dichotomy between IL-18 and IL-1β in GC can be explained by, at least in part, the preferential requirement of epithelial (tumor) cells for IL-18 which directly acts to promote growth of the tumor epithelium, whereas
immune cells predominantly utilize IL-1β to provide a pro-inflammatory microenvironment which can support tumorigenesis.

This notion could also account for our previous observation in \textit{gp130}^{F/F} mice that genetic ablation of the MyD88 adaptor protein, which is crucial for IL-18R signaling, also suppresses growth of the gastric tumor epithelium independent of inflammation (42). Furthermore, in another GC mouse model, Gan, in which inflammation drives gastric tumorigenesis, the ablation of MyD88 in bone marrow-derived immune cells (thus disrupting IL-1β/IL-1R signaling which also requires MyD88) suppressed the tumor-promoting inflammatory microenvironment (43). It is therefore perhaps not surprising that this latter observation contrasts our current bone marrow chimera data in \textit{gp130}^{F/F} mice whereby myeloid cells expressing low levels of IL-18 (and ASC) do not promote gastric tumorigenesis, suggesting that at least this cellular component of the tumor microenvironment does not influence apoptosis and tumor growth in the \textit{gp130}^{F/F} GC model.

While we reveal here a key role for IL-18 as a downstream effector of ASC inflammasomes in GC, our study also raises the intriguing question concerning the identity of the specific PRR(s), as well as the source and nature of its agonist(s) (e.g. microbial- and/or host-derived), that comprises the pro-tumorigenic ASC inflammasome? In the current absence of definitive \textit{in vivo} evidence to answer this question, which for instance requires genetic ablation of specific ASC inflammasome-associated PRRs (e.g. AIM2, NLCR4, NLRP1, NLRP3) in our \textit{gp130}^{F/F} GC model, we refer to our \textit{in vitro} data demonstrating reduced levels of secreted (mature) IL-18 and activated caspase-1 proteins upon CRISPR/Cas9-mediated knock-out of ASC in cultured human GC cells (Fig. 7J). These observations are suggestive of constitutive ASC inflammasome activation driven by the cell intrinsic production and release of inflammasome-activating agonists during culture. Interestingly, cultured human cancer cell lines constitutively release host-derived damage-associated molecular patterns (DAMPs), for
example DNA and HMGB1 (44,45), which can activate the AIM2 and NLRP3 inflammasomes, respectively (46,47). Therefore, it is tempting to speculate that cultured human GC cell lines, and by analogy the gastric tumor epithelium, also have the potential to constitutively release such host-derived DAMPs which promote cellular growth via their specific PRR-associated inflammasome.

It is also worth considering our findings with previous investigations into the role of IL-18 via inflammasome activation in gastrointestinal cancers, which have been restricted to the AOM/DSS-induced CAC model in concert with either Il18−/− mice or the administration of IL-18 to inflammasome-deficient (Casp1−/−) mice. These studies indicate a protective role for IL-18 in the initiating stages of CAC, attributed to high IL-18 expression levels in the intestinal epithelium which maintain barrier integrity through epithelial cell proliferation and survival (48,49). Thus, while this growth-potentiating effect of IL-18 on the intestinal epithelium protects against colonic tumorigenesis, our current findings indicate that, conversely, in the gastric epithelium IL-18 suppresses apoptosis to promote gastric tumorigenesis. In this regard, our current study reveals tissue-specific activities of IL-18, thus supporting the pleiotropic actions previously ascribed to IL-18 on the migration and proliferation of human GC cell lines (9,12,13).

In summary, our current study suggests the existence of a novel pro-tumorigenic ASC/IL-18/NF-κB signaling axis which augments gastric epithelial cell survival in GC. Despite the recent development of numerous small-molecule inhibitors against individual ASC inflammasome components, namely NLRP3, NLRP1, NLRC4 and AIM2 (50), their specificity and efficacy as anti-cancer agents in vivo will require rigorous evaluation in numerous cancer models which take into account the tissue-specific and cell-type contexts which define the multifaceted activities of ASC in cancer. Furthermore, in GC, the potential application of such inhibitors will be influenced by the future identification of specific PRRs which comprise the
disease-associated ASC inflammasome. In light of our findings, and given the current lack of preclinical-validated inhibitors which can block the actions of ASC, therapies which neutralize the biological activity of IL-18 may serve as effective weapons against GC characterized by dysregulated ASC inflammasome-driven IL-18 production.
Acknowledgments

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References


**Figure Legends**

**Figure 1.** Suppressed gastric tumorigenesis in $gp130^{F/F}:Asc^{-/-}$ mice. (A) qPCR expression of $ASC$ (relative to $18S$ rRNA) in gastric tumor (T) and matched, adjacent non-tumor (NT) tissue from 10 Chinese GC patients. ***$P < 0.001$; unpaired t-test. (B) $ASC$ gene expression in T and NT tissues (left graph), and in each T tissue relative to matched NT tissue (right graph), from 18 TCGA GC patients. *$P < 0.05$; unpaired/paired t-tests. (C) Representative 20-24wo $gp130^{F/F}$ (F/F) and $gp130^{F/F}:Asc^{-/-}$ (F/F:Asc-/-) mouse stomachs. Arrows indicate macroscopically visible tumors. Fundic (f), body (b) and antral (a) stomach regions are depicted. (D) Scatter plots depicting total mass (grams; g) of stomachs and gastric tumors, and incidence of tumors in total and by size, from 20-24wo mice. Data are expressed as the mean ± SEM. *$P < 0.05$, ***$P < 0.01$, ****$P < 0.0001$; unpaired t-test. (E) Representative photomicrographs showing H&E-stained whole stomach cross-sections from 20-24wo mice. Tumors are depicted by dotted squares. Scale bars: 1mm. (F) Left panels: representative H&E-stained tumor cross-sections from 20-24wo F/F and F/F:Asc-/- mice. Arrows point to inflammatory cell accumulates. Scale bars: 100µm. Right panels; magnified submucosa areas demonstrating the presence of plasma (P) and lymphocyte (L) inflammatory cells. Scale bars: 50µm. Graph depicts inflammatory scores (0-3; none, mild, moderate, severe) from 6 mice/genotype. Shown in (C) and (E) is 1 from 15-25 representative stomach image/genotype, and in (F) 1 from 6 representative image/genotype.

**Figure 2.** Genetic disruption of ASC does not suppress gastric inflammation in $gp130^{F/F}$ mice. (A) Representative CD45-stained gastric antral tumor cross-sections from 20-24wo $gp130^{F/F}$ (F/F) and $gp130^{F/F}:Asc^{-/-}$ (F/F:Asc-/-) mice (1 from 8 representative image/genotype). Scale bars: 100µm. (B) Quantitative enumeration (mean ± SEM) of CD45-positive cells/high-power field (HPF) in gastric tumor mucosa of 8 mice/genotype. (C) Frequencies of cell populations,
presented as the mean ± SEM, in F/F and F/F:Asc−/− 20-24wo mouse stomachs (6 mice/genotype) as determined by flow cytometry.

**Figure 3.** Suppressed gastric tumorigenesis in gp130<sup>F/F</sup>:Asc<sup>−/−</sup> mice is independent of inflammation and hematopoietic-derived myeloid cells. (A) qPCR expression analyses of inflammatory genes in gastric tumors of 20-24wo gp130<sup>F/F</sup> (F/F) and gp130<sup>F/F</sup>:Asc<sup>−/−</sup> (F/F:Asc<sup>−/−</sup>) mice (8 mice/genotype). Expression data are normalized for 18S rRNA, and are presented from experimental triplicates as the mean ± SEM. (B) qPCR expression analyses of the indicated genes in captured laser microdissected gastric tumor epithelial (Epi) and stroma (Strom) tissue from 20-24 week old F/F mice. Expression data from 5 samples/genotype are shown following normalization for 18S rRNA, and are presented from technical triplicates as the mean ± SEM. **P < 0.05; unpaired t-test. (C) Scatter plot, presented as the mean ± SEM, depicting the total mass (grams; g) of mouse gastric tumors. *P < 0.05, **P < 0.01; one-way ANOVA followed by Tukey’s multiple comparisons test. (D) Representative stomachs from 20-24wo recipient F/F mice reconstituted with autologous F/F (F/F/F/F) or heterologous gp130<sup>F/F</sup>:Asc<sup>−/−</sup> (F/F:F/F:Asc) mouse bone marrow, and recipient F/F:Asc mice reconstituted with autologous F/F:Asc (F/F:Asc<sup>F/F:Asc</sup>) or heterologous F/F (F/F:Asc<sup>F/F</sup>) bone marrow (shown is 1 from 6 representative stomach image/group). Fundic (f), body (b) and antral (a) stomach regions are depicted.

**Figure 4.** ASC promotes gastric tumor cell survival in gp130<sup>F/F</sup> mice. (A) Representative photomicrographs showing PCNA-stained gastric antral tumor cross-sections from 20-24wo gp130<sup>F/F</sup> (F/F) and gp130<sup>F/F</sup>:Asc<sup>−/−</sup> (F/F:Asc<sup>−/−</sup>) mice. Scale bars: 50µm. Graph depicts quantitative enumeration of PCNA-positive cells/high-power field (HPF) in gastric tumor mucosa from mice. (B) Representative photomicrographs of TUNEL-stained antral gastric
tumor cross-sections, along with graph depicting quantitative enumeration of TUNEL-positive cells/20 HPF in gastric tumor mucosa, of mice. TUNEL-positive cells are depicted by arrows. Scale bars: 50µm. (C) Representative photomicrographs of active caspase-8-immunostained antral gastric tumor cross-sections, along with graph depicting quantitative enumeration of active caspase-8-positive cells/20 HPF in gastric tumor mucosa, of mice. Arrows point to active caspase-8-positive cells. Scale bars: 20µm. (D) Representative confocal immunofluorescence photomicrographs of cells stained for cleaved caspase-3 (green), E-cadherin (red, epithelial cell marker) and the nuclear marker 4',6-diamidino-2-phenylindole (DAPI; blue) in cross-sections of 20-24wo F/F and F/F:Asc<sup>−/−</sup> gastric tumors. White circles indicate dual-labelled E-cadherin and cleaved caspase-3 cells. Scale bars: 40µm. Graph depicts stereological quantification of dual-labelled caspase-3/E-cadherin-positive cells in gastric tumors of 20-24wo mice. (E) Representative pNF-κB p65-stained cross-sections through the antral tumor mucosal region of mouse stomachs. Scale bars: 50µm. Graph depicting the percentage of pNF-κB p65 positive cells/area in the gastric tumor mucosa from mice of the indicated genotypes. In A-E, shown is 1 from 8 representative stomach image/genotype, and data in graphs are presented as the mean ± SEM from 8 mice/genotype. **P < 0.01, ***P < 0.001, ****P < 0.0001; unpaired t-test.

**Figure 5.** Upregulated IL-18 production and caspase-1 activation in gp130<sup>F/F</sup> mouse gastric tumors. (A) In antral gastric tissue from 10-12wo and 20-24wo gp130<sup>+/+</sup> (+/+), tumor (T) and non-tumor (NT) tissue from age-matched gp130<sup>F/F</sup> (F/F) mice, shown are Il1b and Il18 mRNA levels by qPCR. Gene expression data are normalized to 18S rRNA, and are presented from technical triplicates as the mean ± SEM. n = 5 samples/group. *P < 0.05, **P < 0.01; unpaired t-test. (B and C) Immunoblots of (B) 10-12wo and (C) 20-24wo +/+, F/F<sup>NT</sup> and F/F<sup>T</sup> gastric antral tissue lysates with anti-IL-18 and anti-IL-1β antibodies detecting both pro/mature forms (24/18kD for IL-18, 31/17kD for IL-1β). Each lane represents an individual mouse.
Protein loading was assessed using α-Tubulin antibody. Graphs depict densitometric quantification of immunoblots from individual gastric tumor tissue lysates (6 mice/genotype) showing pro and mature IL-18 and IL-1β levels relative to α-Tubulin. \( *P < 0.05, \**P < 0.01, \***P < 0.001; \) unpaired t-test. (D) Immunoblots of 20-24wo F/F and F/F:Asc\(^{-/-}\) gastric tumor tissue lysates with antibodies against pro/mature IL-18 and IL-1β, as well as α-Tubulin. Each lane represents an individual mouse. Graph depicts densitometric quantification of immunoblots from individual gastric tumor tissue lysates (6 mice/genotype) showing pro and mature IL-18 and IL-1β levels relative to α-Tubulin. \( *P < 0.05; \) unpaired t-test.

**Figure 6.** IL-18 promotes ASC-mediated gastric tumorigenesis in \( gp130^{F/F} \) mice. (A) Representative stomachs from 20-24wo (F/F) and \( gp130^{F/F}:Il18^{-/-} \) (F/F:Il18\(^{-/-}\)) mice. Arrows indicate macroscopically visible tumors. Fundic (f), body (b) and antral (a) regions are depicted. (B and C) Scatter plots depicting total mass (grams; g) of gastric tumors (B), and incidence of tumors in total (B) and by size (C), from 20-24wo mice. Data are expressed as the mean ± SEM. \( *P < 0.05, \**P < 0.01, \***P < 0.001; \) unpaired t-test. (D) Representative photomicrographs showing H&E-stained whole stomach cross-sections from 20-24wo mice. Dotted squares depict tumors. Scale bars: 1mm. In (A) and (D), shown is 1 from 7 representative stomach image/genotype. (E and F) Representative photomicrographs of TUNEL-stained (E) and caspase-8-stained (F) antral gastric tumor cross-sections of 20-24wo F/F and F/F:II18\(^{-/-}\) mice (shown is 1 from 6 representative image/genotype). TUNEL-positive and caspase-8-positive cells are indicated by arrows. Scale bars: 50\( \mu \)m (E) and 20\( \mu \)m (F). Graphs depict quantitative enumeration, presented as the mean ± SEM, of TUNEL-positive and caspase-8-positive cells/20 high-power fields (HPF) in gastric tumor mucosa of mice (6/genotype). \( \**P < 0.01; \) unpaired t-test.
Figure 7. Augmented epithelial cell IL-18 expression promotes GC cell growth. (A) qPCR gene expression analysis (mean ± SEM) in sorted CD45-positive (immune) or EpCam-positive (epithelial) cells isolated from at least 8 gpi30F/F gastric tumor (T) and non-tumor (NT) tissues. *P < 0.05, **P < 0.01; unpaired t-test. (B) ELISAs for total and mature IL-18 and total IL-1β proteins in gastric tumor and matched non-tumor tissue lysates from 10-15 GC patients are presented as the mean ± SEM. *P < 0.05; unpaired t-test. (C) Linear regression of ASC mRNA and mature IL-18 and IL-1β protein levels in GC patient tumors. R = Pearson’s correlation coefficient. (D) ELISA for secreted IL-18 protein in human GC cell line supernatants (24 hour culture). (E and I) Flow cytometry of apoptotic Annexin-V-positive human AGS cells (E) treated with/without anti-hIL-18 monoclonal antibody (mAb) or (I) transduced with non-targeted control sgRNA (Ctl) and ASC sgRNA (KO). *P < 0.05, **P < 0.01; unpaired t-test. (F-H) Representative images (1 of 6/group) showing colony formation of (F) AGS and (G) MKN1 cells treated with anti-hIL18 mAb, and (H) AGS Ctl and ASC KO cells. Graphs depict colony number/well (6 wells/group) expressed as the mean ± SEM. **P < 0.01, ****P < 0.0001; unpaired t-test. In (E-I), graphs show data (n = 6 experiments/group) that are presented as the mean ± SEM. (J and K) Immunoblots with the indicated antibodies on (J) cell culture supernatants and (J and K) cell lysates from AGS Ctl and ASC KO cells cultured for 24 hours. Shown are 2 independent ASC KO clones.
Supplementary Data Guide

Inflammasome adaptor ASC suppresses apoptosis of gastric cancer cells by an IL-18 mediated inflammation-independent mechanism

Virginie Deswaerte, Paul Nguyen, Alison West, Alison F. Browning, Liang Yu, Saleela M. Ruwanpura, Jesse Balic, Thaleia Livis, Charlotte Girard, Adele Preaudet, Hiroko Oshima, Ka Yee Fung, Hazel Tye, Meri Najdovska, Matthias Ernst, Masanobu Oshima, Cem Gabay, Tracy Putoczki, Brendan J. Jenkins.

Supplementary Table S1. Clinicopathological features and demographics of gastric cancer patients used for expression profiling of inflammasome-related components.

Supplementary Figure S1. Gastric tumor formation in unaltered in 10-12 and 30-34 week old gp130FF mice lacking ASC.

Supplementary Figure S2. Genetic disruption of ASC in gp130FF mice suppresses activation of caspase-1.

Supplementary Figure S3. Genetic disruption of ASC does not suppress gastric inflammation in gp130FF mice, nor expression of angiogenic or cell cycle genes.

Supplementary Figure S4. Elevated apoptotic epithelial cell numbers and reduced NF-κB activation in gastric tumors of gp130FF mice lacking ASC.

Supplementary Figure S5. Gastric tumor formation is unaltered in 10-12 week old gp130FF mice lacking IL-18, or upon genetic disruption of the IL-1R in gp130FF mice.

Supplementary Figure S6. Decrease of apoptotic and pNF-κB-expressing cell numbers,
but no change in proliferation or infiltration of inflammatory cells, in the tumor epithelium of $gp130^{F/F}$ mice lacking IL-18.

**Supplementary Figure S7.** Genetic disruption of IL-18 in $gp130^{F/F}$ mice, whose augmented gene expression is observed in the gastric tumor epithelium of $gp130^{F/F}$ mice, does not interfere with the expression of cell cycle, angiogenesis or inflammatory genes.

**Supplementary Figure S8.** Caspase-1 preferentially processes mature IL-18 production and promotes growth in human gastric cancer cells.
Supplementary Table S1. Clinicopathological features and demographics of gastric cancer patients used for expression profiling of inflammasome-related components.

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\(^1\)TCGA, The Cancer Genome Atlas
**Supplementary Figure S1.** Gastric tumor formation in 10-12 and 30-34 week old gp130\(^{F/F}\) mice lacking ASC. (A) Representative appearance of stomachs from 10-12 week old (wo) gp130\(^{F/F}\) (F/F) and gp130\(^{F/F}\):Asc\(^{+/−}\) (F/F:Asc\(^{+/−}\)) mice (shown is 1 out of 12 representative stomach image/genotype). Arrows indicate macroscopically visible tumors. Fundic (f), body (b) and antral (a) stomach regions are depicted. (B-E) Scatter plots depicting the total mass (grams; g) of stomachs (B) and gastric tumors (C), as well as the incidence of tumors by size (D) and in total (E), from F/F and F/F:Asc\(^{+/−}\) 10-12wo mice. (F-J) Same layout of data as in (A-E), except for 30-34wo F/F and F/F:Asc\(^{+/−}\) mice. Data for each genotype are expressed as the mean ± SEM. **P < 0.01, ***P < 0.001; unpaired t-test.
Supplementary Figure S2. Genetic disruption of ASC in gp130\textsuperscript{F/F} mice suppresses activation of caspase-1. (A) Immunoblots and quantification graphs (n = 5/genotype) of gastric antral tissue lysates from 20-24 week old (wo) gp130\textsuperscript{+/+} (+/+ and gp130\textsuperscript{F/F} (F/F) mice with the indicated antibodies. NT, non-tumor; T, tumor. (B) Immunoblots and quantification graphs (n = 6/genotype) of gastric tumor lysates from 20-24wo F/F and gp130\textsuperscript{F/F}:Asc\textsuperscript{-/-} (F/F:Asc\textsuperscript{-/-}) mice with the indicated antibodies. In both (A) and (B), each lane represents an individual mouse sample. Data for each genotype are expressed as the mean ± SEM. *P < 0.05, **P < 0.01; unpaired t-test.
Supplementary Figure S3. Genetic disruption of ASC does not suppress gastric inflammation in \textit{gp130}\textsuperscript{F/F} mice, nor expression of angiogenic or cell cycle genes. (A) Representative flow cytometry plots showing the proportion of cells positive for CD4/CD8, B220/CD19 and CD11b/F4/80 in the stomachs of \textit{gp130}\textsuperscript{F/F} (F/F) and \textit{gp130}\textsuperscript{F/F:Asc}\textsuperscript{-/-} (F/F:Asc\textsuperscript{-/-}) 20-24 week old (wo) mice (shown is 1 out of 6 representative plot/genotype). Numbers indicate frequency of the gated population. (B-D) Representative B220-stained (B), CD3-stained (C) and CD68-stained (D) cross-sections through the antral tumor region of 20-24wo F/F and F/F:Asc\textsuperscript{-/-} mouse stomachs (shown is 1 out of 8 representative image/genotype). Positive cells are depicted by arrows. Scale bars: 50µm. Graphs depict quantitative enumeration of positive cells per high power (40x) field (HPF) in the gastric tumor mucosa from the indicated mice. Data are presented as the mean ± SEM from n = 8 mice/genotype. (E) Graphs depicting the frequencies of the indicated cell populations in
the perigastric lymph nodes of F/F and F/F:Asc−/− 20-24wo mice (n = 4/genotype) as determined by flow cytometry. Data are presented as the mean ± SEM. (F and G) qPCR expression analyses of angiogenic genes (F) and cell cycle genes (G) in antral gastric tumor tissue of 20-24wo F/F or F/F:Asc−/− mice (n = 8/genotype). Expression data are shown following normalization for 18S rRNA, and are presented from experimental triplicates as the mean ± SEM. *P < 0.05, **P < 0.01; unpaired t-test.
Supplementary Figure S4. Elevated apoptotic epithelial cell numbers and reduced NF-κB activation in gastric tumors of gp130<sup>F/F</sup> mice lacking ASC. (A) Representative confocal
immunofluorescence photomicrographs of cells stained for cleaved caspase-3 (green), E-cadherin (red, epithelial cell marker) or the nuclear marker 4',6-diamidino-2-phenylindole (DAPI; blue) alone in cross-sections of gastric tumor tissues from 6 month old (mo) gp130^{F/F} (F/F) and gp130^{F/F;Asc^-/-(F/F;Asc^-/-(mice. Scale bars: 40μm. (B) Representative confocal immunofluorescence photomicrographs of cells stained for cleaved E-cadherin (green; upper panels), CD45 (green, immune cell marker, lower panels), pNF-κB p65 (red) or DAPI (blue) either alone or merged in cross-sections of gastric tumor mucosal (upper panels) and submucosal (lower panels) regions from 6mo F/F mice. Scale bars: 40μm for low power images, and 25μm for high power images (second merged image from the left). (C) Immunoblots and quantification graph (n = 8/genotype) of gastric tumor lysates from 20-24wo F/F and F/F;Asc^-/-(mice with the indicated antibodies. Each lane represents an individual mouse sample. ***P < 0.001; unpaired t-test. (D) Representative pNF-κB p65-stained cross-sections through the submucosal region of tumor-bearing 20-24wo F/F and F/F;Asc^-/-(mouse stomachs (shown is 1 out of 6 representative image/genotype). Scale bars: 50μm. Graph depicting the percentage of pNF-κB p65 positive cells/area in the gastric tumor mucosa from mice of the indicated genotypes. Data for each genotype (n = 6) are expressed as the mean ± SEM. **P < 0.01; unpaired t-test.
**Supplementary Figure S5.** Gastric tumor formation is unaltered in 10-12 week old \(gp130^{F/F}\) mice lacking IL-18, or upon genetic disruption of the IL-1R in \(gp130^{F/F}\) mice. (A) ELISA for mature IL-18 protein in antral gastric tissue from 20-24wo \(gp130^{+/+}\) (+/+) mice, and tumor (T) and non-tumor (NT) tissue from age-matched \(gp130^{F/F}\) (F/F) or \(gp130^{F/F:Asc^{-/-}}\) (F/F:Asc^{-/-}) mice. Data are presented as the mean ± SEM (n = 4-6 mice per group). *\(P < 0.05\); unpaired t-test. (B) Representative appearance of stomachs from 10-12 week old (wo) \(gp130^{F/F}\) (F/F) and \(gp130^{F/F:Il18^{-/-}}\) (F/F:Il18^{-/-}) mice (shown is 1 out of 11-20 representative stomach image/genotype). Arrows indicate macroscopically visible tumors. Fundic (f), body (b) and antral (a) stomach regions are depicted. (C and D) Scatter plots depicting the total mass (grams; g) of gastric tumors (C), and the total incidence of tumors (D), from F/F and F/F:II18^{-/-} 10-12wo mice. Data for each genotype are expressed as the mean ± SEM. (E) Representative photomicrographs showing H&E-stained whole stomach cross-sections from 10-12wo mice of the indicated genotypes (shown is 1 out of 11-20 representative stomach image/genotype). Adenomatous polyps (tumors) are depicted by the dotted square. Scale bars: 1mm. (F and I) Representative appearance of stomachs from 10-12wo (F) and 20-24wo (I) \(gp130^{F/F}\) (F/F) and \(gp130^{F/F:II1r^{-/-}}\) (F/F:II1r^{-/-}) mice (shown is 1 out of 7-21 (10-12wo) and 1 out of 5-15 (20-24wo) representative stomach image/genotype). Arrows indicate macroscopically visible tumors. Fundic (f), body (b) and antral (a) stomach regions are depicted. (G and J) Scatter plots depicting the total incidence of tumors from F/F and F/F:II1r^{-/-} 10-12wo (G) and 20-24wo (J) mice. Data for each genotype are expressed as the mean ± SEM. (H and K) Representative photomicrographs showing H&E-stained whole stomach cross-sections from 10-12wo (H) and 20-24wo (K) mice of the indicated genotypes (shown is 1 out of 7-21 (10-12wo) and 1 out of 5-15 (20-24wo).
representative stomach image/genotype). Adenomatous polyps (tumors) are depicted by
the dotted squares. Scale bars: 1mm. (L) Anakinra treatment of human gastric cancer cells
has no effect on colony formation. Representative images showing colony formation of
human MKN1 and AGS cells treated with anakinra (shown is 1 out of 4-6 representative
wells/treatment group per experiment). The number of colonies/well are expressed as the
mean ± SEM from 3 separate experiments.
Supplementary Figure S6. Decrease of apoptotic and pNF-κB-expressing cell numbers, but no change in proliferation or infiltration of inflammatory cells, in the tumor epithelium of \( gp130^{FF} \) mice lacking IL-18. (A) Representative pNF-κB p65-stained cross-sections through the antral tumor (upper panels) and submucosal (lower panels) regions of 20-24 week old (wo) \( gp130^{FF} \) (F/F) and \( gp130^{FF}:Il18^{-/-} \) (F/F:Il18^{-/-}) mouse stomachs (shown is 1 out of 6 representative image/genotype). Scale bars: 50µm. Graph depicting the percentage of pNF-κB p65-positive cells/area in the gastric tumor mucosa from mice of the indicated genotypes. (B) Representative photomicrographs showing PCNA-stained cross-sections through the antral tumor-bearing region of stomachs from 20-24wo F/F and F/F:Il18^{-/-} mice. Scale bars: 50µm. Graph depicting quantitative enumeration of PCNA-positive cells per high power (40×) field (HPF) in the gastric tumor mucosa from mice. (C) Left panels: representative H&E-stained cross-sections through the antral tumor region of 20-24wo F/F and F/F:Il18^{-/-} mouse stomachs. Scale bar: 50µm. Right panels; magnified areas demonstrating the presence of inflammatory cell infiltrates in the mucosal epithelium. Scale bar: 20µm. Graph depicting inflammatory scores, based on 0-3 (none, mild, moderate, severe) scoring, from \( n = 6 \) mice/genotype. (D) Representative CD45-stained cross-sections through the antral tumor region of 20-24wo F/F and F/F:Il18^{-/-} mouse stomachs. Scale bar: 50µm. Graph depicting quantitative enumeration of CD45-positive cells per mm\(^2\) area in the gastric tumor mucosa from mice of the indicated genotypes. In (A-D), shown is 1 out of 6 representative image/genotype, and the data in graphs for each genotype (\( n = 6 \)) are expressed as the mean ± SEM. *\( P < 0.05 \); unpaired t-test.
Supplementary Figure S7. Genetic disruption of IL-18, whose augmented gene expression is observed in the gastric tumor epithelium of gp130^{F/F} mice, does not interfere with the expression of cell cycle, angiogenesis or inflammatory genes. (A-C) qPCR expression analyses of the indicated inflammatory (A), cell cycle (B) and angiogenesis (C) genes in antral gastric tumor tissue of 20-24 week old (wo) gp130^{F/F} (F/F) or gp130^{F/F}:Il18^{-/-} (F/F:Il18^{-/-}) mice (n = 5/genotype). Expression data are shown following normalization for 18S rRNA, and are presented from experimental triplicates as the mean ± SEM. (D) qPCR expression analyses of the indicated genes in captured laser microdissected gastric tumor epithelial (Epi) and stroma (Strom) tissue from 20-24wo F/F mice. Expression data from 5 samples/genotype are shown following normalization for 18S rRNA, and are presented from technical triplicates as the mean ± SEM. *P < 0.05; unpaired t-test.
Supplementary Figure S8. Caspase-1 preferentially processes mature IL-18 production and promotes growth in human gastric cancer cells. (A) Representative images (1 of 6/group) showing colony formation of human AGS cells transduced with non-targeted control sgRNA (Ctl) and caspase-1 sgRNA (KO). Graph depicts colony number/well (6 wells/group) expressed as the mean ± SEM. ****P < 0.0001; unpaired t-test. (B) Flow cytometry of apoptotic Annexin-V-positive AGS Ctl and caspase-1 KO cells cultured for 24 hours. *P < 0.05; unpaired t-test. (C) Immunoblots with the indicated antibodies on cell culture supernatants and cell lysates from AGS Ctl and caspase-1 KO cells cultured for 24 hours.