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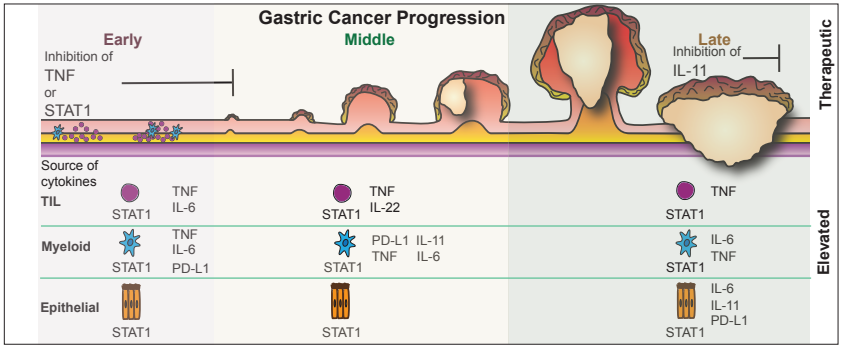
Gastric Cancer Progression
Early
Middle
Late
Inhibition of
TNF
or
STAT1
Inhibition of
IL-11
Therapeutic
Elevated
Source of cytokines
TIL
Myeloid
Epithelial
STAT1
TNF
IL-6
TNF
IL-22
TNF
PD-L1
IL-11
IL-6
TNF
IL-6
PD-L1
IL-6
TNF
IL-6
IL-11
PD-L1

Title:

Loss of NFKB1 Results in Expression of Tumor Necrosis Factor and Activation of STAT1 to Promote Gastric Tumorigenesis in Mice

Short Title:

Loss of TNF prevents invasive gastric cancer in mice.

Authors:

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Abbreviations:

GC: gastric cancer; *H. pylori*: *Helicobacter pylori*; IL: Interleukin, IFN; interferon, IGC: intestinal-type gastric cancer, NF-κB: nuclear factor of kappa B; PD1: programmed death 1, PD-L1: programmed death ligand 1, STAT: signal transducer and activator of transcription, TNF: Tumor Necrosis Factor.

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The authors have no conflicts of interest to disclose.

Author Contributions:

LOR and TP conceived the study, performed experiments, analyzed results, supervised the study and co-wrote the manuscript. JL performed most of the experiments, analyzed results and co-wrote manuscript. MC co-scored and analyzed results. LM performed experiments and analyzed results. MDW analyzed data. YN and AP assisted with experiments. ME and LD provided mouse strains and intellectual advice. AS provided intellectual input, helped interpret data and co-wrote the manuscript.

Abstract:

Background & Aims: Activity of NFkB transcription factors and signaling via STAT are frequently altered in gastric cancer cells. Mice lacking NFKB1 (*Nfkb1*$^{-/-}$ mice) develop invasive gastric cancer and their gastric tissues have increased levels of cytokines, such as interleukin (IL)6, IL22, IL11, and tumor necrosis factor (TNF), as well as increased activation of signal transducer and activator of transcription 1 (STAT1). We investigated whether these cytokines were required for STAT1 activation in gastric tissues of mice and critical for gastric tumorigenesis.

Methods: We crossed *Nfkb1*$^{-/-}$ mice with *Il6*$^{-/-}$, *Il22*$^{-/-}$, *Il11Rα*$^{-/-}$ and *Tnf*$^{-/-}$ mice. Stomach tissues from compound mutant mice were analyzed by histology, immunoblotting and RNA sequencing. Lymphoid, myeloid and epithelial cells were isolated from stomachs and the levels of cytokines were determined by flow cytometric analysis.

Results: *Nfkb1*$^{-/-}$ mice developed gastritis, oxyntic atrophy, gastric dysplasia and invasive tumors, whereas *Nfkb1*$^{-/-}$*Stat1*$^{-/-}$ mice did not, even when followed for as long as 2 years. The levels of *Il6*, *Il11*, and *Il22* and *Tnf* mRNA were increased in the body and antrum of the stomachs from *Nfkb1*$^{-/-}$ mice, from 6 months of age. However, *Nfkb1*$^{-/-}$*Il6*$^{-/-}$, *Nfkb1*$^{-/-}$*Il22*$^{-/-}$ and *Nfkb1*$^{-/-}$*Il11Rα*$^{-/-}$ mice still developed gastric tumors, although the absence of IL11 receptor (IL11R) significantly reduced development of invasive gastric tumors. Stomachs from *Nfkb1*$^{-/-}$ *Tnf*$^{-/-}$ mice exhibited significantly less gastritis and oxyntic atrophy and fewer tumors than *Nfkb1*$^{-/-}$ mice. This correlated with reduced activation of STAT1 and STAT3 and fewer numbers of T cells and B cells infiltrating the gastric body. Loss of STAT1 significantly reduced expression of PD-L1 on epithelial and myeloid (CD11b^{+}) cells in the gastric mucosa of *Nfkb1*$^{-/-}$ mice, indeed to the levels observed on the corresponding cells from wild-type mice.

Conclusions: In studies of gastric tumor development in knockout mice, we found that loss of NFKB1 causes increased expression of TNF in the stomach and thereby drives activation of STAT1, resulting in an inflammatory immune response and the development of gastric cancer. IL11R appears to be required for the progression of gastric tumors to the invasive stage. These

findings suggest that inhibitors of TNF, and possibly also inhibitors of IL11/IL11Rα, might be useful in the treatment of gastric cancer.

KEY WORDS: monocyte, inflammation, programmed cell death 1 ligand 1, inhibitory signal.

Introduction

Gastric cancer (GC) constitutes the third highest cause of cancer-related deaths[1]. Intestinal type GC (IGC) is the most common type of non-cardia adenocarcinoma, and it can be instigated by chronic gastritis[2]. Over many years, chronic inflammatory insults promote mucosal atrophy, intestinal metaplasia and dysplasia. These abnormalities can ultimately lead to adenocarcinoma and invasive/metastatic disease[3]. Current standard-of-care treatments for GC include surgical resection of a portion of the stomach as well as intensive chemotherapy and radiation[4]. These treatments do, however, not address the molecular drivers of this malignant disease and are only rarely curative.

Aberrant activation of the NF-κB and JAK/STAT signaling pathways are known to promote inflammation-associated tumorigenesis within the gastrointestinal tract, including cancers of the colon, pancreas and stomach[5, 6]. We have recently shown that the genetic loss of *Nfkb1*, a member of the NFκB family of transcription factors, results in the development of invasive IGC that, according to the examination by clinical pathologists, faithfully recapitulates the histopathology of human GC[7]. Importantly, polymorphisms in the *NFKB1* gene that reduce its function are associated with IGC in humans (Table S1)[8]. GC in *Nfkb1*$^{-/-}$ mice is driven by sterile inflammation and associated with aberrant STAT1 activity within the non-cardia stomach[7]. While widely considered a tumor suppressor, there is growing evidence that STAT1 can also function as a tumor promoter[9]. We have shown that aberrant STAT1 activation leads to altered expression of ligands of immune check-point regulators, such as PD-L1, within epithelial, lymphoid and myeloid cells in the stomach. Notably, genetic loss of STAT1 inhibited the development of GC in *Nfkb1*$^{-/-}$ mice[7].

The strong association between aberrant STAT activity and solid cancers has precipitated the development of inhibitors of these transcription factors for therapy of these malignancies[10, 11]. Unfortunately, due to the specificity required to target protein-protein and protein-DNA interactions, and the similarity of the different STAT family members, both off-target side-effects and on-target toxicity have hampered the progression of these compounds in clinical trials, with no reports of Phase I trials for GC[10-12]. Consequently, no inhibitors of STAT proteins are approved by the FDA for any cancer, fuelling the development of drugs that act upstream of

STAT, such as inhibitors of their activators, the JAKs[11, 13, 14]. This has led to the FDA approval of two JAK inhibitors (JAK1/JAK2 [Ruxolitinib], JAK1/JAK3 [Tofacitinib]) for the treatment of certain leukemias[15, 16], with clinical trials underway for solid cancers, including GC[11, 13] (NCT01219543, NCT01112397). Unfortunately, on-target toxicity and off-target effects of these drugs remain an issue[11, 13]. Inhibition of cytokines (or their receptors) that activate JAKs and STATs has emerged as an alternative therapeutic strategy[17]. This can be achieved by the administration of monoclonal antibodies or soluble receptors. Notably, such targeting of IL-6 or TNF has proven successful in the treatment of certain inflammatory diseases[17, 18]. Toxicities are limited to on-target effects, meaning that a main clinical challenge is to identify the dominant JAK/STAT activating cytokine(s) within a cancer that should be targeted.

The lack of inhibitors of JAKs[11, 13] or STATs[10-12] that are suitable for the treatment of GC led us to consider therapeutically accessible (i.e. extra-cellular) targets identified in our RNAseq and protein analyses[7,19]. In this study we focus on the STAT1/3 activating cytokines IL-6, IL-22 and IL-11 as well as TNF, since they were elevated in our *Nfkb1*$^{-/-}$ mice and because polymorphisms in their genes and abnormal increases in their expression have been associated with increased risk for IGC and poor outcomes in human patients[20-29].

Materials and Methods

Mice

All animal experiments complied with and were approved by The Walter and Eliza Hall Institute of Medical Research (WEHI) Animals Ethics Committee. *Nfkb1*$^{-/-}$ mice were crossed with *Il6*$^{-/-}$, *Il11Ra*$^{-/-}$, *Il22*$^{-/-}$, *Tnf*$^{-/-}$ or *STAT1*$^{-/-}$ mice (see supplementary materials for original sources). The resulting inter-crossed mice were used to generate *Nfkb1*$^{-/-}$*Il6*$^{-/-}$, *Nfkb1*$^{-/-}$*Il11Ra*$^{-/-}$, *Nfkb1*$^{-/-}$*Il22*$^{-/-}$, *Nfkb1*$^{-/-}$*Tnf*$^{-/-}$ and *Nfkb1*$^{-/-}$*STAT1*$^{-/-}$ mice. Genotyping was performed by polymerase chain reaction using published primers and protocols[7, 30]. All animals were on a C57BL/6 background and housed in specific pathogen-free facilities, free of *Helicobacter* species.

Histopathology

The stomachs of mice were fixed in 80% Histochoice/20% ethanol (v/v) or 10% Formalin for histological analysis after staining with hematoxylin and eosin (H&E). The severity of gastric pathology was scored in the body and antrum by examination of H&E stained sections, as described previously[7]. The following parameters were assessed: chronic gastritis, oxyntic atrophy and dysplasia scale (0-3) or invasion (0-9). For details of pathology scoring see supplementary materials. All photomicrographs were obtained using a 5x, 10x or 20x/NA 0.3 objective lens with a 10x eyepiece on an Axioplan 2 microscope (Carl Zeiss MicroImaging, Inc.).

Purification of lymphoid, myeloid and epithelial cell populations and intracellular staining for cytokines

The stomach body and antra from mice were digested sequentially with 1 mM EDTA, 1 mM DTT (Life Technologies Cat#P2325) then collagenase/dispase (Roche Cat#11097113001) and 0.05% DNAase1 (Roche Cat#10104159001) to release the intra-epithelial leukocytes, as described previously[7]. Cell preparations were combined and stained with the fixable viability stain 700 (BD Horizon Cat#564997) and further stained with surface marker specific monoclonal antibodies and then stained intracellularly (after fixation and permeabilization) for cytokines (see supplementary materials).

Western blot analysis

Lysates were prepared from stomach tissues as described[7] and subjected to immunoblotting (list of antibodies available in supplementary materials). Proteins were visualized on the Odyssey Infrared Imaging System (LI-COR Biosciences) using HRP-conjugated secondary antibodies recommended by the manufacturer.

Gene expression analysis

RNA was extracted from homogenized stomachs using the Qiagen RNeasy Mini Kit (Qiagen Cat#74106). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814). Quantitative gene expression analysis was performed using Taqman reagents (list of primers available in supplementary materials). The CT values obtained were normalized to the *Gapdh* mRNA levels and the Δct values were calculated using the formula: 2^{-} (CT Gene of Interest – CT *Gapdh*).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 7 software. Multiple means comparisons were performed using an unpaired t-test or analysis of variance test (ANOVA) followed by Tukey's test or followed by a Dunnett's Multiple Comparison. Results are graphically presented as the mean ± SEM or log of mean ± SEM.

Results

The absence of STAT1 affords *Nfkb1*$^{-/-}$ mice with long-term protection from GC

We previously reported that the loss of STAT1 prevented gastric pathology in *Nfkb1*$^{-/-}$ mice, while loss of one allele of *Stat3* (complete loss of STAT3 causes embryonic lethality) had no effect[7]. However, *Nfkb1*$^{-/-}$*Stat1*$^{-/-}$ compound mutant animals were only followed for 1 year. To determine whether STAT1 loss only delayed disease progression or was sufficient to abrogate IGC development in *Nfkb1*$^{-/-}$ mice, we aged a cohort of *Nfkb1*$^{-/-}$*Stat1*$^{-/-}$ mice to time points at which we would expect invasive GC (22-28 months). Notably, the absence of STAT1 was able to abrogate all gastric pathology in *Nfkb1*$^{-/-}$ mice aged up to at least 2 years (Figure 1A). Significant reductions in the severity of all parameters of gastric histopathology measured (gastritis, oxyntic atrophy, dysplasia and invasion) at 18 months (Figure 1B) and beyond (Figure 1C, D) were observed, cementing the critical role of STAT1 in this model of GC.

The absence of IL-6, IL-11 receptor or IL-22 does not affect stomach architecture

While STAT1 prevented GC in our murine model (Figure 1), the lack of suitable inhibitors of this transcription factor prompted us to assess activators of STAT1 as potential therapeutic targets[7]. We chose to examine the roles of the STAT1 (and STAT3) activating cytokines: IL-6, IL-11 and IL-22. Firstly, we showed that these cytokines were abnormally elevated in the body or antrum of the stomachs from *Nfkb1*$^{-/-}$ mice from 6 months of age (Figure S1). While IL-6, IL-11 (through IL-11Rα) and IL-22 are activators of the JAK/STAT pathway and have roles in inflammation and immune defence, their actions in the normal development and function of the stomach are not well defined. Therefore, we performed a histological examination of the stomachs of aged mice lacking IL-6, IL-22 or IL-11Rα, comparing them to stomachs from wt and the GC prone *Nfkb1*$^{-/-}$ mice[7]. The stomachs from aged *Il6*$^{-/-}$, *Il11Ra*$^{-/-}$ and *Il22*$^{-/-}$ mice did not display any discernible abnormalities in the gastric body or antrum (Figure S2A) or pathologies associated with the development of GC (chronic gastritis, parietal atrophy, dysplasia or invasive gastric cancer; Figure S2A-B). This reveals that loss of IL-6, IL-22 or IL-11Rα does not impact stomach architecture or induce gastric disease.

The absence of IL-6, IL-11 receptor or IL-22 does not affect gastritis but differentially impacts oxyntic atrophy in *Nfkb1*$^{-/-}$ mice

The sequential pre-neoplastic changes that ultimately lead to GC[3] commence with chronic gastritis (mucosal inflammation) followed by oxyntic atrophy (loss of chief cells and then parietal cells from the oxyntic glands). Since parietal cells produce key signaling molecules that regulate the homeostasis and differentiation of other cell types, including chief cells[31], the loss of cytokines could have wide-ranging impact. To assess whether the absence of IL-6, IL-22 or IL-11Rα affected the development of gastritis or atrophy in *Nfkb1*$^{-/-}$ mice, we conducted a detailed histological analysis of the stomachs from compound mutant mice lacking NF-κB1 and one of these cytokines or IL-11Rα (Figure 2A-D). As previously reported[7], gastritis was detected in *Nfkb1*$^{-/-}$ mice from 6 months of age as focal aggregates (becoming progressively denser) of inflammatory cells within the lamina propria or submucosa in the body or antrum (Figure 2C). These cell aggregates were often in close proximity to blood vessels and associated with mucosal atrophy (Figure 2B). Loss of IL-6, IL-22 or IL-11Rα did not affect the gastritis caused by the absence of NF-κB1 in either the gastric body or antrum at any of the time points examined (Figures 2C-D, S3, S4).

We previously reported that from 6 months of age *Nfkb1*$^{-/-}$ mice developed oxyntic atrophy with increasing severity (loss of parietal cells, hyperplasia of surface mucus cells, atrophic gastritis)[7]. Loss of IL-6 did not alter the oxyntic atrophy caused by the absence of NFκB1 at early stages of disease (6 months, Figure 2D), while the loss of IL-22 mildly augmented this pathology, although no significant impact was seen during late stage disease (Figure 2D). Loss of IL-11Rα, while not affecting oxyntic atrophy during early stages of disease (6 and 12 months), significantly reduced pathology at 18 months of age (Figure 2D). These findings suggest that IL-22 may afford modest protection from atrophy in the gastric mucosa of *Nfkb1*$^{-/-}$ mice during early disease stages, whereas signaling through the IL-11Rα may drive oxyntic atrophy at later stages.

Loss of IL-22 or IL-11Rα had no impact on the accumulation of inflammatory (CD45.2^{+}) cells within the gastric mucosa of *Nfkb1*$^{-/-}$ mice at 6, 12, or 18 months of age (Figure 2E). There were no changes in the accumulation of CD4^{+} or CD8^{+} T cells, Treg cells, NK cells and myeloid cells (Figure 2F-H, S5A-I). The loss of IL-6 caused a significant reduction in both B cells (12 and 18

months; Figure 2F) and myeloid cells ($CD11b^+$) (6 months; Figure S5G), particularly the classical and resident monocyte populations (6 months; Figure 2G,H, S6 gating strategy)

The absence of distinct STAT activating cytokines differentially affects the development of gastric dysplasia in *Nfkb1*$^{-/-}$ mice

Gastric dysplasia refers to the neoplastic transformation of the gastric mucosa and constitutes a precursor to human GC[3], with endoscopic resection of high-grade lesions often necessary to prevent disease progression[32]. Dysplasia is usually associated with inflammation[33, 34] and, of note, this pathology does not develop in animal models of chemical induced oxyntic atrophy where inflammation is absent[35, 36]. We examined whether the absence of IL-6, IL-22 or IL-11Rα could influence the onset or severity of gastric dysplasia in *Nfkb1*$^{-/-}$ mice. As previously reported, dysplastic changes were detected in *Nfkb1*$^{-/-}$ mice from ~12 months[7]. By 18 months of age, the severity score had doubled, with prominent dilation and branching of glands, in addition to loss of normal orientation (Figure 3A,B). Although the loss of IL-22 did not affect the onset or severity of dysplasia in *Nfkb1*$^{-/-}$ mice, a small reduction in this pathology was observed with loss of IL-6 at 12 months of age and with loss of IL-6 or IL-11Rα at 18 months of age (Figure 3B). These findings indicate a minor role for IL-6, and possibly IL-11Rα, at early stages of gastric dysplasia driven by the absence of NFκB1.

The absence of IL-11Rα impedes the development of invasive GC disease in *Nfkb1*$^{-/-}$ mice

Invasive GC in humans is characterized by restriction to the lamina propria or muscularis mucosae[37] or as a protrusion of dysplastic glands through the muscularis mucosae into the submucosa[34]. We have previously shown that the incidence and severity of invasive GC increased progressively in *Nfkb1*$^{-/-}$ mice[7] (Figure 3C,D): ~20% at 12 months, ~50% at 18 months (Figure 3E). While loss of IL-6 or IL-22 did not affect the development of invasive GC in *Nfkb1*$^{-/-}$ mice, the absence of IL-11Rα significantly reduced both its incidence and severity at 12 and 18 months (Figure 3C-E), correlating with the mild decrease in dysplasia (Figure 3B). This suggests that while IL-11Rα has only a limited role in the development of gastritis, atrophy or dysplasia, it does contribute to the progression to invasive GC driven by the absence of NFκB1.

The absence of IL-6, IL-11Rα or IL-22 variably affect STAT signaling in the stomachs of *Nfkb1*$^{-/-}$ mice

We have previously shown that the levels of IL-6, IL-11 and IL-22 are abnormally increased in *Nfkb1*$^{-/-}$ mice[7]. As reported, *Nfkb1*$^{-/-}$ mice displayed an age-associated increase in the levels of *Il6* and *Il11* mRNA in their stomachs (particularly in the stomach body; Figure 4A). The expression of *Il22* mRNA was also elevated in the stomach antrum of *Nfkb1*$^{-/-}$ mice, albeit with considerable variability between individual animals (Figure 4A). We also determined the levels of the non-deleted cytokines in the stomachs from the compound mutant mice to test whether there was a compensatory increase in their expression that may have contributed to the failure of the loss of an individual cytokine to substantially impact GC development. However, we found that the loss of IL-6, IL-22 or IL-11Rα did not alter the expression of the cytokines that had not been genetically deleted in the stomachs of mice on the *Nfkb1*$^{-/-}$ background (Figure 4A).

Our previous studies showed that the absence of NF-κB1 resulted in a progressive increase in the total levels, as well as activity (i.e. phosphorylation) of STAT1 in the gastric mucosa[7]. To investigate whether loss of IL-6, IL-22 or IL-11Rα altered STAT activation in *Nfkb1*$^{-/-}$ mice, we performed Western blot analyses on stomach extracts. We found that the levels of total STAT1 and activated STAT3 were elevated in the stomach body of *Nfkb1*$^{-/-}$ mice, but this was not impacted by the absence of IL-6, IL-22 or IL-11Rα (Figures 4B, S7A). In the stomach antrum of the

Nfkb1$^{-/-}$ mice, total STAT1 and activated STAT1, but not activated STAT3 levels, were increased. This was not impacted by the absence of IL-22, but loss of IL-6 or IL-11Rα resulted in a significant reduction of total and activated STAT1 (Figures 4B, S7B). We confirmed these observations by performing qRT-PCR analysis of the expression of the STAT target gene, *Socs3*, and found it was significantly elevated in the stomach body of *Nfkb1*$^{-/-}$ mice compared to wt controls. The absence of IL-6, IL-11Rα or IL-22 in *Nfkb1*$^{-/-}$ mice did not cause a significant change in the levels of *Socs3* mRNA in the gastric body or antrum, whereas loss of STAT1 greatly reduced its levels (Figure 4C).

We also examined the expression of genes commonly associated with cancer progression, in particular *Cyclin D1*, since its overexpression has been associated with reduced survival in GC

patients[38]. We found that *Nfkb1*$^{-/-}$ mice had abnormally increased levels of *Cyclin D1* mRNA in the stomach body at 6 months of age and mildly increased levels in the antrum at 12 months (Figure S7C). We also examined *c-Myc*, a regulator of cell growth and proliferation that is frequently dysregulated in human GC[39]. The levels of *c-Myc* were found to be only mildly increased in the stomach of *Nfkb1*$^{-/-}$ mice at 12 months of age (Figure S7D). The absence of IL-6, IL-22 or IL-11Rα did not significantly alter the expression of *Cyclin D1* or *c-Myc* in the stomachs of *Nfkb1*$^{-/-}$ mice at either time point (Figure S7C-D). This indicates that these cytokines are not critical for the expression of these cell proliferation driving genes within the gastric mucosa of *Nfkb1*$^{-/-}$ mice.

The absence of TNF inhibits the development and progression of GC in *Nfkb1*$^{-/-}$ mice

While we had anticipated that the loss of STAT activating cytokines would alleviate GC progression, we were also intrigued by our previous RNAseq analysis which revealed abnormally increased levels of the pro-inflammatory cytokine TNF in the stomach of young *Nfkb1*$^{-/-}$ mice, where it was produced by T cells and myeloid cells[7]. This is noteworthy, since *TNFα* gene polymorphisms have been implicated in human GC[26-29] and TNF is elevated in *H. pylori* infected patients[25]. We found that TNF was upregulated in the stomachs of aged *Nfkb1*$^{-/-}$ mice, where it was produced by both T cells (TCRβ^{+}) and other hematopoietic cells (CD45^{+}TCRβ^{-}) (Figure S8A,B). Aged *Nfkb1*$^{-/-}$ mice deficient for IL-6, IL-22 or IL-11Rα also showed abnormally increased levels of TNF in the stomach (Figure S8C). Next, we examined whether *Tnf*$^{-/-}$ mice had discernible abnormalities in the stomach. However, even when aged these animals showed no defects, such as chronic gastritis, parietal atrophy, dysplasia or GC (Figure S9A-B).

In order to determine the role of TNF in the progression of gastritis to GC we generated *Nfkb1*$^{-/-}$ mice deficient in this cytokine (Figure 5). All measured parameters of early gastric pathology, including gastritis and oxyntic atrophy, were significantly less severe in *Nfkb1*$^{-/-}$*Tnf*$^{-/-}$ mice compared to *Nfkb1*$^{-/-}$ mice (Figure 5A,B). Histopathological analysis of aged *Nfkb1*$^{-/-}$*Tnf*$^{-/-}$ mice revealed the substantial impact of loss of TNF on GC development (Figure 6A). Dysplasia and invasion were significantly reduced at 18 months, an age at which invasive GC is prominent

in *Nfkb1*$^{-/-}$ mice (Figures 5B, 6A,B). At 20 - 28 months the protection afforded by loss of TNF was not as profound as that provided by the absence of STAT1 (Figure 1). Highly significant reductions in the advanced parameters of gastric disease (oxyntic atrophy, dysplasia and invasion) were evident in *Nfkb1*$^{-/-}$*Tnf*$^{-/-}$ mice at this advanced age (Figures 5B, 6B). Loss of TNF also markedly reduced the levels of activated STAT1 and STAT3 in the gastric body of *Nfkb1*$^{-/-}$ mice, reaching levels seen in wt mice (Figures 6C, S9C-D). These results demonstrate that the absence of TNF markedly inhibits GC development in *Nfkb1*$^{-/-}$ mice.

The absence of TNF or STAT1 prevents PD-L1 upregulation that is driven by NF-κB1 loss

Detailed flow cytometric analysis of stomachs at advanced disease stages (12, 18 months) revealed that in *Nfkb1*$^{-/-}$ mice, the loss of TNF reduced the overall leukocyte cellularity in the stomach, predominantly affecting T cells (CD4+, CD8+ and Treg) and B cells (Figures 6D, S10A). The absence of TNF in *Nfkb1*$^{-/-}$ mice did not significantly impact the infiltration of the stomach by NK cells or overall myeloid (CD11b^{+}) cells (Figure 6D), however, certain myeloid subpopulations, such as neutrophils, were significantly decreased (Figures S10B, S6).

Tumors, including GC[40], express antigens against which cytotoxic T cells can react. Anti-tumor T cell immune responses are regulated by the balance between co-stimulatory and co-inhibitory signals (immune checkpoint regulators). PD-L1 is the ligand for PD-1, a co-inhibitory receptor on T cells[41] and increased levels of PD-L1 were observed not only in our murine GC model but also in certain sub-types of human GC[39], where this is often correlated with metastasis[42-44]. Since we had previously identified a link between the absence of NFκB1 with upregulation of STAT1 driven expression of PD-L1[7], we examined whether the loss of TNF could, similar to loss of STAT1, impact PD-L1 expression. We confirmed that loss of STAT1 significantly reduced PD-L1 expression in epithelial and myeloid (CD11b^{+}) cells, including classical monocytes, resident monocytes and neutrophils, in the gastric mucosa of 18 month-old *Nfkb1*$^{-/-}$ mice to the levels seen in wt mice (Figure 6E). Loss of TNF had similar impact, albeit with a less marked reduction of PD-L1 expression on neutrophils (Figure 6E). This reveals that the abnormally increased expression of PD-L1 on T cells and myeloid cells in the stomachs of *Nfkb1*$^{-/-}$ mice is dependent on both TNF and STAT1.

In summary, our work establishes a therapeutically targetable link between the absence of NF-κB1 with upregulation of STAT1 as well as TNF pro-tumorigenic functions and the abnormally increased expression of ligands (PD-L1) for inhibitory receptors on T cells.

Discussion

Cytokine/cytokine receptor signaling is a key component in the overall regulation of the immune system. Accordingly, its dysregulation has been linked to many chronic inflammatory diseases, autoimmune disorders and cancer[45, 46]. Pertinently, chronic inflammation has been identified as a key initiator of the development of IGC[3, 45]. Even though activation of the NFκB family of transcription factors has been linked to the control of cytokine and chemokine production in several inflammation-associated cancers, the lack of suitable animal models has made it difficult to study this relationship. To explore the roles of individual cytokines in stomach inflammation and the development of invasive IGC, we used the *Nfkb1*$^{-/-}$ mouse model that faithfully recapitulates all features and stages of human GC. Notably, these mice model the predicted pathogenic polymorphisms in *NFKB1* that have been linked to human GC (Table S1)[8].

We focused on three STAT1/3 activating cytokines, since polymorphisms in the genes for IL-6, IL-11 and IL-22 as well as their over-expression have been associated with increased risk for IGC and poor patient outcomes[20-24]. IL-6 and IL-11 expression are also elevated in the GC of *Nfkb1*$^{-/-}$ mice, while IL-22 is elevated in the serum of sick *Nfkb1*$^{-/-}$ mice[7]. Our results reveal that individually IL-6, IL-22 and IL-11Rα are largely dispensable for GC development in *Nfkb1*$^{-/-}$ mice. This may be due to the fact that these cytokines and their receptors have overlapping functions in activating signaling pathways. This aligns with the observation that loss of IL-6, IL-22 or IL-11Rα on their own had minimal impact on the levels of STAT1 and STAT3 within the gastric mucosa of *Nfkb1*$^{-/-}$ mice. Multiple cytokines are often simultaneously upregulated in both the serum and tumor tissue of patients with advanced GC[22, 47-49], as is the case in the *Nfkb1*$^{-/-}$ mouse model. This may suggest that blockade of multiple STAT-activating pro-inflammatory cytokines and/or their receptors may be necessary to curtail gastritis and GC development[22]. Considering that IL-22 has both a pro-inflammatory role in mucosal immunity and anti-inflammatory functions in the maintenance of tissue homeostasis[50], our findings suggest that loss of the latter may have exacerbated gastric disease in the *Nfkb1*$^{-/-}$ mice. This would indicate that JAK inhibitors that block multiple STAT activating cytokines may not be appropriate for GC therapy.

While gastritis can initiate GC development, inflammation *per se* does not with a 100% incidence result in GC. For instance, while infection with *H. pylori* is the strongest risk factor for human GC, only 3% of infected people develop GC[51]. In the *Nfkb1*$^{-/-}$ mice there is no role for the microflora, as disease occurs as a result of sterile inflammation[7]. However, we have shown that in NFκB1-deficient mice *H. felis* infection did not accelerate GC development[7], and others reported that pre-neoplasia was not enhanced[52]; these findings may indicate a possible mechanistic difference compared to the human disease. Therefore, in addition to infectious pathogens other molecular drivers must also play prominent roles in GC development[53]. We have shown that NFκB1 homodimers and a small amount of NFκB2 were the only NFκB subunits present in the nuclei of gastric epithelial cells of healthy wt mice[7]. Therefore, loss of *Nfkb1* in mice is anticipated to precipitate aberrant NFκB (e.g. involving RELA) and STAT signaling. RNAseq analysis revealed that this occurs at early stages of disease[7], reflecting the likely molecular drivers in GC patients with pathogenic *NFKB1* polymorphisms[8]. We report here that although IL-11/IL-11Rα signaling is dispensable for gastric inflammation and the development of oxyntic atrophy, mucosal atrophy and dysplasia in *Nfkb1*$^{-/-}$ mice, its loss reduced invasive GC disease. Of note, studies of inflammation-associated human gastrointestinal cancers have shown an association between high levels of IL-11 expression and the magnitude of tumor invasion[54-56]. Notably, both IL-11 and IL-11Rα were found to be overexpressed in GC cells and this was linked to increased vessel infiltration and the severity of tumor invasion[56]. Recently, IL-11 was recognized for its role in diseases associated with deregulated mucosal homeostasis, including gastro-intestinal cancers[57]. IL-11 can also increase the proliferation and migration of certain tumor cells[58]. In another murine model of GC, the *gp130*$^{F/F}$ mice, IL-11 was shown to have a more prominent role than IL-6, with the loss of IL-11Rα able to abrogate tumor development[59]. However, *gp130*$^{F/F}$ mice do not develop invasive GC; therefore, the impact of loss of IL-11Rα on this pathology could not be assessed[30]. Our findings suggest that therapeutic targeting of IL-11/IL-11Rα signaling may represent a strategy for the treatment of late stage GC. This may be of relevance not only to patients with pathogenic *NFKB1* polymorphisms, but more generally for inflammation driven GC.

Due to its abnormally increased levels within the immune compartment of *Nfkb1*$^{-/-}$ mice, we examined the role of TNF in GC. Low-dose chronic TNF sustains inflammation and expression

of chemokines and STAT-1-dependent type 1 IFN-response genes[60] and can promote angiogenesis and neoplastic progression in certain cancers[61], but its role in GC has not yet been thoroughly investigated. There is extensive literature suggesting that aberrantly increased TNF can promote the development of at least certain types of cancer[62] and patients with inflammatory diseases who were treated with TNF inhibitory agents were reported to have a reduced risk of cancer[63, 64]. It would be interesting to determine if GC patients with pathogenic *NFKB1* polymorphisms also have abnormally high TNF expression. We report here that loss of TNF inhibited both gastritis and the development of GC in *Nfkb1*$^{-/-}$ mice. This was accompanied by reduced levels of activated STAT1 and STAT3, revealing a link between TNF, STAT activity and GC development. We further explored this relationship in light of the knowledge that the proliferation and metastasis of malignant cells is not only driven by their intrinsic cellular signaling pathways but is also impacted by interactions within the tumor microenvironment. RNAseq studies identified *Tnf* as the most significantly upregulated gene in myeloid cells from the stomachs of young *Nfkb1*$^{-/-}$ mice[7]. Interestingly, compared to *Nfkb1*$^{-/-}$ mice, *Nfkb1*$^{-/-}$*Tnf*$^{-/-}$ mice showed significantly reduced neutrophil infiltration. TNF loss may affect neutrophil recruitment or function within the gastric mucosa, as has been postulated for human gastritis and GC[65]. There are conflicting reports about effects of TNF on neutrophils, either accelerating apoptosis or enhancing survival[66]. Since neutrophils play a major role in linking chronic inflammation to tumorigenesis[67], this may account for the reduced pathology observed in *Nfkb1*$^{-/-}$*Tnf*$^{-/-}$ mice. Our results suggest a role for TNF within the gastric mucosa in regulating the accumulation, and perhaps function of T and B cells, potentially by somehow activating STAT1. Pertinently, loss of TNF prevented T cell suppression by reducing PD-L1 expression on myeloid and epithelial cells within the gastric mucosa of *Nfkb1*$^{-/-}$ mice. This finding correlates with others in demonstrating that TNF causes increased PD-L1 expression on cancer cells[68] and that resistance to anti-PD-1 therapy could be overcome by TNF blockade[69].

In conclusion, our findings support a role for inhibition of IL-11/IL-11Rα signaling in late stage IGC, perhaps using IL11-Mutein, an antagonist of IL11Rα[30, 70]. Most importantly, our findings suggest that TNF or STAT1 may be promising therapeutic targets in GC. TNF would be more tractable, given its extracellular function that can be targeted by blocking antibodies, several of which are already in clinical use for inflammatory conditions[17, 18]. The therapeutic benefit of

TNF inhibitory drugs may extend to a broad range of GCs, not only those with *NFKB1* polymorphisms, albeit with possible limitation to cancers that show abnormally increased levels of TNF. Perhaps blocking both IL-11/IL-11Rα and TNF would be more effective than blocking either cytokine alone, and their impact might be further boosted by cancer immuno-therapy, particularly in GCs that contain large T cell infiltrates and express high levels of PD-L1.

Figure Legends

Figure 1. The absence of STAT1 provides long-term protection from invasive GC in *Nfkb1*$^{-/-}$ mice. (A) Representative images of H&E stained sections of stomachs (>18 months). (B-C) Graphical summary of stomach pathology scoring (B) 18-month-old and (C) >18-month-old mice, *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001; n = number of mice. (D) Representative macroscopic images of H&E stained sections of the body and antrum of stomachs from mice aged 25-28-months. Asterisks = gastric lesions.

Figure 2. Impact of the absence of IL-6, IL-11Rα or IL-22 on the development of pre-neoplastic gastric inflammation in *Nfkb1*$^{-/-}$ mice. Representative images of H&E stained stomach sections showing (A) gastritis (B) oxyntic atrophy in the stomachs of 12-month-old mice. Arrowheads = gastric pathology or features of the mucosa (black = inflammation, blue = blood vessels, red = parietal cell loss, arrows = chief cell loss). (C-D) graphical representation of the scoring of the severity of (C) gastritis and (D) oxyntic atrophy, *p<0.05, **p<0.01, ***p < 0.001, ****p<0.0001; n = number of mice. (E-H) Graphical summary of the hematopoietic cell composition in the stomachs from mice of the indicated genotypes: (E) $CD45^+$ cells, (F) B cells, (G) classical monocytes ($CD45^+CD11b^+Ly6C^{hi}Ly6G^-$), (H) resident monocytes ($CD45^+CD11b^+Ly6C^-Ly6G^-$); n=4-7 mice per genotype per time point; *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001.

Figure 3. Impact of the absence of IL-6, IL-11Rα or IL-22 on the development of dysplasia and invasive GC in *Nfkb1*$^{-/-}$ mice. (A) Representative images of H&E stained stomach sections showing gastric dysplasia in 18-month-old mice. Black arrowheads = dysplastic glands, black arrows = tall columnar cells, red arrows = elongated nuclei, red arrowheads = globoid cells, * cell piling. (B) Graphical summary of the severity of gastric dysplasia; n = number of mice. *p < 0.05, **p < 0.01, ****p < 0.0001. (C) Representative images of H&E stained stomach sections showing invasive GC in 18-month-old mice. Arrowheads = invasive disease. (D) Summary representation of the severity of invasive gastric disease; n = number of mice. *p<0.05, **p<0.01, ***p<0.001. (E) Cumulative graphical representation of the age-related (6, 12, 18 months from B and D) incidence of invasive IGC.

Figure 4. Impact of the absence of IL-6, IL-11Rα or IL-22 in *Nfkb1*$^{-/-}$ mice on the expression of genes associated with the JAK/STAT signaling pathway. (A) Graphical representation of *Il6*, *Il11* and *Il22* mRNA expression in the whole gastric body or antrum of mice of the indicated genotypes at the indicated ages. (B) Representative Western blot analysis of the indicated proteins in the stomach body and antrum in mice of the indicated genotypes at 12 months of age. (C) Graphical representation of *Socs3* mRNA in the body and antrum of mice of the indicated genotypes at 12 months of age or > 12 -18 months (*Nfkb1*$^{-/-}$*Stat1*$^{-/-}$), *p<0.05, **p<0.01, ***p<0.001. (A-C) n=3 mice per genotype.

Figure 5. TNF is essential for the development of pre-neoplastic lesions in *Nfkb1*$^{-/-}$ mice. (A) Representative images of H&E stained sections of the gastric body and antrum from mice of the indicated genotypes at >18 months (range 20-28 months). (B) Graphical summary of pathology scoring data; n = number of mice. **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6. TNF is essential for the development of invasive GC in *Nfkb1*$^{-/-}$ mice. (A) Representative macroscopic images of the gastric body and antrum from mice of the indicated genotypes aged 23-26 months. Arrowheads = gastric lesions. (B) Graphical summary of the severity of GC pathology; n = number of mice. *p<0.05, **p<0.01, ****p<0.0001. (C) Western blot analysis of the indicated proteins in the stomach from 3 mice of each genotype (>18 months of age). (D) Graphical summary of the cellular composition of stomachs from mice of the indicated genotypes. (E) Graphical summary of PD-L1 expression on cells from the stomachs of mice at 18 months; classical monocytes ($CD45.2^+CD11b^+Ly6C^{hi}Ly6G^-$), intermediate monocytes ($CD45.2^+CD11b^+Ly6C^{int}Ly6G^-$), resident monocytes ($CD45.2^+CD11b^+Ly6C^-Ly6G^-$), neutrophils ($CD45.2^+CD11b^+Ly6C^+Ly6G^+$); n=3-7 mice per genotype, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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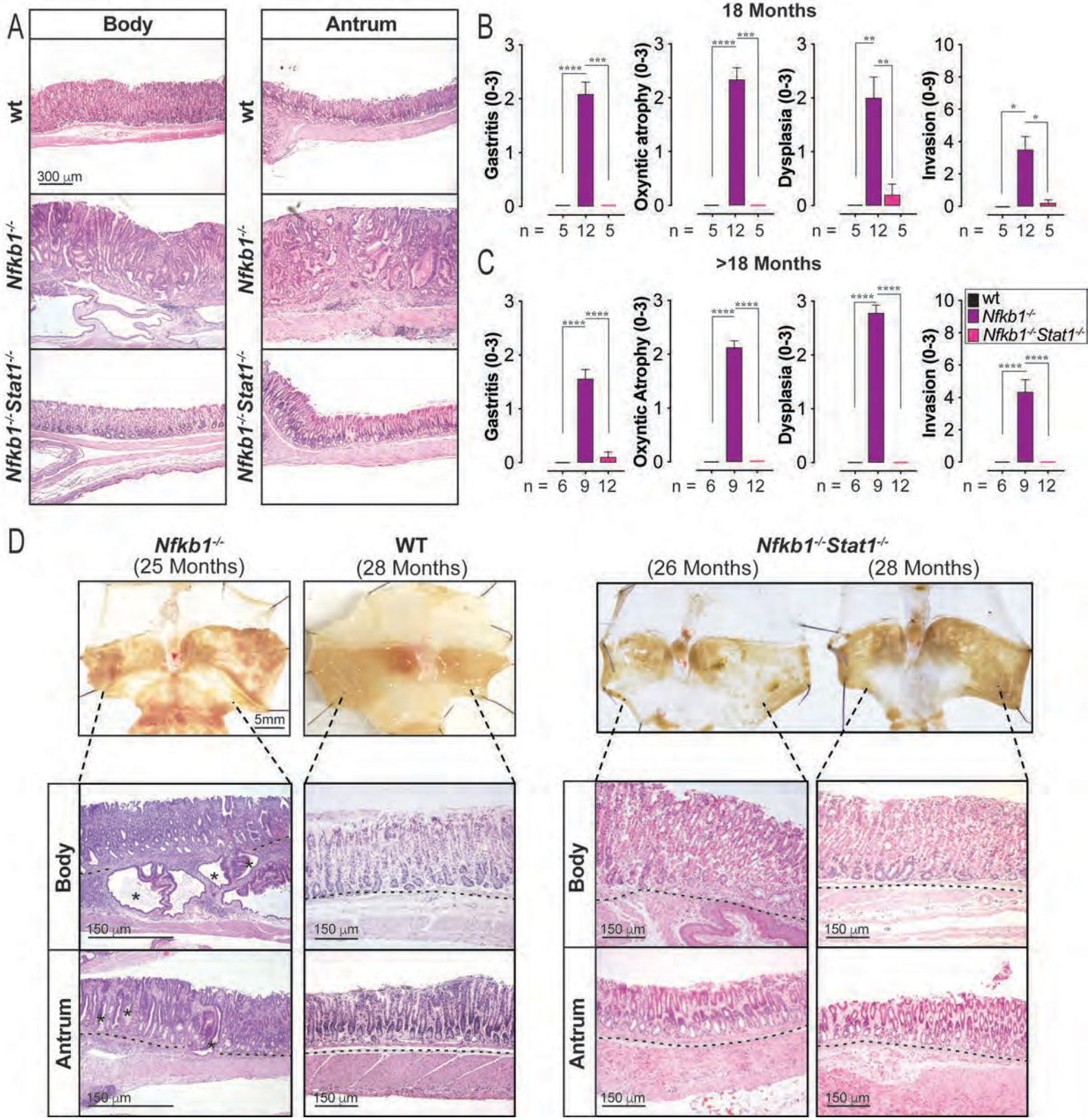
Figure 1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Body
Antrum
wt
Nfkb1-/-
Nfkb1-/-Stat1-/-
300 μm
B
18 Months
Gastritis (0-3)
Oxyntic atrophy (0-3)
Dysplasia (0-3)
Invasion (0-9)
n = 5 12 5
C
>18 Months
Gastritis (0-3)
Oxyntic Atrophy (0-3)
Dysplasia (0-3)
Invasion (0-3)
n = 6 9 12
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Nfkb1-/-
Nfkb1-/-Stat1-/-
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Nfkb1-/-
(25 Months)
WT
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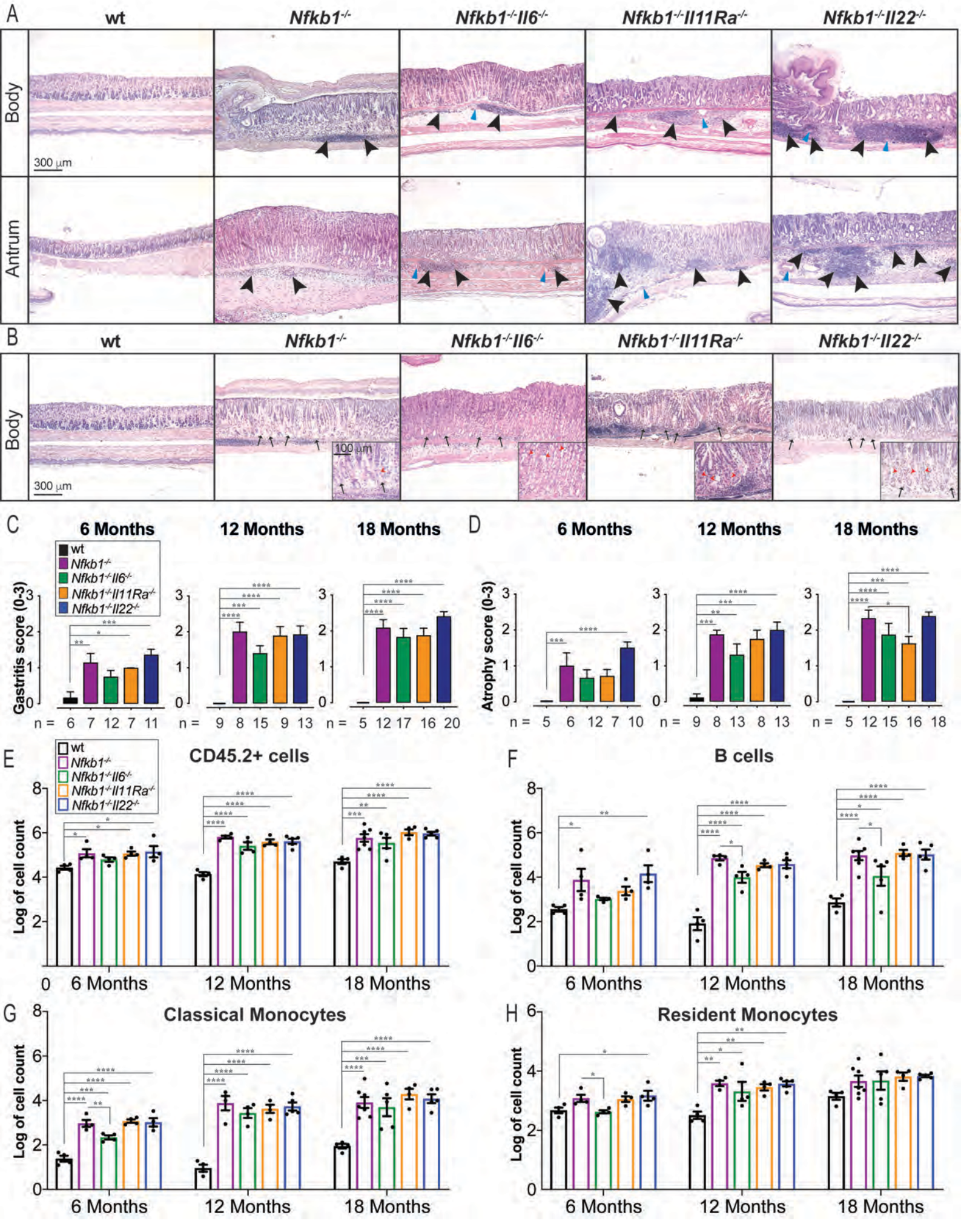
(28 Months)
5mm
Body
Antrum
150 μm

Figure 2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Nfkb1-/-Il22-/-
Body
Antrum
300 μm
B
100 μm
C
6 Months
12 Months
18 Months
wt
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Nfkb1-/-Il6-/-
Nfkb1-/-Il11Ra-/-
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D
Atrophy score (0-3)
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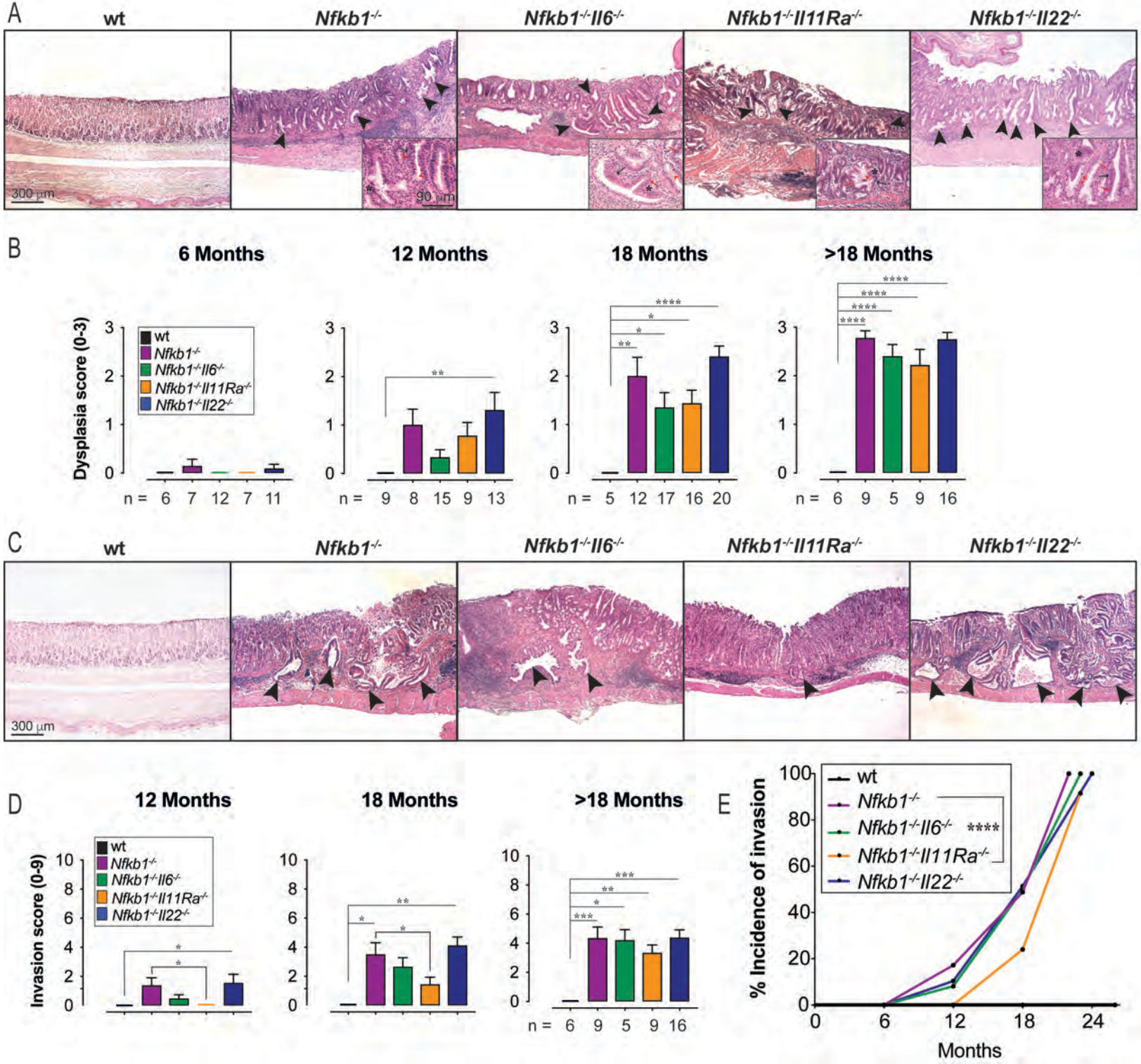
B cells
G
Classical Monocytes
H
Resident Monocytes

Figure 3


A
wt
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Nfkb1-/-Il6-/-
Nfkb1-/-Il11Ra-/-
Nfkb1-/-Il22-/-
300 μm
90 μm
B
6 Months
12 Months
18 Months
>18 Months
Dysplasia score (0-3)
wt
Nfkb1-/-
Nfkb1-/-Il6-/-
Nfkb1-/-Il11Ra-/-
Nfkb1-/-Il22-/-
n = 6 7 12 7 11
n = 9 8 15 9 13
n = 5 12 17 16 20
n = 6 9 5 9 16
C
wt
Nfkb1-/-
Nfkb1-/-Il6-/-
Nfkb1-/-Il11Ra-/-
Nfkb1-/-Il22-/-
300 μm
D
12 Months
18 Months
>18 Months
Invasion score (0-9)
n = 6 9 5 9 16
E
% Incidence of invasion
Months
wt
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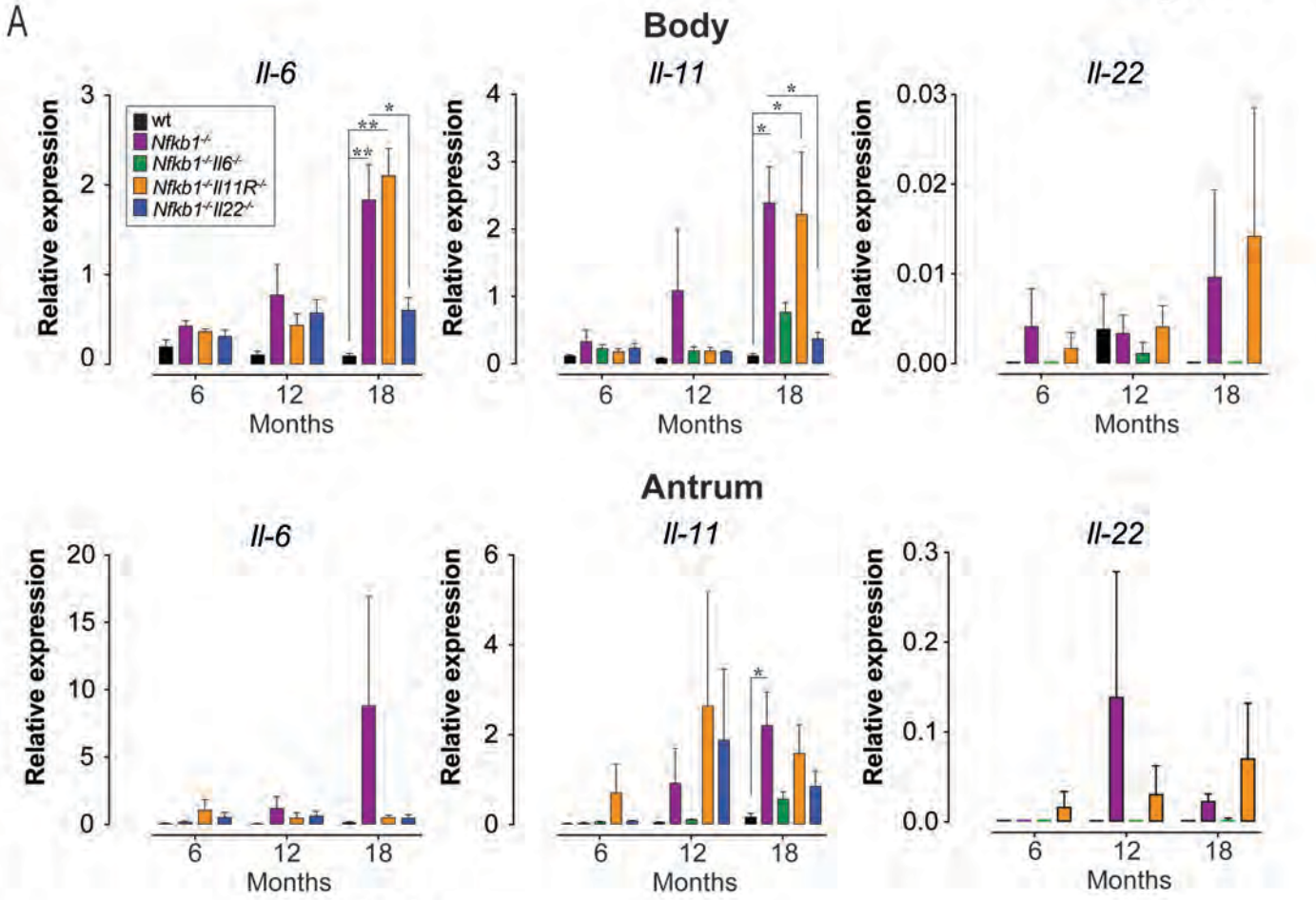
Figure 4


A
Body
Il-6
Il-11
Il-22
Relative expression
wt
Nfkb1-/-
Nfkb1-/-Il6-/-
Nfkb1-/-Il11R-/-
Nfkb1-/-Il22-/-
Months
Antrum
Il-6
Il-11
Il-22
Relative expression
Months

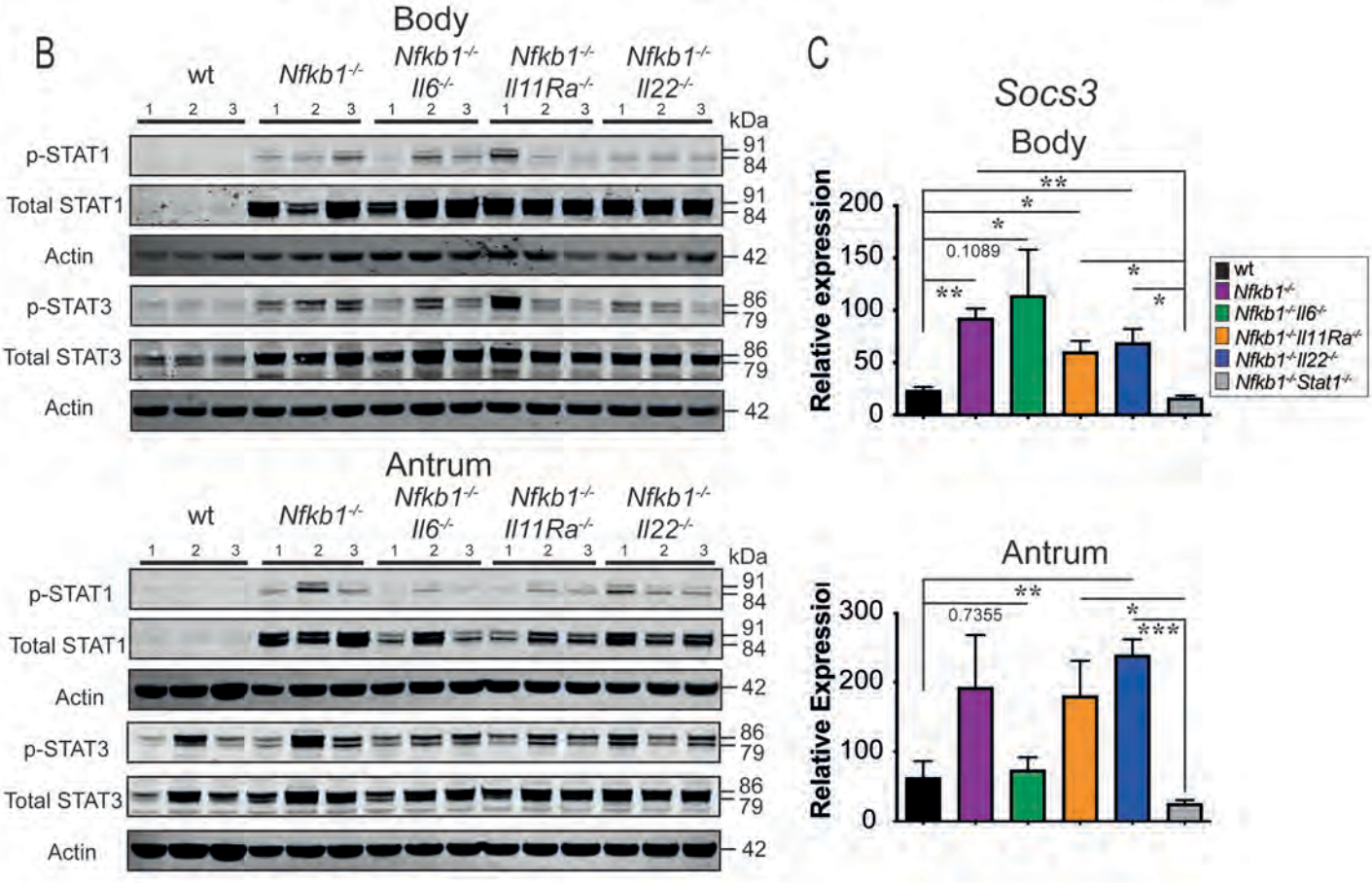

B
Body
wt
Nfkb1-/-
Nfkb1-/- Il6-/-
Nfkb1-/- Il11Ra-/-
Nfkb1-/- Il22-/-
kDa
p-STAT1
Total STAT1
Actin
p-STAT3
Total STAT3
Actin
Antrum
C
Socs3
Body
Relative expression
0.1089
wt
Nfkb1-/-
Nfkb1-/-Il6-/-
Nfkb1-/-Il11Ra-/-
Nfkb1-/-Il22-/-
Nfkb1-/-Stat1-/-
Antrum
Relative Expressio
0.7355

Figure 5

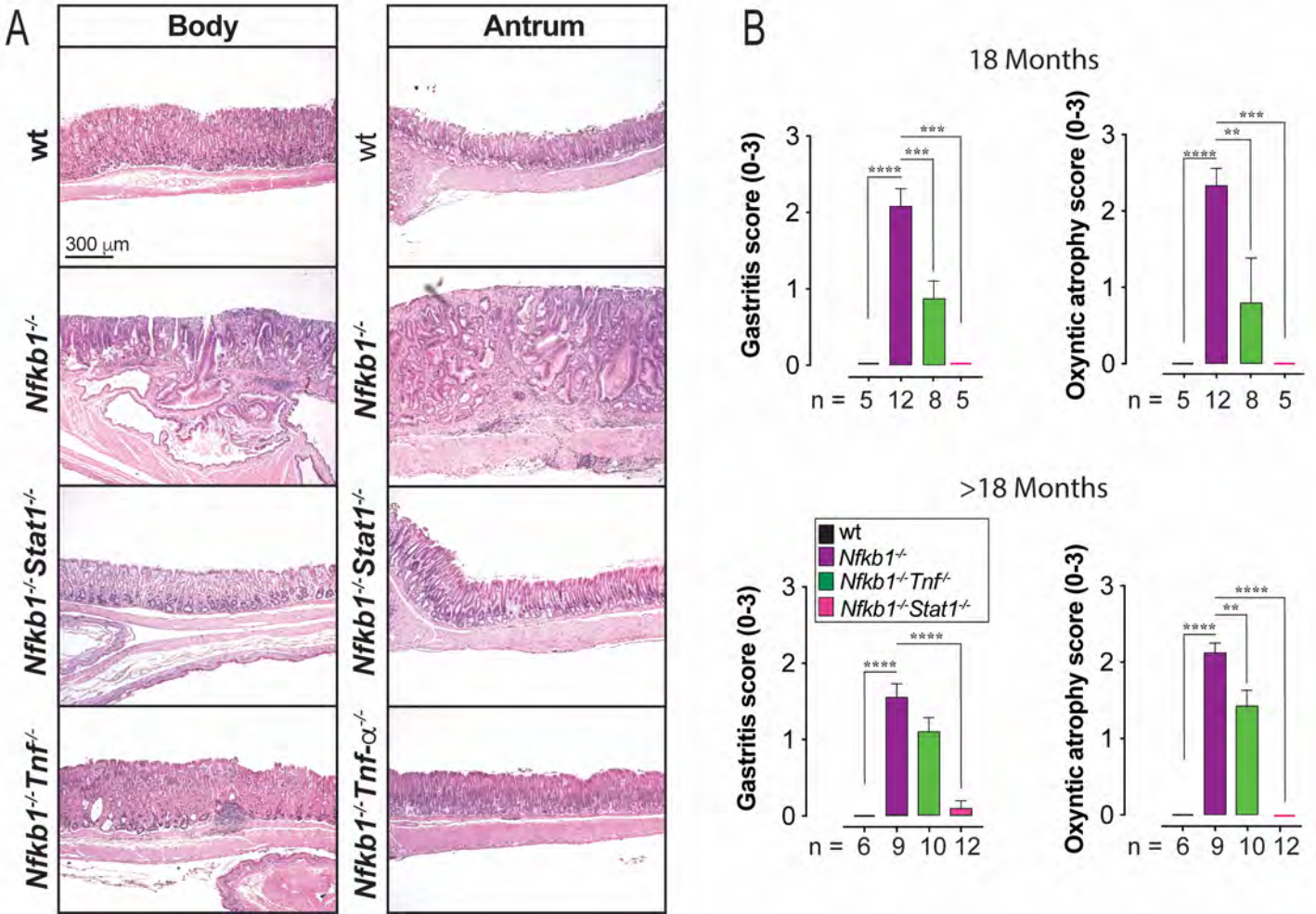
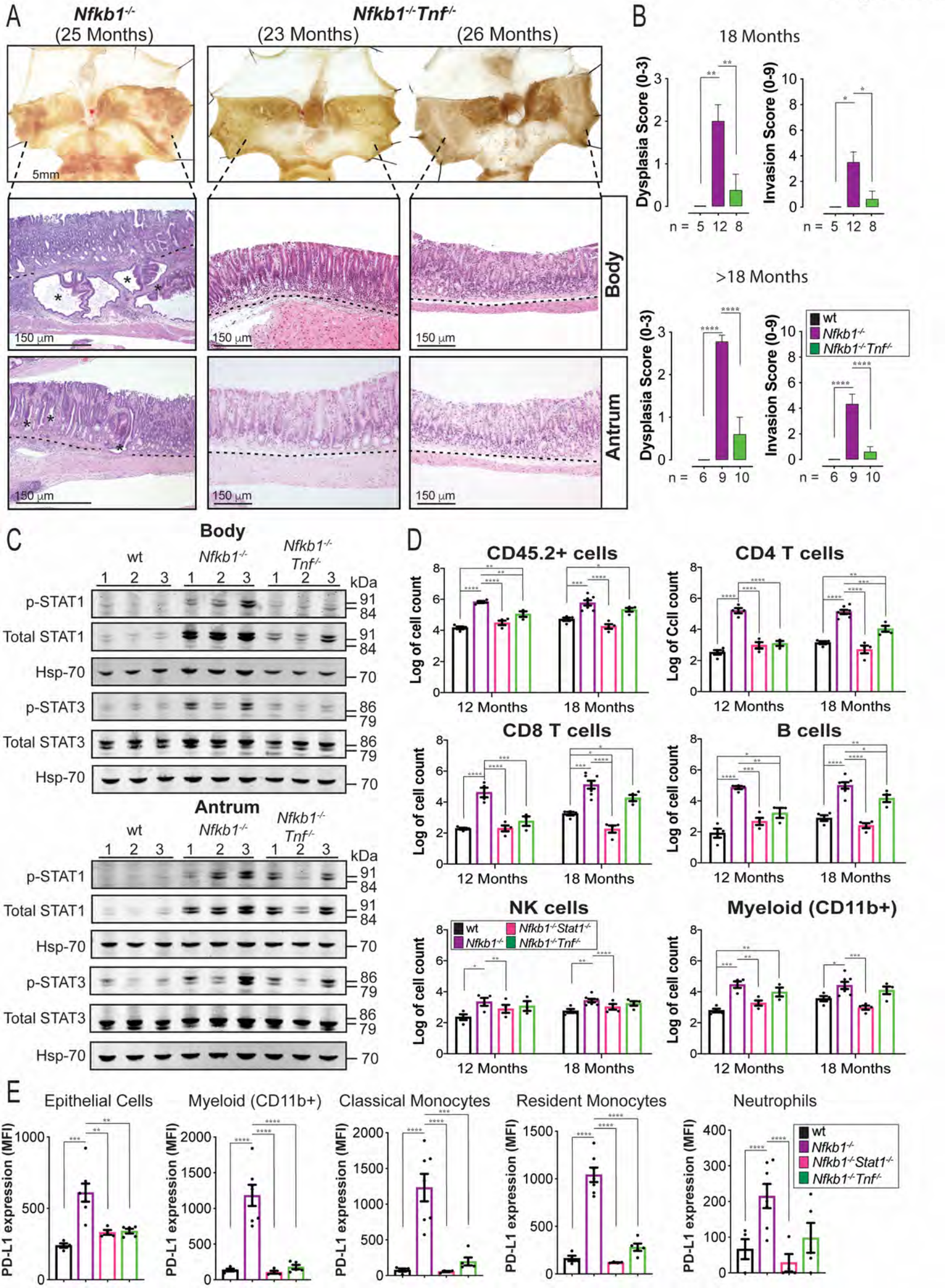
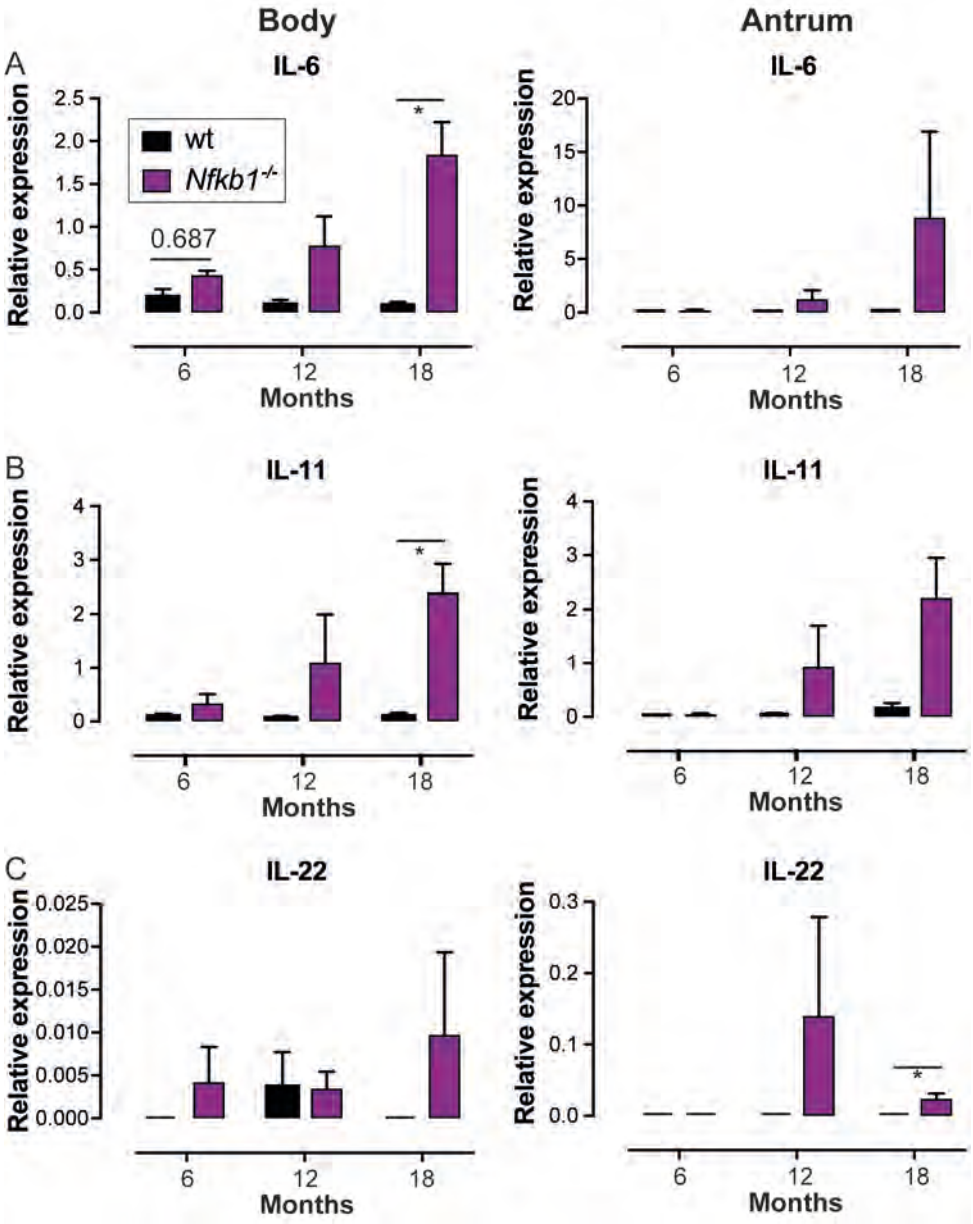

A
Body
Antrum
wt
Nfkb1-/-
Nfkb1-/-Stat1-/-
Nfkb1-/-Tnf-/-
Nfkb1-/-Tnf-α-/-
300 μm
B
18 Months
Gastritis score (0-3)
Oxyntic atrophy score (0-3)
n = 5 12 8 5
>18 Months
wt
Nfkb1-/-
Nfkb1-/-Tnf-/-
Nfkb1-/-Stat1-/-
n = 6 9 10 12

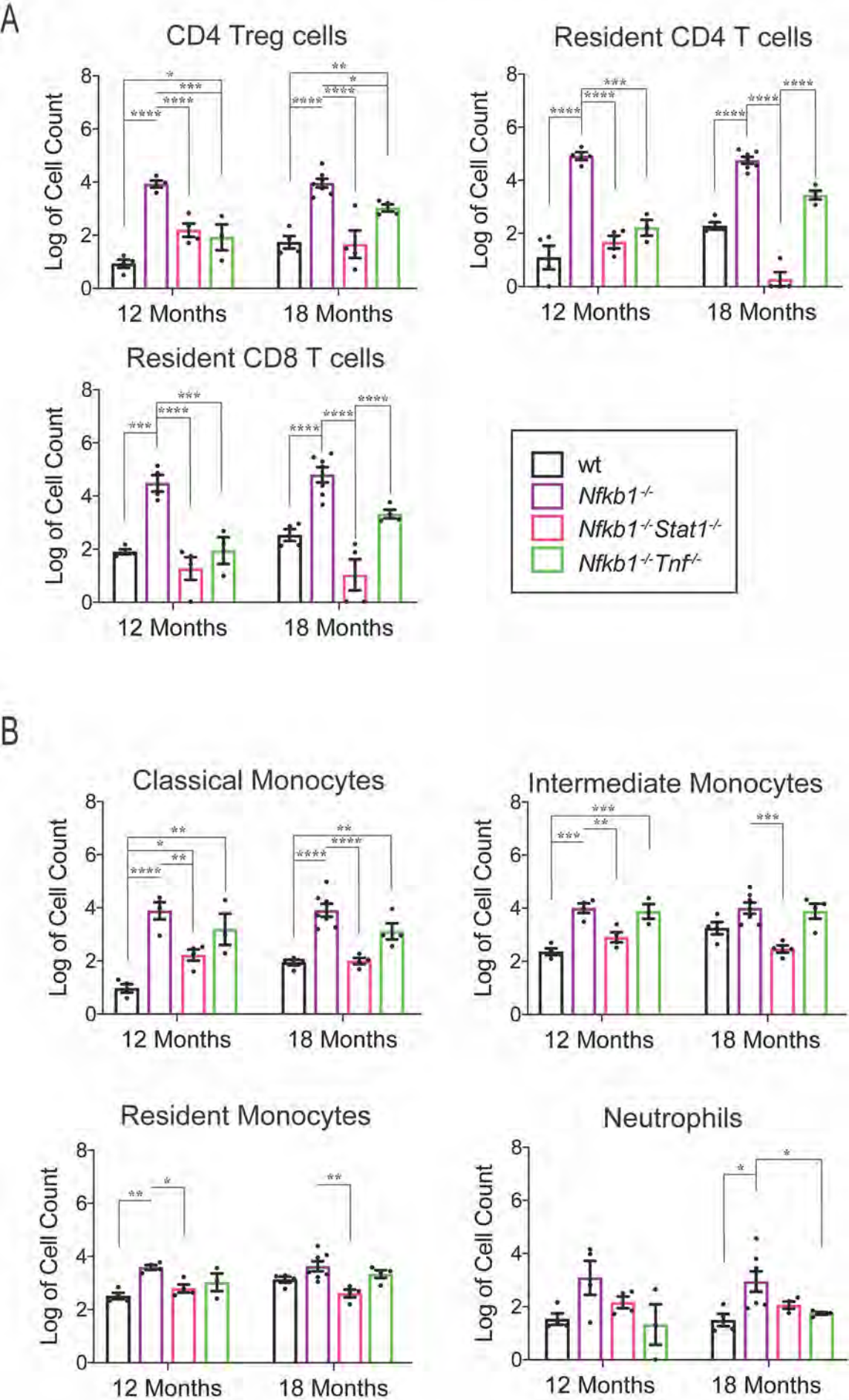
Figure 6


A
Nfkb1-/-
(25 Months)
Nfkb1-/-Tnf-/-
(23 Months)
(26 Months)
5mm
Body
Antrum
150 µm
B
18 Months
Dysplasia Score (0-3)
Invasion Score (0-9)
n = 5 12 8
>18 Months
n = 6 9 10
wt
Nfkb1-/-
Nfkb1-/-Tnf-/-
C
Body
Antrum
wt
Nfkb1-/-
Nfkb1-/-
Tnf-/-
kDa
p-STAT1
Total STAT1
Hsp-70
p-STAT3
Total STAT3
91
84
86
79
70
D
CD45.2+ cells
CD4 T cells
CD8 T cells
B cells
NK cells
Myeloid (CD11b+)
Log of cell count
12 Months
18 Months
wt
Nfkb1-/-
Nfkb1-/-Stat1-/-
Nfkb1-/-Tnf-/-
E
Epithelial Cells
Myeloid (CD11b+)
Classical Monocytes
Resident Monocytes
Neutrophils
PD-L1 expression (MFI)

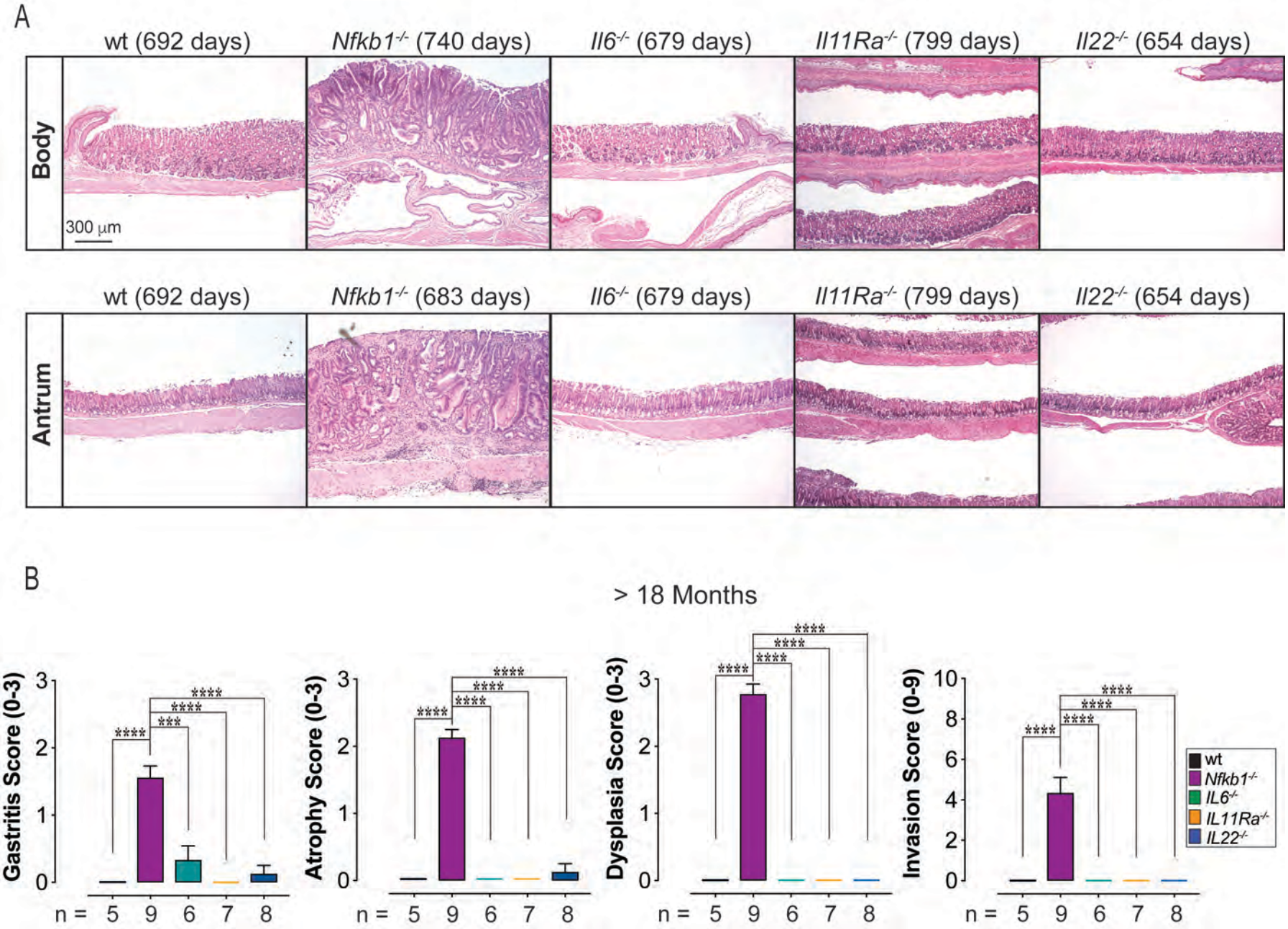
Supplementary Figure 1



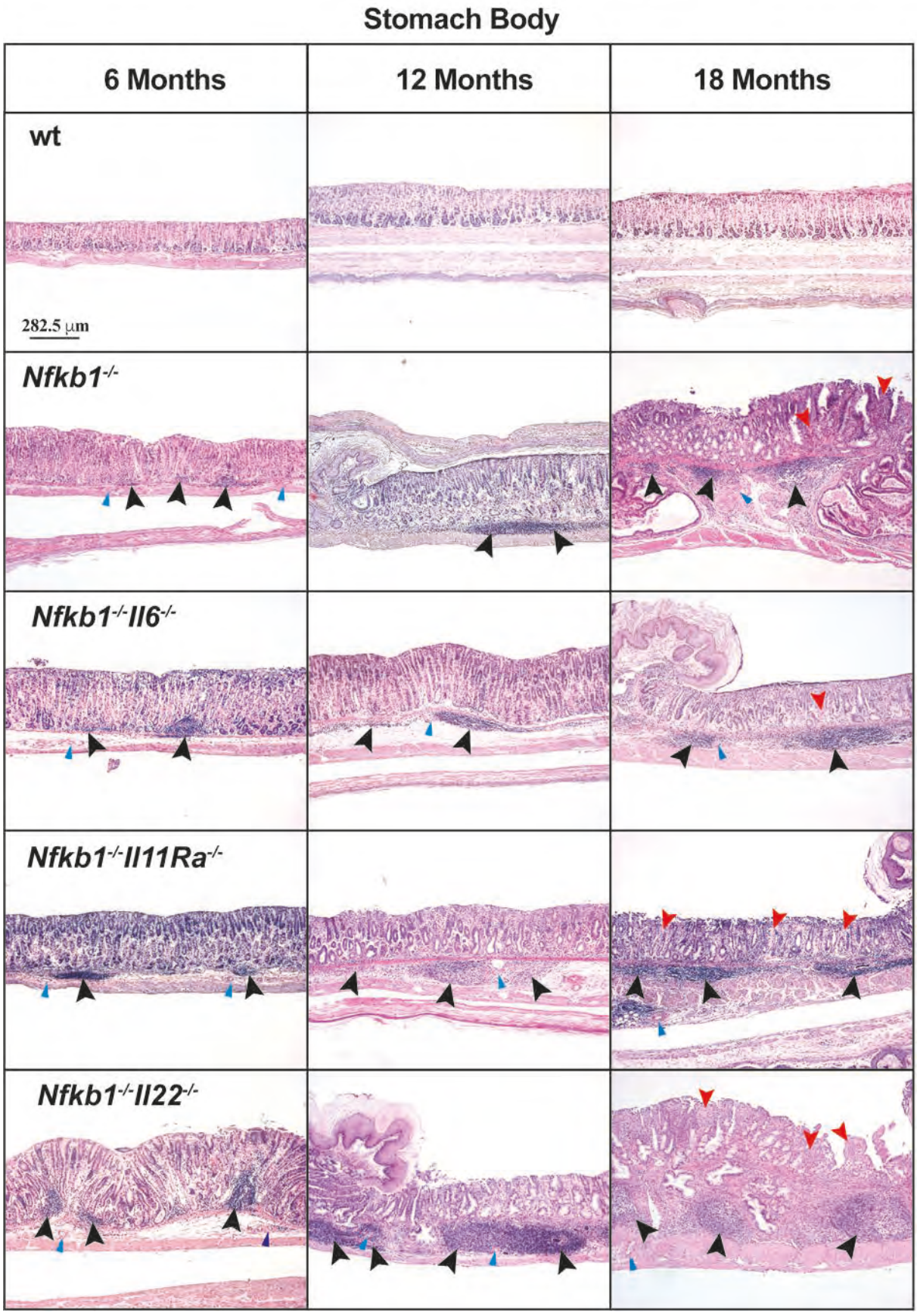
Supplementary Figure 10



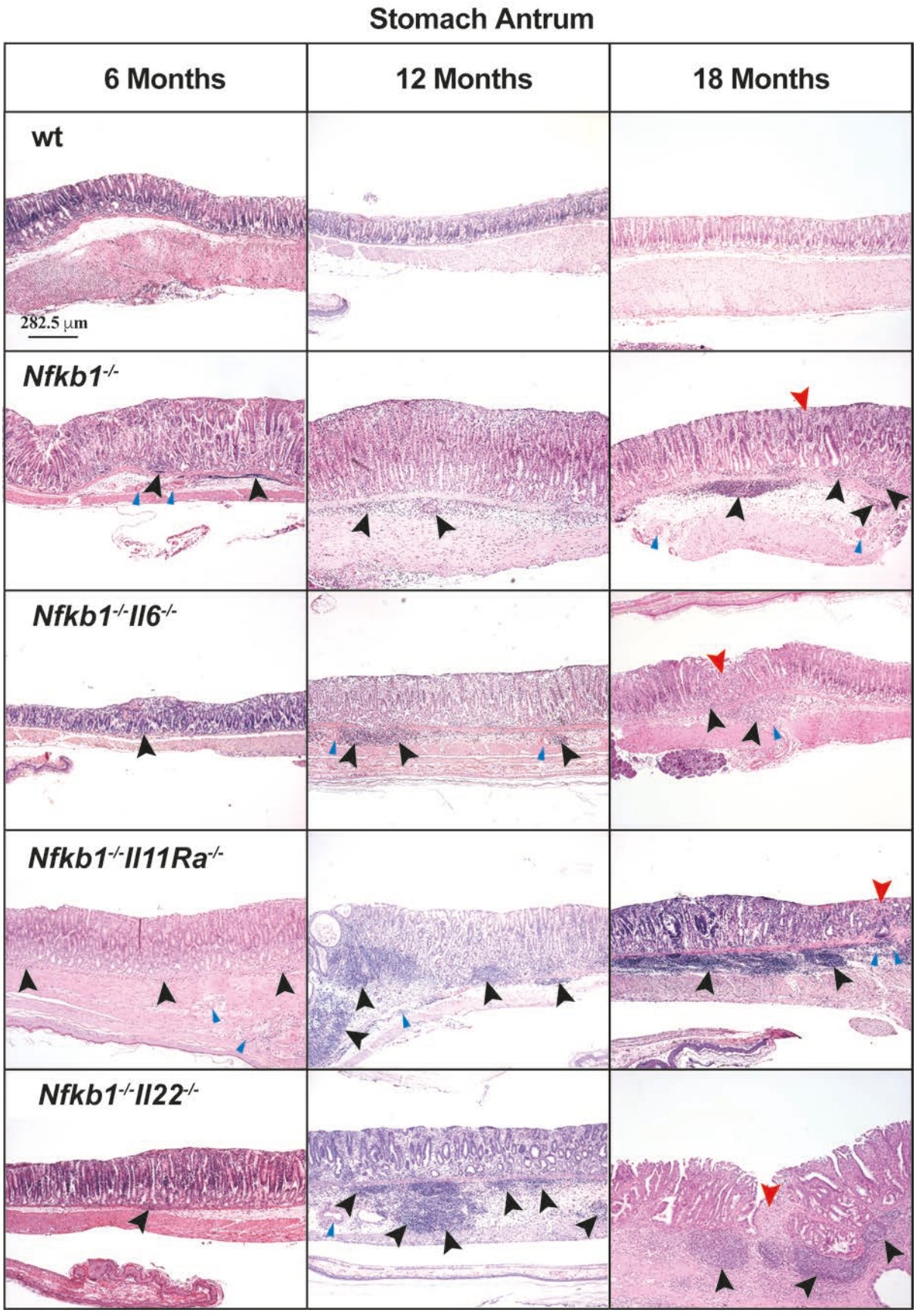
Supplementary Figure 2



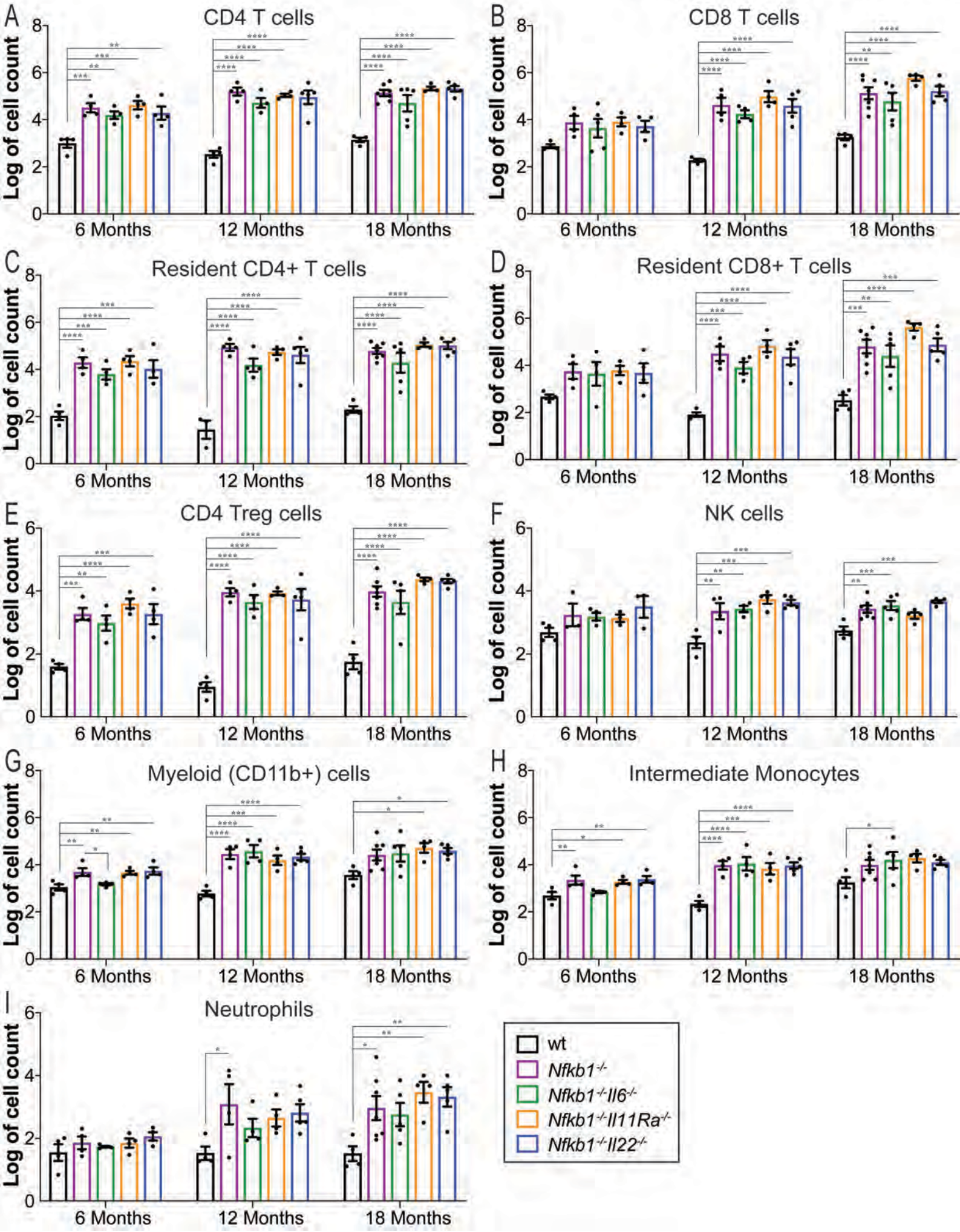
Supplementary Figure 3



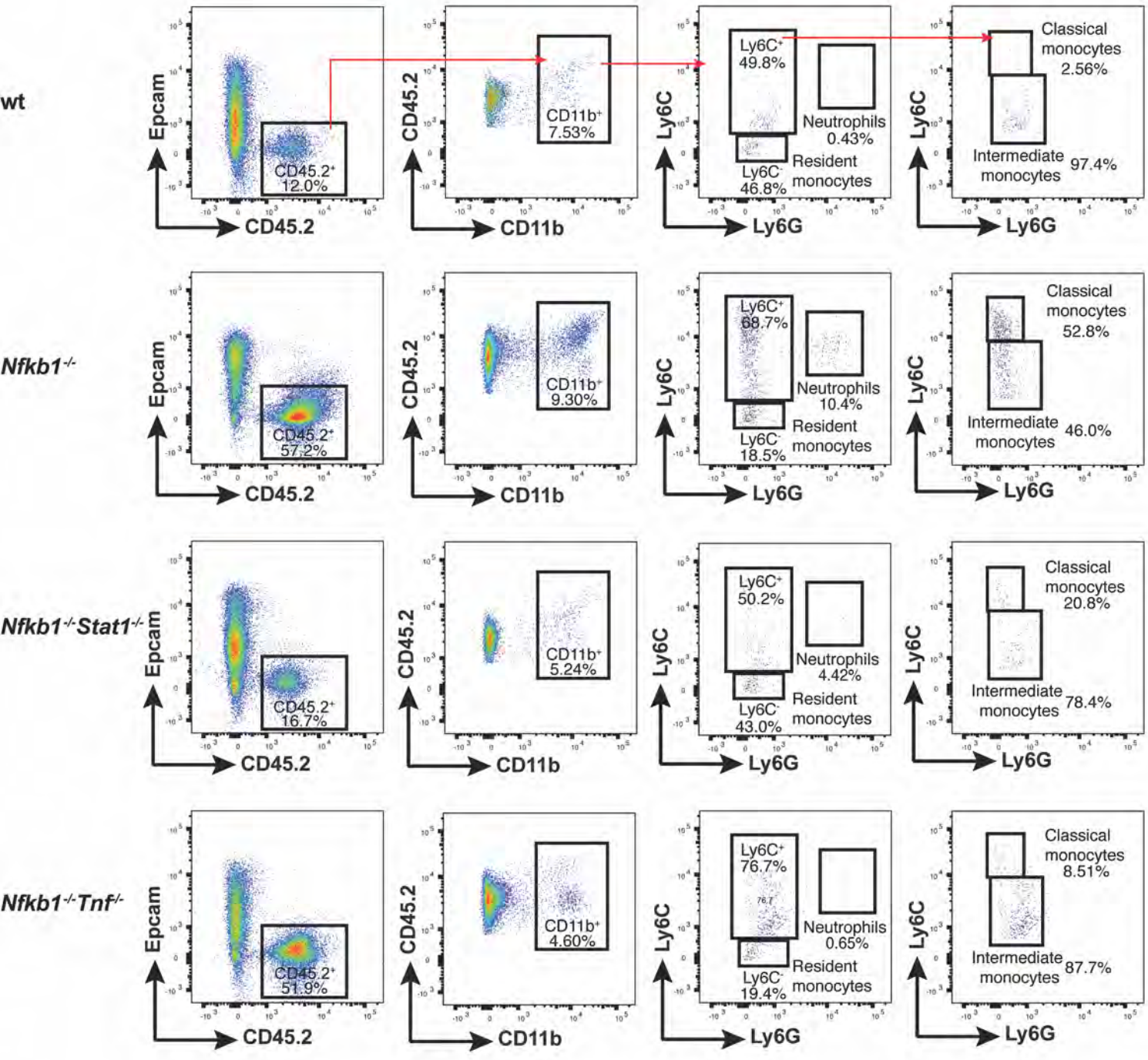
Supplementary Figure 4



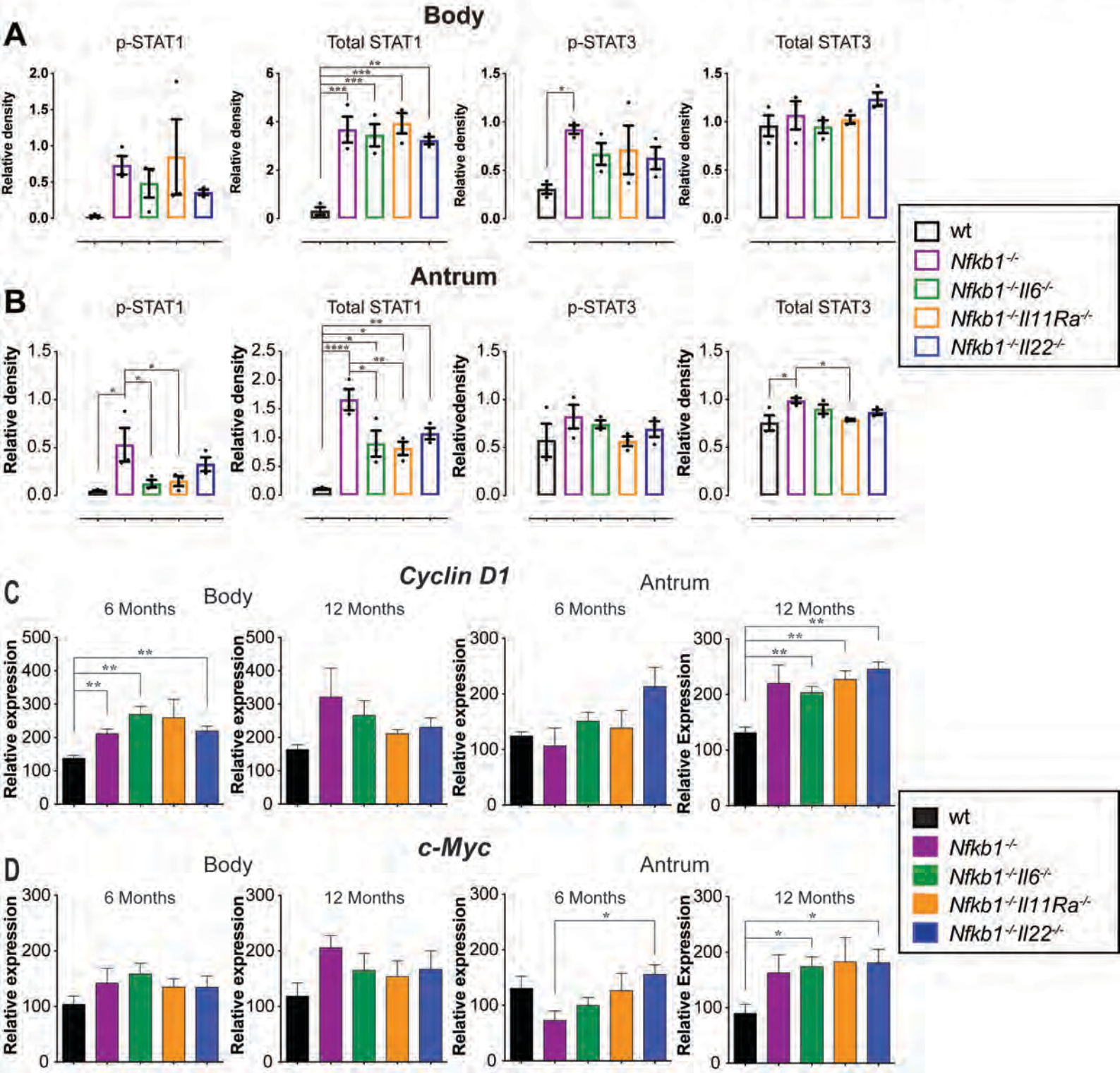
Supplementary Figure 5



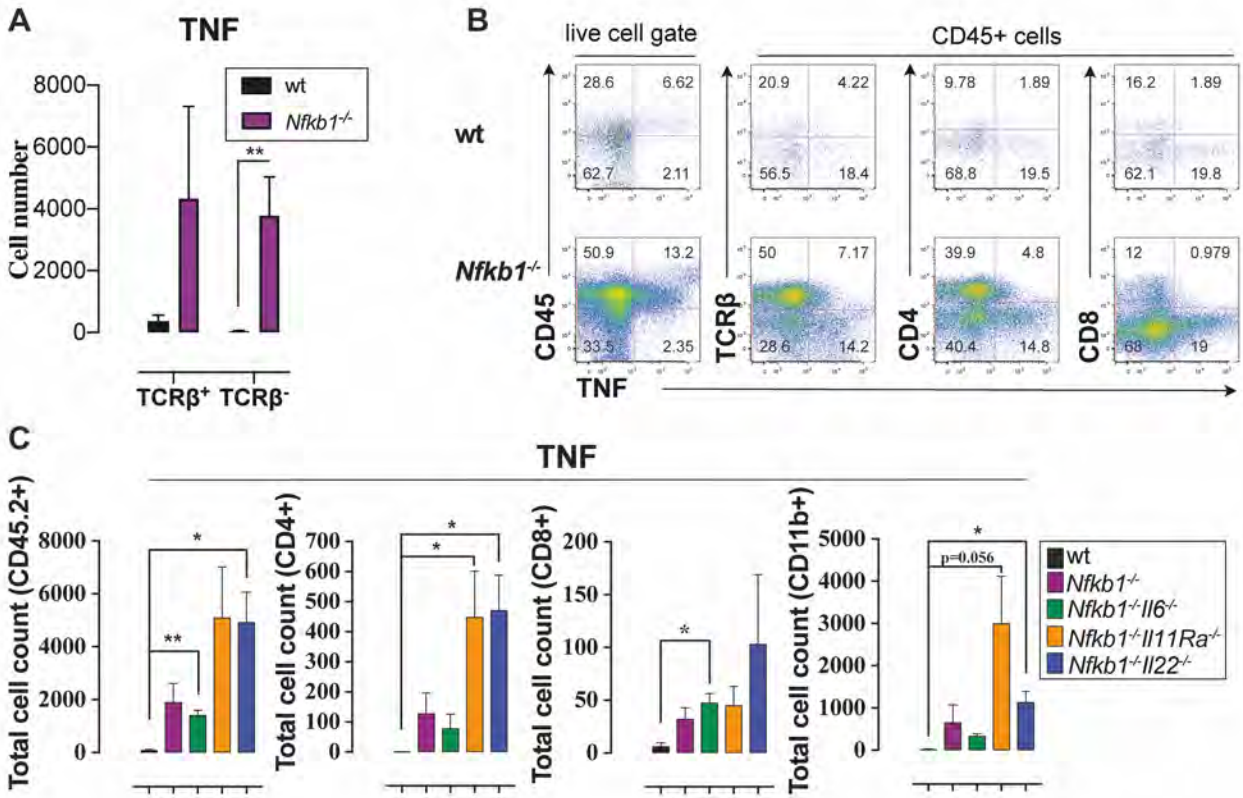
Supplementary Figure 6



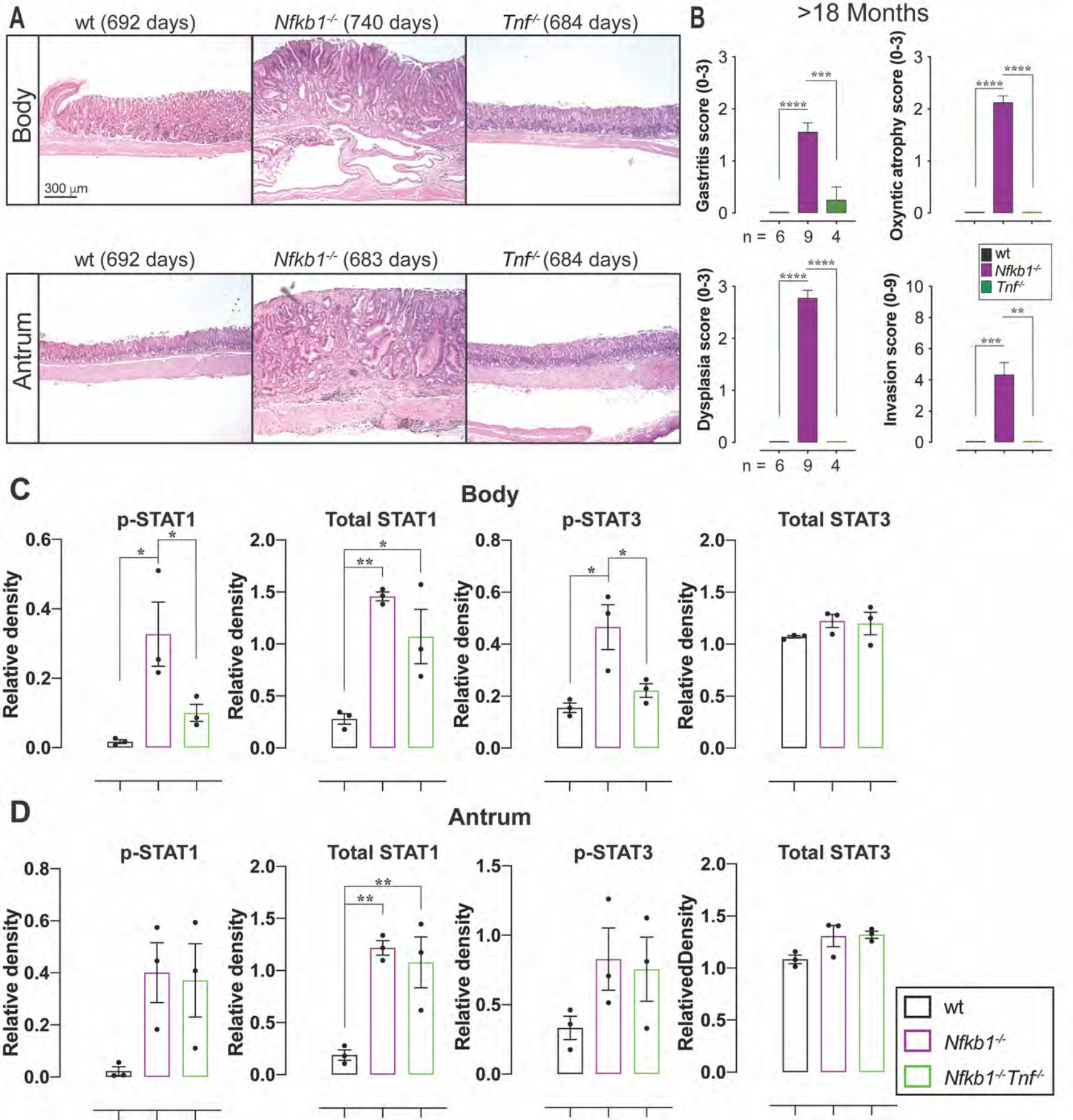
Supplementary Figure 7



Supplementary Figure 8



Supplementary Figure 9



What you need to know:

Background and Context: Activity of NFκB transcription factors and signaling via STAT transcription factors are altered in gastric cancer cells. NFKB1-knockout mice develop invasive gastric cancer and their gastric tissues have increased levels of cytokines, including (IL)6, IL22, IL11, and tumor necrosis factor (TNF), and increased activation of STAT1.

New Findings: Studies of knockout mice showed that loss of NFKB1 causes increased expression of TNF in the stomach and thereby drives activation of STAT1, resulting in an inflammatory immune response and the development of gastric cancer. IL11Rα appears to contribute to the progression of gastric tumors to the invasive stage.

Limitations: These studies were performed in mice; follow-up clinical investigations are needed in humans.

Impact: Inhibitors of TNF and possibly also inhibitors of IL11 or IL11Rα might be useful in the treatment of gastric cancer.

Lay Summary: The authors identified a signaling pathway involving the cytokine TNF and the transcription factor STAT1 that when aberrantly activated causes inflammation and gastric tumor development in mice.