PPARγ-activation increases intestinal M1 macrophages and mitigates formation of serrated adenomas in mutant KRAS mice

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ABSTRACT
To identify novel hubs for cancer immunotherapy, we generated C57BL/6J mice with concomitant deletion of the drugable transcription factor PPARγ and transgenic overexpression of the mutant KRASG12V oncogene in enterocytes. Animals developed epithelial hyperplasia, transmural inflammation and serrated adenomas in the small intestine with infiltration of CD3+ FOXP3+ T-cells and macrophages into the lamina propria of the non-malignant mucosa. Within serrated polyps, CD3+ CD8+ T-cells and phosphorylated ERK1/2 were reduced and the senescence marker P21 and macrophage counts up-regulated, indicative of an immunosuppressive tissue microenvironment. Treatment of mutant KRASG12V mice with the PPARγ-agonist rosiglitazone augmented M1 macrophage numbers, reduced IL4 expression and diminished polyp load in mice. Rosiglitazone also promoted M1 polarisation of human THP1-derived macrophages and decreased Il4 mRNA in isolated murine lymphocytes. Thus, inhibition of the oncogenic driver mutant RAS by PPARγ in epithelial and immune cell compartments may be a future target for the prevention or treatment of human malignancies associated with intestinal inflammation.

Introduction
Inflammatory bowel diseases (IBD), such as Morbus Crohn and Ulcerative Colitis, have been associated in human genome-wide association studies with multiple gene aberrations including those in major oncogenic drivers like KRAS.1 KRAS mutations confer an increased risk for development of precancerous lesions which may progress to cancer after many years. Activating point mutations in KRAS (G12/13D, G12 V e.a.) and NRAS genes are frequently found in patients with sporadic and colitis-associated colorectal cancer (CRC), and introduction of mutated KRAS alleles in mice induces tumors in different organs of the gastrointestinal (GI) tract.2

Overexpression of human mutant KRAS in enterocytes initiates the formation of two morphological subtypes of adenomatous polyps in mice defined by their histological appearance as tubular3 and serrated4 adenomas. The recent molecular reclassification of CRC subtypes in humans assigned the serrated morphology to RAS-pathway mutations.5 A plethora of chemokines, cytokines and nuclear factor kappa B-dependent genes are involved in the initial recruitment of inflammatory cells into the lamina propria by clusters (“field effect”) of RAS-mutated epithelial cells. This fact led to the successful clinical administration of neutralizing antibodies against tumor necrosis factor-alpha (TNFα) (like infliximab).1 Nonetheless, the molecular players which underlie and drive mutant RAS-driven progression of inflammation in the lamina propria to malignant transformation of epithelial cells have only been uncovered in part, and drugable targets which prevent or reverse this transition are urgently needed.

Peroxisome proliferator activated receptor-gamma (PPARγ) is a nuclear transcription factor promoting differentiation of epithelial cells and immune defence in the intestinal tract.6 PPARγ is expressed throughout the GI tract with high levels in the colorectum, though defective in IBD.7 PPARγ inhibits inflammation and, thereby, may prevent cancers associated with chronic inflammation. It is activated by dietary and inflammatory lipids such as nitrated linoleic acid and products derived from nitric oxide (NO) synthases. PPARγ enhances expression of mucosal defensins8 and regulates innate and adaptive immune responses in the intestine, lung and white fat, e.g. by directing macrophage (Mph/MPH) polarization (M1/ M2),9 dampening Th17-driven inflammation10 and up-regulating suppressor cells (e.g. regulatory T-cells11).

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Numerous preclinical studies evinced beneficial metabolic and anti-inflammatory effects of PPARγ, whereas the results on cell proliferation and tumor growth were less consistent. Severe side effects led to the discontinuation of PPARγ-agonists of the glitazone class (pioglitazone) for chronic use in patients with type 2 diabetes mellitus (T2DM), while still holding the promise for applications against lethal diseases including cancer (e.g. melanoma, prostate). Recent pilot clinical studies confirmed therapeutic efficacy of glitazones on liver disease, cerebral stroke and chronic myeloid leukemia, indicative of a potential clinical benefit of combination therapies. The RAS-RAF-MEK1/2-ERK1/2 cascade leads to phosphorylation and posttranslational inactivation of PPARγ, thereby potentially counteracting its protective effect. We therefore raised the hypothesis that constitutively active RAS-ERK1/2 signaling contributes to repression of PPARγ activity in vivo and facilitates progression of inflammation to malignancy.

Results

Generation of PPARγ-deficient mutant KRASG12V transgenic mice

To explore the functional interaction between PPARγ and RAS in the mouse intestinal epithelium in vivo, double transgenic hemizygous C57BL/6j mice for Cre-recombinase and the constitutively active human KRASG12V mutation, both under control of the murine villin promoter, were interbred with homozygous floxed Pparg mice to selectively ablate PPARγ in intestinal epithelial cells (RAS PPARΔIEC) (Fig. 1A). Nomenclature and abbreviations are presented in Table S1. Homozygous progeny with excision of both Pparg alleles in presence of mutant KRAS was not obtained according to expected Mendelian ratios and excluded from the study (Table S2). Cre-recombinase driven by the murine lysozyme promoter yielded viable offspring with deletion of both Pparg alleles in myeloid cells (RAS PPARΔMPI). Kaplan-Meier curves (Fig. 1B) evinced that PPARγ-deficiency in myeloid cells (including macrophages) shortens the life span of mutant KRAS transgenic mice compared with WT mice (p < 0.0001 RAS PPARΔMPI (n = 53) vs. WT (n = 149), log-rank test). Animals succumbed to massive splanchnegaly and myeloproliferative disease and were not further studied. All other genotypes did not reveal any significant differences in survival.

Western blot analysis of GTPase pull-down assays (Fig. 1C) detecting active GTP-bound pan-RAS proteins in whole tissue lysates using RALGDS-GST as a bait confirmed the presence of active RAS in the small intestine (SI). Genomic PCRs on DNA isolated from organs of the respective genotypes corroborated (Fig. 1D) Cre-mediated Pparg exon1/2 excision. FFPE tissue sections of the small and large intestine (colon) from WT and PPARΔIEC mice were then stained by IHC with PPARγ Ab. The strong nuclear staining positivity in enterocytes was consistent with high penetrance and late onset over up to 2 years. Microscopic images of H&E staining in FFPE tissue sections (Fig. 2A) visualized the complete SI (“Swiss roll”) with hyperplastic epithelium and goblet cells. Quantitative analysis of H&E histopathology (n ≥ 4 per genotype) was then performed (Fig. 2B). As a surrogate indicator of transmural inflammation, the distance (i.e. diameter) between the crypt base until the tunica adventitia (serosa) were measured in μm (p < 0.05, Mann Whitney test). In addition, the number of cases (mice) with more or less than 5 polyps and having discrete or extended polyps per total SI were counted (p < 0.05, Fisher Exact test). RAS PPARΔIEC mice developed a more pronounced transmural inflammation (“bowel wall thickening”) and extended hyperplasia in the SI (mainly in the terminal ileum) and the proximal colon as compared with PPARγ-proficient littermates. Upon a latency of > 12 months, hyperplastic polyps (HPP) and serrated adenomas (SA) with goblet cell hyperplasia were frequently observed together with the formation of aberrant crypt foci (ACF) and infrequent sessile serrated adenomas (SSA) with dysplasia and very few high-grade dysplastic tubular adenomas (Ad) (Table S2). Importantly, serrated-type lesions predominated in the lower SI but coexisted with single cases of tubular-type lesions in the duodenum. Mice rarely progressed to macroscopic tumors with flat white morphology at an age of 2 years (not shown). This observation was consistent with the multi-step patterns and molecular subtypes of human GI cancers, where single mutations are insufficient to drive progression from adenoma to carcinoma.

PPARγ-deficiency augments mutant KRAS-mediated immune cell infiltration

To exclude the possibility that gut microbes penetrate the intestinal epithelium in mutant KRAS mice to expose the lamina propria to antigens, FFPE tissues from WT, RAS and RAS PPARΔIEC mice were stained for immunofluorescence microscopy. E-cadherin staining remained at the intercellular membrane (SI), and no nuclear translocation of β-catenin was observed (not shown). RT-qPCR analysis from snap-frozen tissues of WT and RAS mice (n ≥ 3 per genotype, n.s., Mann Whitney test) revealed no significant changes in mRNA expression of the tight junction protein zonula occludens-1 (Zo1). Thus, mutant RAS mice seem to maintain epithelial barrier integrity.

To test whether the epithelial RAS mutation is sufficient to drive immune cell recruitment into the lamina propria, IHC on FFPE tissue sections from WT, RAS and RAS PPARΔIEC mice was done with Abs detecting major leukocyte/lymphocyte protein (Aco) (Fig. 1F).

Western blot analysis of whole tissue lysates also demonstrated reduced PPARγ protein (Fig. 1G). Conversely, phosphorylation of RAS target proteins, such as ERK1/2, AKT and GSK3β, was increased. Thus, this mouse strain allowed us to study the impact of active RAS on the intestinal epithelium on a background of enterocyte-specific deletion of PPARγ.

PPARγ-deficiency exacerbates pre-malignant phenotypes driven by mutant KRAS

Consistent with previous reports, carcinogenesis occurred in RAS mice with high penetrance and late onset up to 2 years. Microscopic images of H&E staining in FFPE tissue sections (Fig. 2A) visualized the complete SI (“Swiss roll”) with hyperplastic epithelium and goblet cells. Quantitative analysis of H&E histopathology (n ≥ 4 per genotype) was then performed (Fig. 2B). As a surrogate indicator of transmural inflammation, the distance (i.e. diameter) between the crypt base until the tunica adventitia (serosa) were measured in μm (p < 0.05, Mann Whitney test). In addition, the number of cases (mice) with more or less than 5 polyps and having discrete or extended polyps per total SI were counted (p < 0.05, Fisher Exact test). RAS PPARΔIEC mice developed a more pronounced transmural inflammation (“bowel wall thickening”) and extended hyperplasia in the SI (mainly in the terminal ileum) and the proximal colon as compared with PPARγ-proficient littermates. Upon a latency of > 12 months, hyperplastic polyps (HPP) and serrated adenomas (SA) with goblet cell hyperplasia were frequently observed together with the formation of aberrant crypt foci (ACF) and infrequent sessile serrated adenomas (SSA) with dysplasia and very few high-grade dysplastic tubular adenomas (Ad) (Table S2). Importantly, serrated-type lesions predominated in the lower SI but coexisted with single cases of tubular-type lesions in the duodenum. Mice rarely progressed to macroscopic tumors with flat white morphology at an age of 2 years (not shown). This observation was consistent with the multi-step patterns and molecular subtypes of human GI cancers, where single mutations are insufficient to drive progression from adenoma to carcinoma.
populations (n ≥ 3 per genotype). Elevated immune cell counts in the lamina propria with single intraepithelial lymphocytes (IEL) and macrophages were recorded (S2). Quantitative analysis of staining intensity and frequency (Fig. 2C) scores revealed an increase (WT < RAS < RAS PPARΔIEC) in Tregs (FOXP3+) ("p < 0.05 vs. RAS PPARΔIEC, Kruskal Wallis test). Macrophages (F4/80+) and pan-T-cells (CD3+) displayed a similar trend, whereas pan-B-cell (CD45R/B220+) counts were unchanged. Thus, epithelial PPARγ-deficiency augmented immune cell infiltration driven by epithelial mutant KRAS.

Figure 1. Characteristics and validation of mouse genotypes. A, Breeding scheme. Nomenclature and abbreviations are presented in Table S1. B, Kaplan-Meier survival curves. PPARγ-deficiency in myeloid cells such as macrophages (MPh/MPH) but not in the intestinal epithelium (IEC) shortens the life span compared with WT mice ("p < 0.0001 RAS PPARΔMPH (n = 53) vs. WT (n = 149), log-rank test). Legend: "1" = event (death); "0" = alive or censored. C, Western blots of pull-down assays detecting active GTP-bound pan-RAS proteins in mouse whole tissue lysates compared to total pan-RAS (input) using RALGDS-GST as a bait. Representative gels are presented. KRASG12V-mutant human CRC cell line SW480 was a positive control. D, Pparg exon1/2 excision. Genomic PCRs were conducted on DNAs isolated from organs of the respective genotypes. Representative agarose gels are presented. Expected amplicon sizes are indicated. E, PPARγ protein expression. FFPE tissue sections from WT and PPARΔIEC mice colon (Co) were stained with PPARγ Ab for immunohistochemistry (IHC). Magnification 200x. F, PPARγ target gene expression. Total RNA was extracted from snap-frozen small intestine (SI). CT-values from RT-qPCRs on acyl-CoA oxidase (Aco) were normalized to B2-microglobulin (B2 m) and calculated as -fold ± S.E. (n = 3 per genotype, "p < 0.05, Mann Whitney test). G, PPARγ and RAS target protein expression. Whole tissue lysates (from Co and SI) were subjected to Western blotting. Representative gels and quantitative analysis are shown. O.D. values of bands in gels were normalized to HSP90 and calculated as -fold ± S.E. compared with WT mice (n = 2 per genotype, "p < 0.05, Wilcoxon signed rank test).
Mutant KRAS promotes senescence and immune escape in serrated polyps

Prolonged RAS-activation can result in oncogene-induced senescence (OIS). Since most polyps did not progress to carcinoma, we performed IHC on tissue sections of RAS and RAS PPAR\textsuperscript{ΔIEC} mice (n=3 per genotype) with Abs detecting P-ERK1/2 as a marker of active RAS-signaling and P21 (CIP1/WAF1) as a marker for OIS (Fig. 3A). IHC staining underwent quantitative analysis for absolute numbers of cases having high
(scores +2,+3) or low (scores 0,+1) positivity in polyps vs. the non-malignant mucosa (\(^p < 0.05\), Fisher Exact test). Polyps were summarized from the following morphologies; hyperplastic polyp (HPP), serrated adenoma (SA) and sessile serrated adenoma (SSA). Aberrant crypt foci (ACF) and tubular adenomas (Ad) were infrequent and excluded from the analysis. Notably, the staining positivity (Fig. 3B) of P-ERK1/2 in polyps was reduced, while P21 was increased compared with the non-malignant mucosa, indicative of an arrest of RAS-ERK1/2-driven proliferation in favour of RAS-induced senescence. No difference was stated between polyps of the two genotypes (not shown). These data suggested that PPAR\(\gamma\) is dispensable once adenoma formation has been initiated by mutant RAS, in contrast to its immunoregulatory role in the hyperplastic pre-malignant mucosa where it counteracts mutant RAS effects.

Immune evasion develops during progression of pre-malignant lesions to cancer. Therefore, we separately analyzed the immune cell infiltration of the normal non-malignant mucosa vs. polyps. Quantitative analysis of IHC (Fig. 3C) on tissue sections of RAS and RAS PPAR\(\gamma\) mice (n=3 per genotype, \(^p < 0.05\), Fisher Exact test) revealed that the staining positivity of macrophages (F4/80+) remained high in polyps, while T-cell (CD3+) counts were reduced therein, indicative of a potential immune evasion mechanism in the lesions (S3). Again, there was no difference between polyps of the two genotypes (not shown), indicating that PPAR\(\gamma\) is sufficient for counteracting RAS-directed immune cell infiltration in the normal mucosa but is unable to prevent immune escape upon progression to adenoma.

**RAS-mediated phenotypes are attenuated by treatment of mice with PPAR\(\gamma\)-agonist**

Based on the previous findings that genetic targeting of PPAR\(\gamma\) reshapes mutant RAS-driven immune cell infiltration into the non-malignant intestinal mucosa, we next tested the reciprocal approach using pharmacological activation of PPAR\(\gamma\) by rosiglitazone (rosi). WT and RAS mice were fed a chow diet enriched with rosi (n\(\geq\)10 mice per treatment group and genotype) corresponding to an approx. dose of 25 mg/kg\ day for 4 months. Microscopic images of H&E staining in tissue sections visualized drug efficacy in “Swiss rolls” showing the complete SI with extended vs. discrete serrated polyps in chow and rosi-treated animals (Fig. 4A). Quantitative analysis of H&E histopathology (Fig. 4B) failed to record any differences in transmural inflammation (n\(\geq\)6 mice per group, n.s., Mann Whitney test). The number of cases (mice) having more or less than 4 polyps per total SI (n.s., Fisher Exact test) were not changed either. Instead, the number of mice having extended polyps per total SI (p = 0.0505, Fisher Exact test) showed a trend for reduction by rosi. Similar results were obtained if all genotypes were subjected to statistical comparison (S4 and Table S2). Notably, rosi did not unfold its weight gain adverse effect in mice with mutant RAS, as evident from the quantitative analysis of body and spleen weights (n\(\geq\)10 per group, \(^p < 0.05\) vs. ROSI, One-way ANOVA) (Fig. 4C). IHC on tissue sections of WT, WT+ROSI, RAS and RAS+ROSI mice with Ab detecting Ki67 in the non-malignant mucosa and subsequent quantitative analysis (Fig. 4D) (n\(\geq\)4 per group, \(^p < 0.05\) vs. ROSI, Kruskal Wallis test) corroborated that PPAR\(\gamma\)-agonist reduced proliferation in the small intestinal crypts. Conclusively, these data showed that pharmacological activation of PPAR\(\gamma\) attenuated polyp formation in the murine SI.

**PPAR\(\gamma\)-activation increases M1 macrophages in the murine small intestine**

To assess the type of inflammation observed in the lamina propria of RAS mice, total RNA was extracted from snap-frozen mice SI, cRNA was hybridized to cDNA microarrays, and differential gene expression was analyzed as described in. Overall, genes related to inflammation and immunity were upregulated in the SI tissue of RAS mice compared with WT animals (S5). To validate the array data, RT-qPCRs were conducted (n = 6 per genotype, \(^p < 0.05\), Mann Whitney test) (Fig. 5A). Notably, the M1 marker iNOS (Nos2) was decreased, while all other inflammation and immunity-related mRNAs tested were increased in RAS vs. WT mice, indicative of a chronic inflammatory microenvironment with immunosuppressive characteristics.

To investigate the impact of PPAR\(\gamma\)-activation on the immune response profile, tissue sections of RAS and RAS+ROSI mice were then stained for IHC (Fig. 5B). Quantitative analysis revealed a trend that lamina propria infiltration of macrophages (F4/80+) into the non-malignant mucosa was slightly increased by rosi (n = 3 per group, p = 0.100, Mann Whitney test), whereas T-cells remained unaffected. To further distinguish between immune cell subsets, tissue sections of RAS and RAS+ROSI mice were stained with Abs detecting CD4+ for Th and CD8+ for Tc cells and iNOS+ for M1 and STAB1+/CD206+ for M2 polarized macrophages (Fig. 5C). Consistent with the above described RT-qPCR results, iNOS (M1) positivity was reduced in the non-malignant mucosa of RAS vs. WT mice. Conversely, lamina propria infiltration of M1 (iNOS+) macrophages into the non-malignant mucosa was increased by rosi (n\(\geq\)4 per group, \(^p < 0.05\) vs. ROSI, Mann Whitney test). Likewise, there was a trend for decreased M2 (CD206+/STAB1+) counts by rosi, whereas T-cell amounts were unchanged under all conditions. Representative images are shown in S6 and S7. These findings proposed that the RAS mutation supports an immune evasive M2-type, whereas PPAR\(\gamma\) activation favors a “killer” M1-type microenvironment.

To determine, whether immune cell counts were also changed in mutant RAS-driven serrated polyps, quantitative analysis of IHC staining was conducted in RAS and RAS+ROSI mice (n\(\geq\)3 mice per group and genotype, \(^p < 0.05\) vs. ROSI, Mann Whitney test) (Fig. 6A). Overall, the presence of CD8+ Tc (but not CD4+ Th) cells was reduced in polyps vs. the non-malignant mucosa, underscoring the existence of an immune evasion mechanism driven by mutant Kras. Nonetheless, rosi could not restore T-cell numbers within polyps. Instead, a trend was stated for a rise in M1 (iNOS+) and a decrease in M2 (STAB1+) positivity by rosi. Taken together, PPAR\(\gamma\)-agonist seems to rewire the M1/M2-type immune response by counteracting the effects of mutant Kras.

To characterize the inflammatory microenvironment in more detail, custom-made PCR arrays were designed (S8). Expression profiling of 84 genes involved in the communication between
tumor cells and cells related to inflammation and immunity was conducted to reveal differential regulation of genes by the PPARγ-agonist. Total RNA was extracted from snap-frozen mouse SI from RAS and RAS+ROSI mice, and cDNA (n = 1 per group) was used as an input for the Mouse Cancer Inflammation & Immunity Crosstalk RT2 Profiler PCR Array as detailed in the Supplementary Methods. For hit validation of immune-related genes differentially regulated by PPARγ-agonist, total RNA was extracted from snap-frozen SI from RAS and RAS+ROSI mice (Fig. 6B) and subjected to RT-qPCR analysis (n = 9-10 per group, *p < 0.05, Mann Whitney test). Rosi significantly down-regulated the Th2 marker cytokine IL4 and exerted a trend to up-regulate the Th1 marker...

Figure 3. Mutant KRAS promotes senescence and immune escape in serrated polyps independently of PPARγ. A, IHC on tissue sections of RAS and RAS PPARγIEC mice (n≥3 per genotype) with Abs detecting P-ERK1/2 and P21 as a marker for oncogene-induced senescence (OIS). Left: P-ERK1/2 positivity was reduced in polyps (arrow “p”) compared to the strong signal in crypts and basal regions of villi in the adjacent mucosa (arrow “m”); Right: P21 was increased in apical tips of polyps and interspersed lamina propria cells. No difference was observed between polyps of the two genotypes (not shown). B, Quantitative analysis of IHC in A. Number of mice having high (scores +2,+3) or low (scores 0,+1) positivity in polyps vs. non-malignant mucosa (*p < 0.05, Fisher Exact test). C, IHC using Abs against B-cells (B220/CD45R), T-cells (CD3), Tregs (FOXP3) and macrophages (F4/80) was calculated as in B (*p < 0.05, Fisher Exact test). Macrophages remained high in polyps, T-cells were reduced.
chemokine Cxcl10 \((p = 0.08)\). As a positive control, induction of the PPARγ-target gene Aco was detectable as well (not shown). These data suggested that PPARγ participates in reprogramming of the host immune response against mutant RAS-driven oncogenic effects by enforcing a “killer”-type tumor-attacking mode of macrophages which may extend to T-cell subpopulations (please see model in Fig. 7).

**PPARγ-activation increases M1 markers in human THP1-derived macrophages**

To test this hypothesis in an appropriate *in vitro* model, we employed human THP1-derived macrophages. Phorbolester (PMA) enhanced mRNA expression of markers for differentiation (CD14) and polarization to M1 (TNFA) and M2
Figure 5. RAS-mediated phenotypes are attenuated by PPARγ-agonist. A, Immune marker gene expression in mouse tissues. Total RNA was extracted from snap-frozen SI of WT and RAS mice. CT-values from RT-qPCRs normalized to β2 m were calculated as \( \text{fold} \pm \text{S.E.} \) (n = 6 per genotype, \( ^* p < 0.05 \) WT vs. RAS for all genes, Mann Whitney test). B, IHC on tissue sections of RAS and RAS+ROSI mice using Abs detecting T/B cells and total macrophages in the non-malignant mucosa. Data are means \( \pm \text{S.E.} \) (n = 3 per group, \( p = 0.100 \), Mann Whitney test). C, PPARγ-activation increases intestinal M1 macrophage numbers. IHC with Abs detecting subpopulations of macrophages. Data are presented as in B (n = 4 per group, \( ^* p < 0.05 \) vs. ROSI, Mann Whitney test).
(CD206) after treatment with cytokine cocktails as detailed in the Supplementary Methods (S9). Differentiated polarized macrophages (“M”) were then co-cultured with SW480 human CRC cells (“TU”) with and without rosi (“R”) (at 10μM) for 48h. RT-qPCRs were conducted on RNA extracted from both cell types. M1 but not M2 macrophages reduced mRNA expression for markers of proliferation (cyclin D1/CCND1), cell survival (BCL2), senescence (P21/CIP1) and metabolism (ACO) in co-cultured tumor cells (n = 3, *p < 0.05 M1 vs. M2, Two-way ANOVA). Consistent with our observation in mice, PPARγ-agonist also down-regulated M2 (CD163) but up-regulated M1(TLR4) marker expression in differentiated polarized macrophages co-cultured with tumor cells (n = 3, *p < 0.05 vs. rosi, Two-way ANOVA). In turn, tumor cells reduced M1 but not M2 marker expression in co-cultured macrophages. This reciprocal cross-talk provided additional experimental evidence that PPARγ-activation enforces an M1-dependent macrophages response leading to reduced tumor cell viability.

**PPARγ-activation decreases Th2 response markers in murine primary intestinal lymphocytes**

For further validation using an *ex vivo* primary cell model, lamina propria lymphocytes (LPLs) were isolated from SI of WT and RAS mice as detailed in the Supplementary Methods (S10). Flow cytometry (FC) confirmed the identity and purity of the LPL population. LPLs from WT mice were isolated and stained with Abs and viability dye (7AAD) as indicated in Table S3. First, lymphocytes were identified by gating based on forward and side scatter, followed by selection of single and live cells. CD3+ cells were selected, and CD4+ and CD8+ T-cell subpopulations identified and displayed as percentages from dot plots. LPLs were CD4+ (n = 7, *p < 0.05 CD4+ vs. negative,

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**Figure 6.** Immune-related marker genes/proteins are differentially regulated by PPARγ-agonist. A, IHC on tissue sections of RAS and RAS+ROSI mice using Abs detecting subpopulations of T-cells (top) and macrophages (bottom) in polyps vs. the non-malignant mucosa. Data are means ± S.E. (n=3 per group and genotype, *p < 0.05 vs. ROSI, Mann Whitney test). B, Total RNA was extracted from snap-frozen SI of RAS and RAS+ROSI mice. CT-values from RT-qPCRs normalized to B2 m were calculated as -fold ± S.E. (n = 9-10 per group, Mann Whitney test). If4 (*p < 0.05) was reduced by rosi, there was a trend for Cxcl10 ( p = 0.08) up-regulation.
Kruskal Wallis test), whereas intraepithelial lymphocytes (IELs) were CD8+. RT-qPCRs demonstrated that LPLs from KRAS mutant animals displayed elevated marker mRNAs for the Th2 immune response compared with WT (n = 5 per genotype, \(p < 0.05\) WT vs. KRAS, Two-way ANOVA), confirming our PCR results from mixed tissue samples. LPLs from WT mice (n = 6) were then incubated with or without rosi (10 \(\mu\)M) for 16h before RNA extraction. RT-qPCRs again showed that PPAR\(\gamma\)-agonist decreased the mRNA levels of the Th2 cytokine Il4 \(ex\) \(vivo\) in isolated intestinal lymphocytes as before \(in\) \(situ\) in intestinal tissues of rosi-treated WT mice. A similar trend was obtained from lymphocytes isolated from spleens (n = 3). Taken together, PPAR\(\gamma\) seems to be able to shape immune cell response profiles in tissue environments and isolated primary cells.

**Discussion**

In this study, we demonstrate that PPAR\(\gamma\)-deficiency exacerbates mutant KRAS-driven formation of serrated polyps, a phenotype which is mitigated by treatment with PPAR\(\gamma\)-agonist. At least one allele of intestinal epithelial PPAR\(\gamma\) was necessary for viability of KRAS-mutant mice, underscoring the indispen-
sable role of this nuclear receptor in embryonic development.\(^6\) KO of both PPAR\(\gamma\) alleles in myeloid cells also reduced the life span of KRAS-mutant mice, indicating that PPAR\(\gamma\) in mono-
cytes/macrophages and related cell types (e.g. dendritic cells and granulocytes) is important for health in adult animals. These survival data pointed at a functional crosstalk of epithelial cell compartments. We therefore asked whether PPAR\(\gamma\) is able to protect against mutant KRAS-imposed oncogenic stress in the intestine.

Mutant KRAS triggers recruitment of innate immune cells\(^{22}\) and Tregs\(^{23}\) to mouse organs including the pancreas and lung facilitating carcinogenesis within an immunosuppressive microenvironment. Likewise, epithelial\(^{24}\) and T cell-specific KO\(^{25}\) of PPAR\(\gamma\) in mice reshapes the immune response and exacerbates experimental colitis. Macrophage-specific KO of PPAR\(\gamma\) yielded corresponding results in other tissues (e.g. liver\(^{26}\) and lung\(^{27}\)). Consistent with these findings, we observed that KRAS-mutant mice with epithelial PPAR\(\gamma\)-deficiency suffered from infiltration of T-cells, both total CD3+ lymphocytes and FOXP3+ Tregs, and F4/80+ macrophages into the lamina propria of the non-malignant, yet hyperplastic, mucosa of the SI, resembling an IBD-like chronic inflammation.

To test if oncogene-induced chronic inflammation can be prevented, we activated PPAR\(\gamma\) systemically in mutant KRAS mice by a pharmacological approach as opposed to its cell-

restricted genetic deletion in epithelial and myeloid compart-

ments. Unexpectedly, long-term dietary administration of the PPAR\(\gamma\)-ligand rosiglitazone (25mg/kg’d for 4 months) to KRAS-mutant mice did not impact on pan-T (CD3+), Th/Tc (CD4+ /CD8+) or Treg (FOXP3+) cell infiltration rates, but rather augmented total (F4/80+) macrophages and M1 (iNOS+) counts at the expense of M2 (CD206+/STAB1+) cell numbers. Hence, PPAR\(\gamma\)-activation seems to enforce an immune “switch” in the lamina propria, favoring a “tumor-

attacking” rather than an immunosuppressive, tumor-promot-

ing microenvironment.

In line with previous reports\(^4\) and the recent molecular class-

ification of human CRC,\(^5\) mutant KRAS-animals not only developed chronic inflammation but also multiple serrated polyps associated with oncogene-induced senescence (OIS). ERK1/2 phosphorylation was strongly reduced within basal (crypt) and apical (villus) serrated regions of polyps accompanied by an increase of the cyclin-dependent kinase inhibitor (CDKI) P21(WAF1/CIP1), indicative of a senescence barrier that protects against progression to carcinoma. Notably, macrophages remained within polyps and expressed both M1/M2 markers. Conversely, T-cells were excluded from serrated lesions as compared with the adjacent normal, yet hyperplastic, small intestinal mucosa. In human CRC, KRAS mutations correlate with MYC-dependent up-regulation of sirtuin-1 (SIRT1),\(^{28}\) a deacetylase of P53 and PPAR\(\gamma\), leading to their inactivation. Both, mutant RAS\(^4\) and PPAR\(\gamma\)\(^6\) up-regulate P21 and promote senescence in mice, and P53-mediated cell cycle checkpoints are often by-passed due to mutation during pro-

gression of adenoma to carcinoma. We may thus conclude that mutant KRAS-driven serrated polyps harbor a senescence barrier, which consists of OIS leading to up-regulation of CDKIs and immunological escape evident by removal of CD8+ T-cells and retention of macrophages within the lesions. However, the underlying molecular players which connect genetic and immune aspects of senescence (e.g. SASP) remain to be defined. Notably, the PPAR\(\gamma\)-agonist did not recover T-cell counts within serrated polyps, but instead tipped the balance from a M2/Th2 towards a M1/Th1 marker profile both in polyps and the pre-malignant mucosa and reduced overall polyp load in the SI. Addressing PPAR\(\gamma\) may thus be a strategy for preven-

tion or intervention of inflammation-associated malignancies.

PPAR\(\gamma\) has been firmly established as an anti-inflammatory factor, e.g. by epigenetic trans-repression of NFkB/AP1-dependent pro-inflammatory genes in immune cells,\(^{29}\) and its agonists exert (pre)clinical benefit in IBD\(^\circ\) and other inflamm-

ation-associated conditions (i.e. atherosclerosis, asthma, meta-

bolic syndrome). PPAR\(\gamma\) is also a master regulator of
macrophage differentiation, polarization and function towards the M2 phenotype,\textsuperscript{9} e.g. in resident macrophages in the white adipose tissue of diabetic mice\textsuperscript{30} and in murine and human tumor-associated macrophages (TAMs).\textsuperscript{31} The presence of TAMs usually predicts poor prognosis in cancer patients,\textsuperscript{32} whereas epithelial PPARγ expression correlates with improved survival in human CRC,\textsuperscript{33} indicative of compartment-specific function of PPARγ in enterocytes vs. macrophages. This concept was further supported by studies in lung\textsuperscript{44} and breast\textsuperscript{45} cancer mouse models.

In contrast, recent research underscored a more versatile role for PPARγ in context- and cell type-dependent reprogramming of the immune response. For example, pathogen elimination is facilitated by PPARγ-polarized macrophages via enhanced phagocytosis and release of NO, reactive oxygen species, cytotoxic cytokines and altered glucose metabolism (e.g. against *Pseudomonas*,\textsuperscript{36} *Mycobacterium*,\textsuperscript{37} *Trypanosoma*,\textsuperscript{38} *Leishmania*,\textsuperscript{39} *Plasmodium*\textsuperscript{40} and *Candida*\textsuperscript{41}) in mice and in patients with chronic granulomatous disease (CGD).\textsuperscript{42} Non-self recognition of tumors as an “internal pathogen” may be exploited by the same mechanism. PPARγ-activation by agonists reverses M2-mediated cytotoxic T-lymphocyte (CTL) suppression\textsuperscript{43} in favour of M1-dependent phagocytosis and killing activities. Specifically, this treatment augmented removal of apoptotic and dead cells by efferocytosis.\textsuperscript{44} Conversely, inhibition of PPARγ (by overexpression of a dominant-negative mutant) in myeloid-lineage cells induced systemic inflammation, immune-suppression and tumorigenesis,\textsuperscript{45} confirming our data using genetic deletion of PPARγ. This fine-tuning of challenge clearance by a delicate balance of M1/M2-type macrophages activities contributes to the resolution of inflammation and prevents chronic tissue damage, and may thus counteract transition to malignancy, which can otherwise be regarded as “a wound that never heals”.

Based on the existing literature and our data (Fig. 7), we draw the following model: PPARγ activation reduces expression of the Th2 cytokine IL4 (e.g. via transrepression of NFAT\textsuperscript{29}) in LPLs leading to defective STAT3/6 signaling in IL4-target cells, impairing Th2/M2 differentiation and polarisation, thereby indirectly favoring the development of Th1/M1 “killer” cell phenotypes. PPARγ in IECs inhibits proliferation and promotes senescence together with RAS in a cell-intrinsic fashion,\textsuperscript{4} thus counteracting the progression of benign adenomas to cancer. The cross-talk between macrophages and enterocytes may comprise additional soluble factors such as CXCL10, a marker chemokine of the host’s Th1-orientated immune response induced by PPARγ-agonist and a prognostic factor in cancer patients.\textsuperscript{46} Pro- and anti-inflammatory cytokines in turn regulate PPARγ expression and activities, corroborating mutual cellular communication networks.\textsuperscript{29}

Additional experiments have to specify the nature of PPARγ-target cell subpopulations for anti-diabetic insulin-sensitizers of the glitazone class within the complex mucosa-associated intestinal immune system that differs considerably between rodents and humans. In sum, our preclinical data propose that PPARγ-activation reprograms the host’s immune response against mutant KRAS-driven oncogenic effects by enforcing “killer”-type macrophages. The results in mice were statistically significant, but the magnitude of the biologic effect renders the biologic significance for translation to humans uncertain and may limit applications in patients. Nonetheless, our work may relate to a possible clinical relevance. Cancer immunotherapies are designed to restructure the immunosuppressive tumor microenvironment towards a tumor-attacking mode. A high “immunoscore”, i.e. infiltration of CTLs and immunogenicity markers (e.g. MHC and B7 molecules), predicts favorable prognosis in CRC.\textsuperscript{47} However, clear endpoints and response prediction markers for checkpoint inhibitor Abs are lacking. Current strategies against T-cell/tumor interfaces may be complemented with macrophage-based therapies, and repurposing and combination with established drugs already approved for other diseases is encouraged.\textsuperscript{48} Therein, PPARγ-agonists may have a perspective as potential candidates for future immunotherapies.

**Methods**

**Animals**

Wildtype (WT) C57BL6/J and transgenic (tg) mice with an activating human KRASG12V mutation were obtained from Charles River (Lyon, France) and KPI,\textsuperscript{9} respectively. Floxed and Cre transgenic mice were from Jackson Labs (Bar Harbor, ME). Intestine-specific disruption of Pparg gene function was achieved by excision of loxp sites (abbrev. FL) flanking exons 1 and 2\textsuperscript{49} followed by interbreeding with mice transgenic for Cre recombinase\textsuperscript{20} and KRASG12V\textsuperscript{30} both under the control of the murine villin promoter in intestinal epithelial cells (enterocytes) [C57BL6/J-Tg(Vil-KRAS‘G12V)Robine/J x B6.129-Ppargtm2Rev/J] by using mice expressing Cre recombinase under the murine lysozyme promoter\textsuperscript{51} [C57BL6/J-Tg(Vil-KRAS‘G12V)Robine/J x B6.129-Ppargtm2Rev/J x B6.129 P2-Lyz2tm1(cre)Ifo/J]. Nomenclature and abbreviations for genotype combinations are listed in Table S1. Mice were housed in a specific pathogen-free animal facility on a 12 h day/night light cycle and fed water and chow diet *ad libitum* (by day) as published previously.\textsuperscript{20} All experiments were conducted in agreement with ethical approvals from the governmental authorities of Baden-Württemberg, Karlsruhe, Germany (35-9185.82/G-176/12, 35-9185.81/G-146/15).

**Reagents, primers and antibodies**

Additional information is provided in Supplementary Methods and Tables S3/S4.

**Immunohistochemistry (IHC)**

Ab and hematoxylin & eosin (H&E) stainings on formalin-fixed paraffin-embedded (FFPE) tissue sections were done as detailed in.\textsuperscript{52} Antigen retrieval was performed by heating in antigen unmasking solution H-3000 (Vector Laboratories, Burlingame, USA) and blocking with H2O2, if not stated otherwise. Abs were diluted as detailed in Table S3, and staining was
processed as recommended by the Vectastain ABC kit (Vectorlabs). The substrate 3,3'-diamino benzidine (DAB) (Vectorlabs) was employed for detection followed by hematoxylin counter-staining. Frequency and intensity of staining positivity was determined in epithelial and lamina propria (stroma) cells. The staining scores were defined as: 0+ = negative (0–25%), 1+ = weak (25–50%), 2+ = moderate (50–75%), 3+ = strong (75–100%). Percentage was calculated as IHC positive cells divided by total number of cells within the field. Signals were quantified observer-blinded at a standard bright-field microscope using Image J (image.nih.gov/ij) (n > 20 signals per field; n = 5 fields per image). The field area was defined by the morphology of a given crypt-villus unit and measured in square mm (mm²).

Software and statistics

Data from in vivo and in vitro studies are displayed as means ± S.E. from at least 3 independent experiments from different cell passages or individuals (frozen samples from mice). The cut-off values for dichotome analysis of IHC staining scores (0 to 3+) were calculated as ≥2.0 for tumor and stroma positivity. Optical densities (OD) of bands in gels from Western blots and PCRs were measured using automated imaging devices and quantified with Image J (image.nih.gov/ij). Data were normalized to house keeping genes or proteins as indicated in the legends to figures and calculated as -fold or % compared to control. Statistical analysis was done with Graphpad Prism (version 4.0, La Jolla, CA). All tests were unpaired and two-sided if not stated otherwise. P-values < 0.05 were considered significant (*).

Abbreviations

Ab antibody
ACF aberrant crypt foci
Ad adenoma
APC allophycocyanin
BSA bovine serum albumin
CD cluster of differentiation
Cre recombinase
CTL cytotoxic T-lymphocyte
CRC colorectal cancer
ERK extracellular signal-regulated protein kinase
FC flow cytometry
FCS fetal calf serum
FL floxed gene (loxP site inserted)
FFPE formalin-fixed and paraffin-embedded
FITC fluorescein isothiocyanate
G tumor grade
GI gastrointestinal
GSEA gene set enrichment analysis
H&E hematoxylin and eosin
HPP hyperplastic polyp
IEC intestinal epithelial cells
IEL (intestinal) intraepithelial lymphocytes
IL interleukin
IFN interferon
IHC immunohistochemistry
LPL lamina propria lymphocytes
Lysozyme
LPS lipopolysaccharide
M1/2 macrophage polarization type 1/2
NT normal (intestinal) tissue
MAPK mitogen-activated protein kinase
MEK macrophage
MPH/Mph optical density
OD
OIS oncogene-induced senescence
P21 cyclin-dependent kinase inhibitor (WAF1/CIP1)
PE phycocytin
PMA phorbol 12-myristate 13-acetate
PPARγ peroxisome proliferator-activated receptor gamma
PPARδIEC Rous sarcoma oncogene (mouse genotype) VilKRASG12V
PPARδMPH Rous sarcoma oncogene (gene name)
RAS (gene name) Rous sarcoma oncogene
RAS (mouse genotype) VilKRASG12V
RTK receptor tyrosine kinase
SCF subcellular fractionation
SI small intestine
(S)SA (sessile) serrated adenoma
T2DM type 2 diabetes mellitus
TCL total cell lysate
tg transgene
Tc cytotoxic T-cells
Th helper T-cells
Treg regulatory T-cells
TNF tumor necrosis factor
TU tumor tissue
Vil Vilin
WT wildtype
WT (mouse genotype) Pparg+/+//FL/FL x LyzCre

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Transcript profiling

Data from CDNA microarrays were deposited at Gene Expression Omnibus (GEO) with the accession number GSE47772 under the following link: https://www.ncbi.nlm.nih.gov/gds/?term=GSE47772[Accession]

Author contributions

All authors cooperated and contributed to, critically reviewed and approved the manuscript. EB, WR and ME defined the research theme. TG, CW, PW, FH, SH, TF, SY and KP designed methods and carried out the experiments. EB, TG, CW, PW, SH and KPJ analyzed the data and interpreted the results. EB wrote the paper. KPJ provided mouse strains and performed bioinformatic analysis. TiG and JK provided, analyzed and interpreted the results. EB, TG, CW, PW, SH and KPJ analyzed the data and performed bioinformatic analysis. TiG and JK provided, analyzed and interpreted immunostainings.

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